In vitro growth and development of the sweet medicinal plant *Stevia rebaudiana* Bertoni

by

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Declaration

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Abstract

Stevia rebaudiana Bertoni is a plant native to the Amambay region located north-east of Paraguay in South America. *S. rebaudiana* is a natural, sweet perennial herb that contains *ent*-kaurene diterpene glycosides in its leaves. There are over 9 *ent*-kaurene diterpene glycosides and stevioside is the most abundant but rebaudioside A is the sweetest. *S. rebaudiana* also commonly known as *Stevia* is recognized to have great economic and scientific value around the world due to its sweetness and reported therapeutic properties. As a result, it is cultivated commercially in certain parts of the world. This, however, excludes southern Africa. South Africa has an opportunity to cultivate *S. rebaudiana* as a new crop for the agricultural sector. The aim of this study was to establish a protocol to determine the best treatment for optimal seed germination using acid scarification, smoke-water, a combination of acid scarification and smoke-water and gibberellic acid. To study the macronutritional requirements of *S. rebaudiana* plants utilizing nitrogen and phosphate manipulation *in vitro*. To determine if *in vitro* derived plant extracts differ in metabolite profiles regarding the main bio-actives (diterpene glycosides) using a metabolomic approach that involved the application of LC-MS and GC-MS technology. To determine the effects of drought and salinity stress on the growth of *S. rebaudiana* using different concentrations of (w/v) polyethylene glycol 6000 (PEG 6000) and sodium chloride (NaCl) as osmotica.

This plant exhibits a low seed germination rate which is a great challenge towards large scale propagation thus making its production expensive. Using a tissue culture system as a propagation study tool, germination of *Stevia* seeds was tested using 1% (w/v) 2, 3, 5-triphenyl tetrazolium chloride solution in this study. This showed a low viability of 19%. *S. rebaudiana* seeds were subjected to four variables namely: smoke water extract, chemical scarification using 70% (v/v) sulfuric acid for 30 seconds, a combination of smoke water extract and 70% (v/v) sulfuric acid and gibberellic acid were tested as a means of improving germination *in vitro*. The smoke treatment was highly efficacious in producing a significant germination percentage ($P < 0.05$) while seeds scarified using 70% (v/v) H_2SO_4 had the lowest germination rate.

To test the effect of macronutrients (nitrogen and phosphate), various levels of nitrogen and phosphate were added to the growth medium. Thereafter, liquid chromatography-mass spectrometry was used to analyze the effects on the metabolomic profile. All other 85 nutritional elements were kept similar to the control which contained similar concentrations as Murashige and Skoog (1962) medium (MS) with both nitrogen (NH_4NO_3 at 20.61 mM and KNO_3 at 18.79 mM) and phosphate (KH_2PO_4 at 1.25 mM). Two distinct clusters were revealed after principal component analysis of the metabolite profiles. The orthogonal partial least squares discriminant analysis was also applied. This allowed the organization of the clusters into two distinct groups. Steviol hydrate, stevioside hydrate and rebaudioside A contributed significantly to the distinct separation of phosphate-treated plants from the nitrogen-treated plants. The clustering suggests different chemical influences at enzyme and gene level on secondary metabolism resulting in different chemical profiles. Reducing the nitrogen level to half (0.5 N) in the MS medium led to the tallest plants. Reduction in the roots was observed with increasing levels of nitrogen and phosphate. I further assessed the effects of drought and salinity stress by using polyethylene glycol 6000 (PEG) and sodium chloride (NaCl) at different concentrations, respectively. Higher concentrations of PEG 6000 (7.5 and 10%) and NaCl (75 and 100 mM) resulted in a decline in both *ent*-kaurene diterpene glycosides and terpenes present in the treated *Stevia* leaves. Headspace solid phase microextraction gas chromatography spectrometry revealed an abundance of α-pinene, β-pinene and sabinene in all treated plants except in the plants exposed to 10% PEG 6000 which showed no growth. The addition of PEG 6000 decreased the concentrations of rebaudioside A and stevioside significantly.

In conclusion, this study has revealed the importance of nitrogen and phosphate in the manipulation of *ent*-kaurene diterpene glycoside production in *Stevia* microplants, setting a platform to test these effects *ex vitro*.

Opsomming

Stevia rebaudiana Bertoni is ‘n inheemse plant van die Amambay streek, Noord-Oos van Paraguay in Suid-Amerika. *S. rebaudiana* is ‘n natuurlike soet, meerjarige krui wat *ent*-kaurene diterpeen glikosiedes bevat in die blare. Daar is meer as 9 *ent*-kaurene diterpeen glikosiedes waarvan steviosied die mees volop is, maar rebaudiosied A is die soetste. *S. rebaudiana* (*Stevia*) is bekend vir sy merkwaardige ekonomiese en wetenskaplike waarde wêreldwyd, as gevolg van sy soetheid en berigte terapeutiese eienskappe. As gevolg word dit kommersiëel vervaardig in sekere dele van die wêreld. Hierdie sluit alhoewel nie Suid-Afrika in nie, en dus is daar ‘n geleentheid vir vervaardiging van *S. rebaudiana* as ‘n nuwe kropgewas in die lanbousektor.

Die doel van hierdie studie was om 'n protokol op te stel vir die beste behandeling vir optimale ontkieming met behulp van suurbeskadiging, rookwater, 'n kombinasie van suurbeskadiging en rookwater en gibberelliensuur. Om die makronutriënt vereistes van *S. rebaudiana* plante te bestudeer deur gebruik te maak van *in vitro* stikstof en fosfaat manipulasie. Om te bepaal of plant ekstrakte wat *in vitro* versamel is, verskil in metaboliet profiele met betrekking tot die belangrikste bio-aktiewe molekules (diterpeen glikosiede) met behulp van 'n metabolomiese benadering wat die toepassing van LC-MS en GC-MS tegnologieë gebruik. Om die uitwerking wat droogte en soutgehalte stres het op die groei van *S. rebaudiana* te bepaal met behulp van verskillende konsentrasies van (w/v) poliëtileenglikol 6000 (PEG 6000) en natriumchloried (NaCl) as osmotika.

Hierdie plant het laë ontkiemings-sukses, wat ‘n groot uitdaging is vir groot-skaal boerdery, en as gevolg produksie duur maak. Na aanvang van ‘n tetrasolium toets met gebruik van 1% (w/v) 2, 3, 5-trifeniel tetrasolium chloried oplossing, was ‘n weefselkultuur sisteem gebruik as ‘n wetenskaplike werktuig om ontkieming-sukses van *Stevia* saad te toets. Lewensvatbaarheid na hierdie toets was slegs 19%. *S. rebaudiana* sade was behandel met vier veranderlikes naamlik: rookwaterekstrak; chemiese skade met gebruik van 70% (v/v) swaelsuur vir 30 sekondes; ‘n kombinasie van rookwaterekstrak en 70% (v/v) swaelsuur and gibberelliensuur. Hierdie veranderlikes was getoets as ‘n metode vir die verbetering van *in vitro* ontkieming. Die rook

behandeling was hoogs effektief met ‘n beduidende ontkieming persentasie ($P < 0.05$), terwyl saaddoppe wat verswak was met 70% (v/v) swaelsuur, die laagste ontkiemings-sukses gehad het.

Om die effek van makronutriënte te toets, was verskeie vlakke van stikstof en fosfaat by die groeimedium gevoeg. Dit was gevolg deur vloeistofchromatografie-massaspektrometrie te gebruik om die effekte op die metaboliese profile te ontleed. Al die ander 85 voedingstowwe was behou soortgelyk aan die kontrole, wat dieselfde konsentrasie Murashige and Skoog (1962) medium (MS) met albei stikstof (NH_4NO_3 van 20.61 mM en KNO_3 van 18.79 mM) en fosfaat (KH_2PO_4 van 1.25 mM). Twee onderskeie groepe was merkbaar na hoofkomponentanalise van die metaboliese profiele. Die ortogonale gedeeltelike kleinste kwadrate diskriminantontleding was ook gebruik. Dit het die groepering van twee onderskeie groepe moontlik gemaak. Steviol hidraat, steviosied hidraat en rebaudiosied A het beduidende bedrae tot die verdeling van fosfaat-behandelde plante van die stikstof-behandelde plante. Die groepering stel verskeie chemiese invloede op ensiem en geen vlak vir sekondêre metabolism, wat lei tot unieke chemiese profiele. Die halvering van die stikstof konsentrasie (0.5 N) in die MS medium, het gelei tot die hoogste lengte plante. Verminderde wortellengte was waargeneem deur verhoogde vlakke van stikstof en fosfaat. Die effekte van droogte en soutgehalte stres was geëvalueer deur gebruik van poliëtileenglikol 6000 (PEG) en natriumchloried (NaCl) teen verskeie konsentrasies, onderskeidelik. Hoër konsentrasies van PEG 6000 (7.5 en 10%) en NaCl (75 en 100 mM) het gelei tot ‘n vermindering in beide *ent*-kaurene diterpeen glikosiedes en terpene teenwoording in *Stevia* blare. Kopspasie vastestoffase mikroekstraksie gaschromatografie spektrometrie het ’n oorvloed van α-pineen, β-pineen en sabineen in alle behandelde plante waargeneem behalwe dié blootgestel aan 10% PEG 6000, wat geen groei aangetoon het nie. Die byvoeging van PEG 6000 het die konsentrasies van rebaudiosied A en steviosied drasties verminder.

Hierdie studie het die belang van stikstof en fosfaat in die manipulasie van *ent*-kaurene diterpeen glikosied vervaardiging in *Stevia* mikroplante bewys, wat ‘n platform skep om hierdie effekte *ex vitro* te toets.

Scientific outputs

Conference presentations (Appendix A)

Posters

Magangana, T.P. and Makunga, N.P., 2015. Phytochemical and molecular analyses of *Stevia rebaudiana* extracts generated from different cultivation methods. Indigenous Plant Use Forum 18th Annual Conference 2015, Clanwilliam, South Africa.

Magangana, T.P. and Makunga, N.P., 2016. The effect of various factors on seed germination and the influence of abiotic stresses on growth productivity, physiology and differences in metabolite profiles (diterpene glycosides) of *Stevia rebaudiana* Bertoni. South African Association of Botanists Conference 2016, Hosted at the Business School Complex on the campus of the University of the Free State, Bloemfontein, South Africa.

Oral

Magangana, T.P. and Makunga, N.P., 2016. Effect of nitrogen and phosphate on the growth productivity and biochemicals of *Stevia rebaudiana.* South African Association of Botanists 42nd Annual Conference 2016, Hosted at the Business School Complex on the campus of the University of the Free State, Bloemfontein, South Africa.

Dedication

I would like to dedicate this publication to my God who has been my rock throughout this study; my parents Mr. E.D Magangana and Mrs. B.F Bingwa-Magangana, my siblings Zimkita, Nobuntu and Matongo Magangana, my fiancé Moses Masimba Chisvino and my son Samuel. Last but not least my beautiful niece, Ande Magangana. Thank you for your support, without you I would not be where I am today. You have been such a blessing to me.

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List of acronyms

%	Percent
º C	Degree Celsius
ANOVA	Analysis of variance
BA/BAP	6-Benzyladenine
B.C	Before Christ
BHA	Butylated hydroxyaniosole
BHT	Butylated hydroxytoluene
CAF	Central Analytical Facility
CDP	(-)-Copalyl diphosphate
CDPS	(-)-Copalyl diphosphate synthase
cm	Centimetres
CMK	4-(Cytidine5' diphospho)-2-C-methyl-D-erythritol kinase
DMADP	Dimethylallyl diphosphate
dS m^{-1}	DeciSiemens per metre
DXP	1-Deoxy-D-xylulose-5-phosphate
DXPR	1-Deoxy-D-xylulose-5-phosphate reducto-isomerase
DXS	1-Deoxy-D-xylulose-5-phosphate synthase
EI	Electronic impact
EtOH	Ethanol
eV	Electron volt
FDA	Food and Drug Administration
g	Gram(s)
GA_3	Gibberellic acid
GC-MS	Gas chromatography mass spectrometry
GGDP	Geranylgeranyl diphosphate
Gly	Glucose

Ha	Hectare
HDR	(E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase
HDS	(E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase
HSD	Honesty Significance Difference
HS-SPME	Headspace solid phase microextraction
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IDP	Isopentenyl diphosphate
K	Potassium
Kg	Kilograms
Kn	Kinetin
KO	Kaurene oxidase
kPa	Kilopascal
KS	(-)-Kaurene synthase
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
L	Litre
m	Metres
M	Molar
mABP	Mean arterial blood pressure
MCT	4-(Cytidine5’ diphospho)-2-C-methyl-D-erythritol synthase
MDS	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase

MeOH	Methanol
MEP	2-C-methyl-D-erythritol-4 phosphate
mM	Millimolar
mm	Millimetres
MS	Murashige and Skoog (1962)
MS	Mass spectrometry
MVA	Mevalonate
m/ z	Mass to charge ratio
NaCl	Sodium chloride
NADP H	Nicotinamide adenine dinucleotide phosphate oxidase
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance spectroscopy
P	Phosphorus
PCA	Principal component analysis
PEG	Polyethylene glycol
PGR	Plant growth regulators
pH	Potential of hydrogen
Qtof	Quadrupole time-of-flight
Rha	Rhamnose
rpm	Revolution per minute
S	South
TDZ	Thidiazuron
TOF	Time of flight

TPA	12-0-tetradecanoylphorbol-13-acetate
UHPLC- UV	Ultra high pressure liquid chromatography- ultraviolet
UV	Ultra violet
v/v	Volume per volume
WHO	World health organization
w/ w	Weight per weight
w/ v	Weight per volume
Xyl	Xylose
Zn	Zinc
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar

Chapter One

1. General introduction

1.1 The global need for sweet foods

Humans have sought sweet foods and sugar-sweetened beverages throughout history. Man is said to have an innate preference for sweetness (Pepino and Mennelia, 2006). Intake of sweet substances has risen considerably among all age groups over the years. This is a direct result of many factors in terms of preference for sweeteners such as: an individual's early experience, genetics, race and/or ethnicity, medication use, nutritional deficiencies, metabolic changes, otitis and addictions (Hayes *et al*., 2008; Levine *et al*., 2003; Mennelia *et al*., 2010). It is also believed that evolution has played a part in high sweet preference as it has molded the types of foods preferred or discarded by children. In fact the ability to detect or respond to taste is well developed before birth and builds up through time (Granchrow *et al*., 2003). Humans have well developed sensory systems which give them the ability to prefer sweet tasting foods over potentially toxic ones with a bitter taste (Ventura and Mennelia, 2011). These sweet tasting substances are also interpreted by the brain as a sign of reward or as means of pain relief (Ventura and Mennelia, 2011).

There is a great demand for sweet foods and sugar-sweetened beverages today. High levels of sugar (referring to sucrose here) is associated with many health risks such as cardiovascular disease, weight gain, development of obesity, diabetes and dental caries (Gasmalla *et al*., 2014; Pradhan *et al*., 2014; Ventura and Mennelia, 2011). There has thus been a movement to adopt other alternatives for use as sweeteners worldwide.

In Japan, *Stevia* has been used commercially since the 1970s, with no reported negative effects (Lemus-Mondaca *et al*., 2012 Singh and Rao, 2005; Yadav *et al*., 2011). Since then, the use of *Stevia* has spread around the world. In Canada, the plant is sold as a tea ingredient (Ramesh *et al*., 2006) and in Europe, it is used as an ornamental (Lemus-Mondaca *et al*., 2012). Farmers in India have been encouraged to cultivate *Stevia* due to the high potential demand. This is a result of the studies done by the International Diabetes Federation and Madras Diabetes Research

Foundation which revealed that India had a dramatic increase in people with Type 2 diabetes in 2011 of 62.4 million as compared to 2010 where 50.8 million people were recorded as having this disease (Pradhan *et al*., 2014). It is also cultivated in China, Taiwan, Thailand, Korea, Brazil, Malaysia, Israel, Ukraine, Phillipines, California, Mexico and all over South America (Ghosh *et al*., 2008; Lemus-Mondaca *et al*., 2012; Shivanna *et al*., 2013; Thomas and Glade, 2010; Yadav *et al*., 2011).

Stevia rebaudiana Bertoni (Asteraceae family) could become a profitable alternative new crop in southern Africa that would be of great benefit as more people are looking for healthier alternatives of foods to live longer, maintain or lose weight, to have more active lifestyles and would be beneficial to the economy of the country (South Africa). *S. rebaudiana* has been used for over a century as a non-caloric, non-carcinogenic and non-allergic sweetener and for its extensive therapeutic properties. The medicinal and commercial value which this plant has, has led to its urgent demand for large scale production in various parts of the world. *S. rebaudiana* is not grown in southern Africa on a commercial scale. There is potential to establish it as a crop in this region which could lead to a new agricultural industry that may be of benefit to South Africa's economy. To meet this aim, it becomes essential to explore propagation systems to study the influence of environmental conditions on the key bio-actives.

S. rebaudiana has been recognized to have great economic and scientific value around the world due to its sweetness and reported therapeutic properties. Its popularity has spread to various parts of the world including South Africa, where it has been recently launched in 2012 in the form of Canderel green. However, this valuable plant is not cultivated in South Africa and in other parts of southern Africa on a commercial scale. Its popularity in other parts of the world offers an opportunity for South Africa to produce it commercially.

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Chapter Two

2. Literature review

2.1 History of *Stevia rebaudiana* Bertoni

Stevia rebaudiana Bertoni (Figure 2.1) is related to sunflowers, chrysanthemums and marigolds (Khattab *et al*., 2015; Lemus-Mondaca *et al*., 2012; Singh and Rao, 2005; Yadav *et al*., 2011). It belongs to a group of annual and perennial herbs, sub-shrubs and shrubs (Yadav *et al*., 2011). This herbaceous perennial shrub has been recorded as being used since the 1500s by the Guarani Indian tribe of Paraguay and Brazil as a natural sweetener. They called the plant *Ka'a he'e* which means 'sweet herb/grass' (Brandle *et al*., 1998; Laura *et al*., 2006; Suttajit *et al*., 1993; Yadav *et al*., 2011). They used it to counteract the bitter taste of plant based medicines and beverages such as yerba maté and as a flavour enhancer (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). The genus *Stevia* contains over 240 species that are native to South America, Central America and Mexico; but only two species contain *ent*-kaurene diterpene glycosides which give the plants their sweet and therapeutic properties namely *S. rebaudiana* and *S. phlebophylla* (Brandle and Telmer, 2007; Brandle *et al*., 1998; Lemus-Mondaca *et al*., 2012; Ranjan *et al*., 2011;; Yadav *et al*., 2011). Steviol glycosides are also found in *Rubus chingii* which belongs to the Rosaceae family native to China (Brandle and Telmer, 2007). The genus *Stevia* was first researched and named after a Spanish botanist and physician named Petrus Jacobus Stevus (1500-1556). However, it was only in 1887 an Italian botanist, Dr. Moisés Santiago Bertoni, gave the herb its first botanical description (Laura *et al*., 2006; Yadav *et al*., 2011). Formally known as *Eupatorium rebaudianum*, but later changed to *Stevia rebaudiana* (Bertoni) in 1905 by Dr. Bertoni who named the "new" variety of the genus in honor of a Paraguayan chemist named Rebaudi, who became the first to extract the plants sweet components (De Oliveira *et al*., 2004; Laura *et al*., 2006; Ranjan *et al*., 2011 ; Yadav *et al*., 2011). The extract was only purified in 1931 to produce stevioside (Lemus-Mondaca *et al*., 2012; Yadav *et al*., 2011). In 1964, commercial cultivation of the plant proceeded in Paraguay whilst in Japan it began in the late 1960s (Brandle *et al*., 1998). Since then, the plant has been of peculiar interest in certain regions of the world due to its commercialization. This includes places

like Brazil, Argentina, Canada, China, Korea, Taiwan, Malaysia, Thailand, Ukraine, the Philippines, Hawaii, California, Tanzania, Abkhazia, United Kingdom and Russia (Brandle *et al*., 1998; Ghosh *et al*., 2008; Lemus-Mondaca *et al*., 2012; Shivanna *et al*., 2013; Thomas and Glade, 2010; Yadav *et al*., 2011).

Figure 2.1 Morphology of *S. rebaudiana*, **A** and **B** show the leaves. **C** and **D** show the flowers and roots respectively.

2.2 Geographical distribution

S. rebaudiana is native to the Amambay region located north east of Paraguay near the Brazilian border between latitudes 23º and 24º S in South America, which also includes the zone of San Pedro, Yhu, Jejui Guaza and near the source of the river called Monday which is a border area between Brazil and Paraguay (Brandle *et al*., 1998; Chatsudthipong and Muanprasat, 2009; Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Thomas and Glade, 2010; Yadav *et al*., 2011). This sun-loving perennial herb grows best in warm, humid conditions (Ramesh *et al*., 2006; Yadav *et al*., 2011) and is found particularly in tropical and subtropical regions of the world and is suggested to have a higher leaf recovery in the subtropics than in the tropics (Ramesh *et al*., 2006; Yadav *et al*., 2011). It belongs to a group of annual and perennial herbs, sub-shrubs and shrubs that occur in mountainous areas, open forests, dry valleys, borders of rivers and is commonly found on the outskirts of the marshland of sandy, infertile, acid soils of Paraguay (Brandle *et al*., 1998; Ramesh *et al*., 2006; Yadav *et al*., 2011). *Stevia* is prone to moisture stress and thrives in sites with adequate drainage and sites not subjected to prolonged irrigation. It has a daily average water requirement of 2.33 mm and is also prone to salinity stress as it cannot tolerate saline soils (Ramesh *et al*., 2006; Yadav *et al*., 2011). Determining the effect of these two stresses on the growth of the plant and on the *ent*-kaurene diterpene glycosides found on the leaves would be beneficial in the potential commercialization of the plant in southern Africa, which is known to contain a variety of soil and weather patterns.

The genus *Stevia* contains more than 240 species of herbaceous, shrub and sub-shrub plants which are found in several other regions of the world (Lemus-Mondaca *et al*., 2012; Ranjan *et al*., 2011). It is also cultivated in the United Kingdom under greenhouse conditions as a leaf crop and this is because it cannot survive the winter climate (Ramesh *et al*., 2006). There are approximately 90 varieties of *S. rebaudiana* world-wide and these varieties may be suitable for specific climactic conditions (Lemus-Mondaca *et al*., 2012). In its native land, this plant still remains a rare shrub. Although field cultivation is possible, it is complicated by 1) low or poor seed germination; 2) a few number of individuals being obtained from a single plant; and, 3) high labour input (Lemus-Mondaca *et al*., 2012; Thiyagarajan and Venkatachalam, 2012).

2.3 Botanical description

S. rebaudiana can grow up to 50-120 cm tall (Brandle *et al*., 1998; Ramesh *et al*., 2006) or up to 1 m tall (Lemus-Mondaca *et al*., 2012; Singh and Rao, 2005). The plant contains sessile leaves with an estimated growth span of 3-4 cm. These leaves may be elongate-lanceolate or speculate shaped having a blunt-tipped lamina, with a margin which is serrate from the middle to the tip and entire below (Figure 2.1 A and B). It contains small (7-17 mm), pentamerous white flowers with pale purple throat corollas (Figure 2.1 C) (Brandle *et al*., 1998; Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). These may be composite surrounded by involucres of the epicalyx. Its capitula are contained in loose, mainly irregular, sympodial cymes. The plant also contains 2 to 6 tiny white florets which are born in small corymbs usually arranged in loose panicles (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). Flowering may begin after 4 leaves have formed, but may take a month for the different developmental flower stages to occur (Yadav *et al*., 2011). It is self-compatible and thought to be an insect pollinated shrub (Ramesh *et al*., 2006; Yadav *et al*., 2011). Certain genotypes of *S. rebaudiana* have been reported to have agamospermy (Yadav *et al*., 2011). There are 5 small anthers and its pollen is reported to be highly allergenic (Ramesh *et al*., 2006; Yadav *et al*., 2011). The stem is woody with a weak pubescent at the bottom and with the rhizome containing slightly branching roots. *S. rebaudiana* has an extensive root system with fine roots found around the soil-surface and the thicker roots extending to the deeper parts of the ground (Figure 2.1 D) (Ramesh *et al*., 2006). *S. rebaudiana* seeds are very small and are found in all-ribbed spindle-shaped slender achenes (3 mm) with each containing 20 persistent pappus bristles. Each seed contains a tiny endosperm and are dispersed in the wind using hairy pappus (Brandle *et al*., 1998; Yadav *et al*., 2011).

2.4 Therapeutic properties

The diterpene glycosides are suggested to be responsible for many therapeutic properties. They may be used as immunomodulatory agents, acting as immunosuppressants or immunostimulators depending on the effect they have on the immune system (Lemus-Mondaca *et al*., 2012). Hence, they have the ability to regulate one or more immune functions in the body (Chatsudthipong and Muanprasat, 2009; Lemus-Mondaca *et al*., 2012). The plant itself has been used as a traditional

medicine for the prevention of different ailments such as ulcers in the gastrointestinal tract, for the treatment of cancer, anti-gingivitis, dental caries, for hypertension, for its anti-diarrhoeal properties, diuretic properties, as an anti-inflammatory, to maintain weight or for weight loses and the *Stevia* leaf extract is reported to lower the blood sugar level by up to 35.2% within 6 to 8 hours of ingestion making it beneficial for diabetic patients (Carbonell-Capella *et al*., 2013; Ghosh *et al*., 2008; Lemus-Mondaca *et al*., 2012; Razak *et al*., 2014; ; Tadhani *et al*., 2007).

The leaves of *S. rebaudiana* also contain other secondary plant constituents which include flavonoids, alkaloids (such as steviamine), water-soluble chlorophylls and xanthophylls, hydroxycynnamic acids, neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements (Lemus-Mondaca *et al*., 2012; Michalik *et al*., 2010; Tadhani *et al*., 2007). Flavonoids and other phenolic substances have been suggested to play a preventive role in the development of cancer and heart disease (Tadhani *et al*., 2007). The leaves and callus of *S. rebaudiana* are also a great source of antioxidants which are suggested to have specific health effects beneficial to those suffering from coronary heart disease and cancer (Tadhani *et al*., 2007; Yadav *et al*., 2011). The leaves and roots of *S. rebaudiana* produce also fructo-oligosaccharides which are used as storage compounds. These storage components are suggested to play a role as prebiotics and are important for diabetes control (Braz de Oliveira *et al*., 2011).

2.5 Anti-microbial activity

S. rebaudiana is suggested to play a role in inhibiting the growth of certain bacteria and other infectious organisms (Lemus-Mondaca *et al*., 2012; Singh and Rao, 2005; Suttajit *et al*., 1993). This helps in explaining its traditional use by the Guarani tribe in Paraguay and Brazil who used the *Stevia* leaves to treat wounds, sores and gum disease. It also helps those susceptible to yeast infections or reoccurring streptococcal infections, which are two conditions which seem to worsen through the use of white sugar (Lemus-Mondaca *et al*., 2012; Singh and Rao, 2005; Suttajit *et al*., 1993). In some studies the microbial activity of various extracts of *S. rebaudiana* have been investigated taking special interest in some selected microorganisms such as *Salmonella typhi, Escherichia coli, Aeromonas hydrophila, Vibrio cholera, Bacillus subtilis, Staphylococcus aureus* and others have been examined (Lemus-Mondaca *et al*., 2012; Razak *et al*., 2014).

2.6 Pharmacological activity

Anti-oxidants have been shown to prevent oxidative damage caused by free radicals. This is achieved by interfering with the oxidation process reacting with the free radicals, chelating catalytic metals and also acting as oxygen scavengers (Lemus-Mondaca *et al*., 2012). The anti-oxidant activity of medicinal plants depends on the concentration of the individual anti-oxidant entering into the composition. Several synthetic anti-oxidants exist in the market such as butylated hydroxyaniosole and butylated hydroxytoluene but they are not safe (Lemus-Mondaca *et al*., 2012). Hence, they are prohibited in many parts of the world. *S. rebaudiana* contains a great degree of anti-oxidant activity that hinders the formation of $_{DL}$-α-tocopherol. Its anti-oxidant activity is a result of scavenging of free radical electrons and superoxides (Lemus-Mondaca *et al*., 2012).

S. rebaudiana is a high-potency sweetener which is more than 250 or even 450 times sweeter than sucrose and serves as a good alternative for diabetic patients (Chatsudthipong and Muanprasat, 2009; Geuns, 2003). It is also suggested to have anti-hypertensive effects, whereby it has the ability to decrease the mean arterial blood pressure in both humans and animals. High human consumption of the plant may result in the reduction of both systolic and diastolic blood pressure (Chatsudthipong and Muanprasat, 2009).

The *S. rebaudiana* extract known as isosteviol contains anti-inflammatory and anti-tumor effects and is actively involved in retarding three different types of human cancer cells and inhibiting inflammation induced by 12-0-tetradecanoylphorbol-13-acetate which is known to also induce formation in mammalian cells (Chatsudthipong and Muanprasat, 2009). The effectiveness of *S. rebaudiana* is ascribed to its diterpene glycoside compound activity.

Regular use of *S. rebaudiana* decreases the content of sugar, radionuclides and cholesterol in the blood, improves cell regeneration and blood coagulation, suppresses neoplasmic growth and strengthens blood vessels, plays a role as an anti-gingivitis agent (Lemus-Mondaca *et al*., 2012). It is a non-toxic, non-mutagenic, non-carcinogenic and non-allergic sweetener. Stevioside has shown really low toxicity in mice, rats and hamsters (Geuns, 2003). However, it is suggested to cause some allergic reactions to people sensitive to plants of the Asteraceae family and pregnant women are also advised not to consume the plant.

2.7 Propagation methods

S. rebaudiana seed has a low fertility rate (Razak *et al*., 2014; Yadav *et al*., 2011) thus propagation by seed is not efficient with rates ranging below 10% (Miyazaki and Wantabe 1974); 36.3% (Goettemoeller and Ching, 1999) and others show a 41% rate (Raji and Osman, 2011) while others successfully germinate with high rates such as 67.33% (Abdullateef *et al*., 2015). As these reports are not congruent, testing the viability of a seed lot becomes key. Often propagation by seed does not allow the production of homogenous populations and this results in great variability of important features related to intensity of its sweetness and chemical composition. Propagation by cuttings is possible but may be prone to a lack of genetic diversity and potentially increase insect and disease weakness in the new *Stevia* plant (Khalil *et al*., 2014; Yücesan *et al*., 2016). To reiterate, vegetative propagation is also limited by a low number of individuals that can be obtained simultaneously from a single plant (Janarthanam *et al*., 2010; Razak *et al*., 2014; Yadav *et al*., 2011). Tissue culture is known to produce plants that often mature quickly. It can also be adopted for commercial production of plants in demand which may easily establish *ex vitro* (Colling *et al*., 2009) for large-scale farming. There are several protocols for micropropagation of *S. rebaudiana*, where different clones or varieties of the same species demonstrate different behaviours in *in vitro* culture (Table 2.1) (Razak *et al*., 2014).

Table 2.1 Optimized plant growth regulation treatments for *S. rebaudiana*.

Treatment	**Details**	**Researchers**
Thidiazuron (TDZ) 0.5 mg/L	Increasing number of shoots (3.00 ± 0.57), shoot length (2.20 ± 0.11 cm) and number of leaves (33.00 ± 2.88) in *S. rebaudiana* in 21 days. This induced a better response than 6-benzyl adenine (BA) in shoot regeneration.	Singh and Dwivedi, 2014
Kinetin (Kn) 9.3 μM + Adenine sulphate 40 mg/L	Best shoot proliferation (maximum numbers of shoots: 65 shoots/ explant) in *Stevia.* In 7 days the emergence of shoot buds was observed in nodal explants after inoculation. Results suggest that kinetin, combined with Adenine sulphate, improves the process of organogenesis. Adenine sulphate is known to enhance natural cytokinin biosynthesis.	Khan *et al*., 2014
6-benzyl adenine (BA) + Indole-3-acetic acid (IAA)	The synergistic effect of these two hormones at concentrations of 1 mg/L + 0.5 mg/L respectively, were shown to give the best results for shoot induction when observed in 40 days, i.e.: shoot tip explant (16.20 ± 0.37) and nodal explant (14.00 ± 0.31)	Anbazhagan *et al*., 2010
BAP (0.5mg/L) + Kn 0.25 mg/L	Highest shoots were observed in MS medium supplemented with 0.5 mg/L BAP and 0.25 mg/L Kn (7.82 ± 0.7) after four weeks of cultivation. They suggested in their publication that Kn is less effective at inducing multiple shoots when compared to BAP.	Razak *et al*., 2014

BA (1.5 mg/L) + GA_3 (0.5 mg/L)	Produced 90% shoot production in *S. rebaudiana*	Khalil *et al*., 2014
6-benzyladenine (BA) (2 mg/L) + 2,4-dichlorophenoxyacetic acid (2,4-D) (2 mg/L)	Best callus induction on 84.6% of the explants	
6-Benzyle adenine (BA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) both at 2.0 mg/L).	Leaf explants were placed on MS medium and exposed to various spectral lights to obtain the best callus induction. The control light at 16/8 hours produced best results of 92.73% callogenic response.	Ahmad *et al*., 2016

As glycoside profiles are subject to change due to several reasons such as geographic area, state of plant maturity, environmental conditions as well as from harvesting and processing, a number of techniques have been used in the past to determine and quantify steviol glycosides. Some of the common methods include liquid chromatography (LC), capillary zone electrophoresis, bi-dimensional ultra-high performance liquid chromatography-ultraviolet detector (UHPLC-UV), micellar kinetic capillary electro-phoresis, nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) with mass spectrometry. In all the above mentioned approaches, LC coupled with mass spectrometry (MS) or ultraviolet (UV) detection is the most preferred approach in quantifying individual steviol glycosides (Montoro *et al*., 2013). Head space-solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) on the other hand, has been used on many compounds for the extraction of volatile compounds and semi-organic compounds from environmental, biological and food samples (Vas and Vékey, 2004). However, to cultivate *Stevia* in southern Africa a region with vast weather and soil variation, and experiencing drought and many other abiotic stresses, we need to understand the requirements to grow it as a crop. It becomes important to study the effect of these different environmental stresses and look at the impact of the biochemicals associated with *S. rebaudiana*.

2.8 Phytochemical and nutritional constituents of *S. rebaudiana*

Plants are an important source of active natural products which contain a wide range of structural and biological properties. *S. rebaudiana* contains over one hundred chemicals which have been identified (Ranjan *et al*., 2011).

2.8.1 *Ent*-kaurene diterpene glycosides

Ent-kaurene diterpene glycosides are also termed steviol glycosides which are tetracyclic diterpenes which originate from a similar kaurenoid precursor as gibberellic acid (Brandle and Telmer, 2007; Yadav *et al*., 2011). The diterpene known as steviol is an aglycone of the sweet glycosides found in *S. rebaudiana*. Stevioside has a chemical formula of C_{38} H_{60} O_{18} and can be converted by hydrolytic cleavage into a sugar (glucose in this case) and a non-sugar component referred to as an aglycone (Khattab *et al*., 2015; Lemus-Mondaca *et al*., 2012). There are over

eight *ent*-kaurene diterpene glycosides found in the *Stevia* leaf namely: stevioside, rebaudioside A-F, steviolbioside and dulcoside (Brandle and Telmer, 2007; Geuns, 2003; Lemus-Mondaca *et al*., 2012; Pezzuto *et al*., 1985; Yadav *et al*., 2011). These zero-calorie *ent*-kaurene diterpene glycosides are the major constituents in the leaves and when ingested are not metabolized to produce energy (Bondarev *et al*., 2010; Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Thomas and Glade, 2010). The thermostable diterpene glycosides present in *S. rebaudiana* leaves include stevioside, rebaudioside A-F, dulcoside A and B, steviolbioside, steviol, isosteviol and dihydroisosteviol (Chatsudthipong and Muanprasat, 2009; De Oliveira *et al*., 2004; Lemus-Mondaca *et al*., 2012; Liu *et al*., 2010; Mondal *et al*., 2012). Stevioside is the predominant sweetener which is 300 times sweeter than sucrose (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006). Rebaudioside A is 400 times sweeter than sucrose and is the second most abundant sweetener with a superior taste quality than stevioside (Braz de Oliveira *et al*., 2011; Liu *et al*., 2010).

The stevioside chemical structure was shown to be an *ent*-kaurene diterpene glycoside in 1952 (Lemus-Mondaca *et al*., 2012). It is the most abundant diterpene glycoside in all eight *ent*-kaurene diterpene glycosides. It is a white, crystalline odourless powder that contains an aglycone which is a steviol moiety and three molecules of glucose and differs in the residues of carbohydrate found in position C13 and C19, this depends on the number of saccharides present and whether these saccharides contain glucose or rhamnose (Figure 2.2) (Gasmalla *et al*., 2014; Khattab *et al*., 2015; Lemus-Mondaca *et al*., 2012). Steviol is the most common aglycone backbone of the sweet steviol glycosides (Chatsudthipong and Muanprasat, 2009; Lemus-Mondaca *et al*., 2012). All these diterpene glycosides found in *S. rebaudiana* have a β-glucopyranosyl linkage in their structures and are known to belong to the *ent*-13-hydroxykaur-16-en-19-oic acid (Chaturvedula and Prakash, 2011; Montoro *et al*., 2013). Stevioside accounts for 4-13% (w/w) of the dried *S. rebaudiana* leaves (Table 2.2) (Lemus-Mondaca *et al*., 2012; Yadav *et al*., 2011). It is responsible for the undesirable bitter aftertaste, which can be described as a 'licorice' taste which is not enjoyed by the majority of the people and tends to decrease its acceptability (Brandle and Telmer, 2007; Khattab *et al*., 2015; Singh and Rao, 2005; Yadav *et al*., 2011). In many countries studies are underway to improve the taste of stevioside (Singh and Rao, 2005). It has been reported that this undesirable aftertaste can be rectified by enzymatic modification of stevioside by pullanase, isomaltase, β-galactosidase or dextrin saccharase

(Chatsudthipong and Muanprasat, 2009; Lemus-Mondaca *et al*., 2012; Liu *et al*., 2010). The synthetic conversion of stevioside to rebaudioside to eliminate the bitter aftertaste is an alternative method used to combat the lingering aftertaste (Singh and Rao, 2005). Equal portions of stevioside and rebaudioside A are reported to also eliminate the risk of any undesirable aftertaste (Yadav *et al*., 2011). Amino acids such as $_{L}$-alanine and glycine have also been reported to be effective in reducing the negative aftertaste of stevioside by reducing non-enzymatic browning which is a result of the Maillard reaction (Khattab *et al*., 2015). Stevioside and its derivatives are reported to hinder aphid feeding of the aerial parts of *S. rebaudiana* plants, which suggests action as chemical defense against herbivore predators (Brandle and Telmer, 2007; Ramesh *et al*., 2006).

Rebaudioside A is the second abundant (2-4% w/w) diterpene glycoside found in the leaves of *S. rebaudiana* (Lemus-Mondaca *et al*., 2012; Yadav *et al*., 2011). It contains a superior taste than all the *ent*-kaurene diterpene glycosides present in *Stevia*. This is due to the extra glucose unit it possesses (Figure 2.3 A) as compared to the three stevioside possesses (Figure 2.3 F) (Chatsudthipong and Muanprasat, 2009; Kumar *et al*., 2012; Lemus-Mondaca *et al*., 2012; Liu *et al*., 2010; Yadav *et al*., 2011). Moreover, due to similar chemical structures of the steviol glycosides, the purification of rebaudioside A is a difficult task. Due to factors like genotype and the environment, the concentrations of the steviol glycosides may differ (Brandle and Telmer, 2007). For instance, in rebaudiosides the sweetness of the glycoside is dependent on the number of sugars attached to the aglycone (differential glycosylation), which means an increase in the number of sugars results in the increase in sweetness (Brandle and Telmer, 2007; Lemus-Mondaca *et al*., 2012). Although this may be true, it has a negative effect on the amount of the rebaudioside level present in the plant (Brandle and Telmer, 2007). Unlike stevioside with its aftertaste, rebaudioside A has a pleasant flavour and is of particular interest due to desirable sweetness (Singh and Rao, 2005; Yadav *et al*., 2011). However, stevioside is still considered the main glycoside which is a sugar substitute and commercial sweetener (Chatsudthipong and Muanprasat, 2009). Both diterpene glycosides can be degraded into their aglycone steviol by rat intestinal microflora (Gardana *et al*., 2003); and, in pigs stevioside is also completely degraded into steviol (Geuns, 2003). However, degradation of these compounds is not possible in the digestive enzymes from the gastro-intestinal tract of man and a variety of animals (Gardana *et al*., 2003; Geuns, 2003).

Other diterpene glycosides namely dulcoside A (Figure 2.3 E) and B, rebaudioside B-F, steviolbioside, isosteviol and dihydroisosteviol are of minor concern as they occur is minute concentrations (1-2%) in the leaves (Table 2.2) (Kumar *et al*., 2012; Montoro *et al*., 2013). It is reported that steviolbioside and rebaudioside B (Figure 2.3 B) are not true constituents of *S. rebaudiana* but rather are formed by partial hydrolysis during the extraction process (Khalil *et al*., 2014). The rest of the steviol glycosides (besides stevioside and rebaudioside A) are for taste, as they remain undigested with no nutritional value (Geuns, 2003; Lemus-Mondaca *et al*., 2012; Yadav *et al*., 2011). A comparison in the sweetness of sucrose to the rest of the diterpene glycosides found in *S. rebaudiana* leaves is reported in several articles. Table 2.2 shows the differences in structure and a comparison in sweetness between the steviol glycosides and to sucrose (Chatsudthipong and Muanprasat, 2009; Geuns, 2003; Lemus-Mondaca *et al*., 2012). Changes in the composition of steviol glycosides present in the plant may occur. This may be a result of the geographic area that the plant is growing in, the level of plant maturity, environmental conditions, harvesting and processing of the plant (Montoro *et al*., 2013; Ramesh *et al*., 2006).

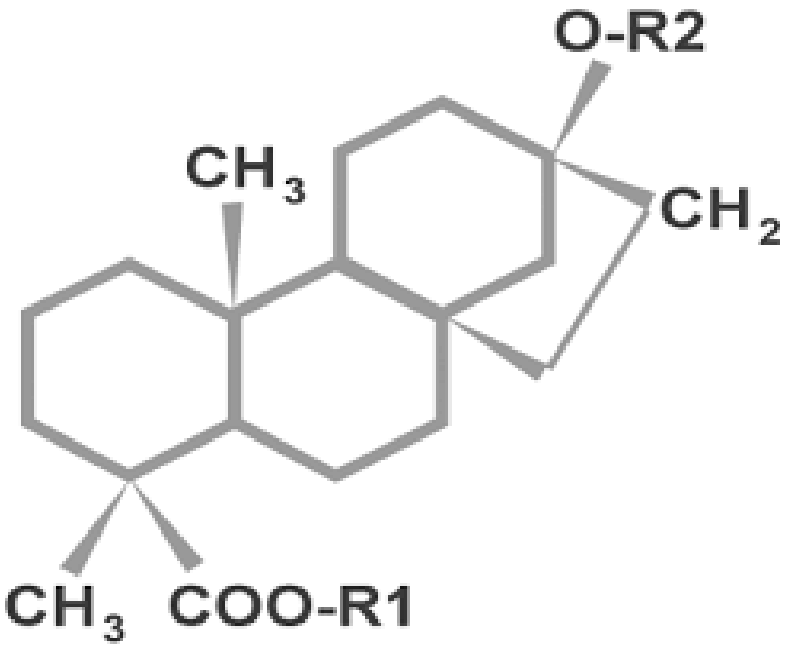

Figure 2.2 Similar backbone structure (steviol) of *S. rebaudiana* which differs in the residues of carbohydrates in C13 (R1) and C19 (R2). Adapted from Ashwell (2015).

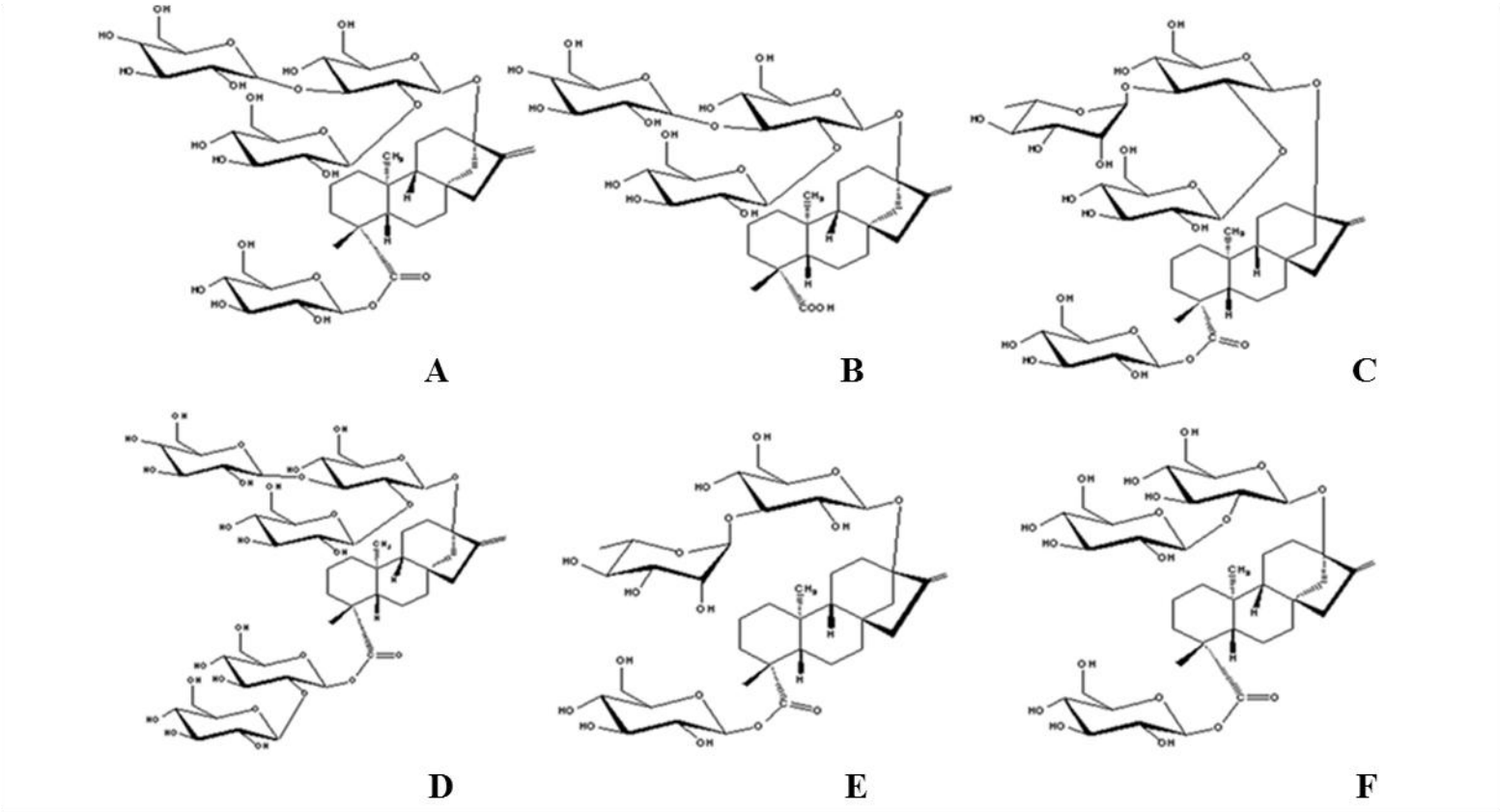

Figure 2.3 Chemical structures of the steviol glycosides present in *S. rebaudiana*: **A**. Rebaudioside A. **B**. Rebaudioside B. **C**. Rebaudioside C. **D**. Rebaudioside D. **E**. Dulcoside A and **F**. Stevioside (Soufi *et al*., 2016).

Table 2.2 Structure of the major glycosides of *S. rebaudiana* leaves. Gly, Xyl and Rha represent, respectively, glucose, xylose, and rhamnose sugar.

Compound name	**R1**	**R2**	**Sweetness intensity: compared to sucrose**
Steviol	H	H	
Steviolbioside	H	β –Glc- β –Glc (2→1)	100-125 times
Stevioside	β-Glc	β–Glc- β –Glc (2→1)	250-300 times
Rebaudioside A	β –Glc	β–Glc- β –Glc (2→1) | β –Glc (3→1)	250-450 times
Rebaudioside B	H	β –Glc- β –Glc (2→1) | β –Glc (3→1)	300-350 times
Dulcoside B (Rebaudioside C)	β –Glc	β-Glc-α-Rha (2 →1) | β –Glc (3→1)	50-120 times
Rebaudioside D	β –Glc- β –Glc (2→1)	β–Glc- β –Glc (2→1) | β –Glc (3→1)	250-450 times
Rebaudioside E	β –Glc- β –Glc (2→1)	β –Glc- β –Glc (2→1)	150-300 times
Rebaudioside F	β –Glc	β-Glc-β-Xyl (2 →1) | β –Glc (3→1)	150-300 times
Dulcoside A	β –Glc	β-Glc-α-Rha (2 →1)	50-120 times

Adapted from Geuns (2003); FAO (2007) and Lemus-Mondaca *et al*. (2012). .

2.8.2 Glycoside content in different plant parts

S. rebaudiana contains more than eight steviol glycosides which are responsible for its edulcorant properties in different plant parts. There is a greater amount of steviol glycoside content in the leaves of *S. rebaudiana* compared to the rest of the other organs (flowers, stem, seeds and roots) (Singh and Rao, 2005; Yadav *et al*., 2011). This is important as the leaves are the major sweet bearing parts of the plant and high ratios of cultivated *S. rebaudiana* leaves represent a high glycoside content which is more desirable (Ghosh *et al*., 2008; Singh and Rao, 2005). Bondarev and company (2010) reported that the roots contain less than 0.1% steviol glycoside content while Yadav *et al*. (2011) stated that absolutely no steviol glycosides is found in the roots of *S. rebaudiana*. The reason for this is linked to chloroplasts as the main organelle that is important in precursor synthesis. In summary, parts such as stems and roots have lowered to no synthesis of steviol glycosides (Singh and Rao, 2005).

The leaves are suggested to serve as the main tissue for both synthesis and primary accumulation of stevioside compounds (Singh and Rao, 2005; Yadav *et al*., 2011). The plant is an obligate short-day plant and long-days sustain vegetative growth with significant increase in the leaf biomass and steviol glycoside content (Ceunen and Geuns, 2013; Ghosh *et al*., 2008; Singh and Rao, 2005; Yadav *et al*., 2011). *S. rebaudiana* is also influenced by temperature variability. Its yield develops well between 15-30 ºC and it can occasionally tolerate temperatures between 0-3 ºC (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Singh and Rao, 2005). Yadav *et al*. (2011) reported that the ideal climate for *Stevia* consists of semi-humid subtropical temperatures which range from -6 to 43 ºC, with an average of 23 ºC. There is a difference in steviol glycoside content between the upper and lower leaves of *S. rebaudiana*, this occurs during the long days of vegetative growth. The upper leaves gradually increase accumulation of sweet chemicals near the late vegetative stage. Steviol glycoside levels can be approximately four times greater than the lower leaves (Ceunen and Geuns, 2013).

The lower mature leaves of *S. rebaudiana* have fewer glands per leaf area than the upper, younger leaves, thus a positive correlation between gland distribution density and steviol glycoside level exists (Bondarev *et al*., 2010). This means that a greater amount of steviol glycoside is present in the younger upper leaves than the lower leaves (Bondarev *et al*., 2010).

However, Yadav *et al*. (2011) argue that the steviol glycosides tend to accumulate in the tissues as the plant matures resulting in the older leaves becoming sweeter than the younger upper leaves. During the flowering stage, steviol glycoside levels decrease as compared to the late vegetative phase or flower budding. Plants grown under short-day conditions were reported to show significant variation in steviol glycoside levels only during the flowering phase when apical leaves showed significant decline in steviol glycoside level compared to lower leaves (Ceunen and Geuns, 2013). The stems of *S. rebaudiana* plants have little steviol glycoside content and it has been reported that as the stem matures they lose colour and the little steviol glycosides remaining are also lost (Singh and Rao, 2005; Yadav *et al*., 2011). However, they are reported to contain some flavour enhancers, odourisers and other agents which can be used in improving food stuffs or alcoholic beverages (Singh and Rao, 2005).

2.8.3 Biosynthetic pathway of steviol glycoside using 2-C-methyl-D-erythritol-4 phosphate (MEP) pathway

The biosynthetic pathway of steviol glycosides has been of interest to many researchers and steviol glycosides are synthesized through the 2-C-methyl-D-erythritol-4 phosphate (MEP) as pathway (Figure 2.4) (Geuns, 2003; Kumar *et al*., 2012; Totté *et al*., 2000). However, researchers were not able to conclude on the involvement of mevalonate (MVA) pathway. It was later shown by Madan *et al*. (2010) in steviol biosynthesis. The MEP pathway involves the synthesis of 1-deoxy-D-xylulose-5-phosphate (DXP) by condensation of pyruvate and D-glyceraldehyde 3-phosphate. This step is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Geuns, 2003; Kumar *et al*., 2012; Yadav *et al*., 2011). The DXP is later rearranged and catalyzed by the enzyme 1-deoxy-D-xylulose-5-phosphate reducto-isomerase (DXPR) through NADPH dependent reduction to form MEP (Geuns, 2003; Kumar *et al*., 2012; Totté *et al*., 2000). A great deal still needs to be done concerning the initial steps of steviol biosynthesis. Yadav *et al*. (2011) reported that dimethylallyl diphosphate (DMADP) is not a committed precursor of isopentenyl diphosphate (IDP), that synthesis may have risen from different synthesis routes. However, Kumar *et al*. (2012) indicated that the conversion of MEP to isopentenyl diphosphate (IPP) and DMADP occurs in these plants. This step is facilitated by five enzymes namely: 4-(cytidine5' diphospho)-2-C-methyl-D-erythritol synthase (MCT), 4-(cytidine5' diphospho)-2-C-methyl-D-

erythritol kinase (CMK), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS), (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS), and (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) which are encoded by *MCT*, *CMK*, *MDS*, *HDS* and *HDR* genes respectively (Brandle and Telmer, 2007; Kumar *et al*., 2012; Liao *et al*., 2016).

One molecule of DMADP condenses with three molecules of IDP with the help of geranylgeranyl diphosphate synthase to form an essential intermediate of biosynthesis for most isoprenoids which is termed geranylgeranyl diphosphate (GGDP) (Brandle and Telmer, 2007; Kumar *et al*., 2012; Yadav *et al*., 2011). This essential intermediate is further cyclisized to (-)-copalyl diphosphate (CDP) by (-)-copalyl diphosphate synthase (CDPS). This is followed by the production of (-)-kaurene from CDP by ionization-dependent cyclization which is catalyzed by (-)-kaurene synthase (KS). This rearrangement leads to an *ent*-kaurene which is subsequently oxidized by kaurene oxidase (KO) at the C19 position forming an *ent*-kaurenoic acid (Brandle and Telmer, 2007; Kumar *et al*., 2012; Totté *et al*., 2000; Yadav *et al*., 2011). The *ent*-keurenoic acid at position C13 is hydroxylized by the enzyme kaurenoic acid hydroxylase to form steviol. The two oxygenated functional groups of steviol namely C13 alcohol and C19 carboxylate are then able to present attachment sites for sugar side chains that establish the characteristics of the different glycosides.

The C13 alcohol undergoes glucosylation forming firstly steviol-monoside followed by steviol-bioside. While C19 carboxylate is also glucosylated to form stevioside, this stevioside undergoes glucosylation to form rebaudioside A which is where the pathway ends (Brandle and Telmer, 2007; Kumar *et al*., 2012; Totté *et al*., 2000; Yadav *et al*., 2011). The inclusion of the uridine diphosphate (UDP) rhamnose moiety to steviol-monoside increases the chances of obtaining rhamnosylated glycosides (Yadav *et al*., 2011).

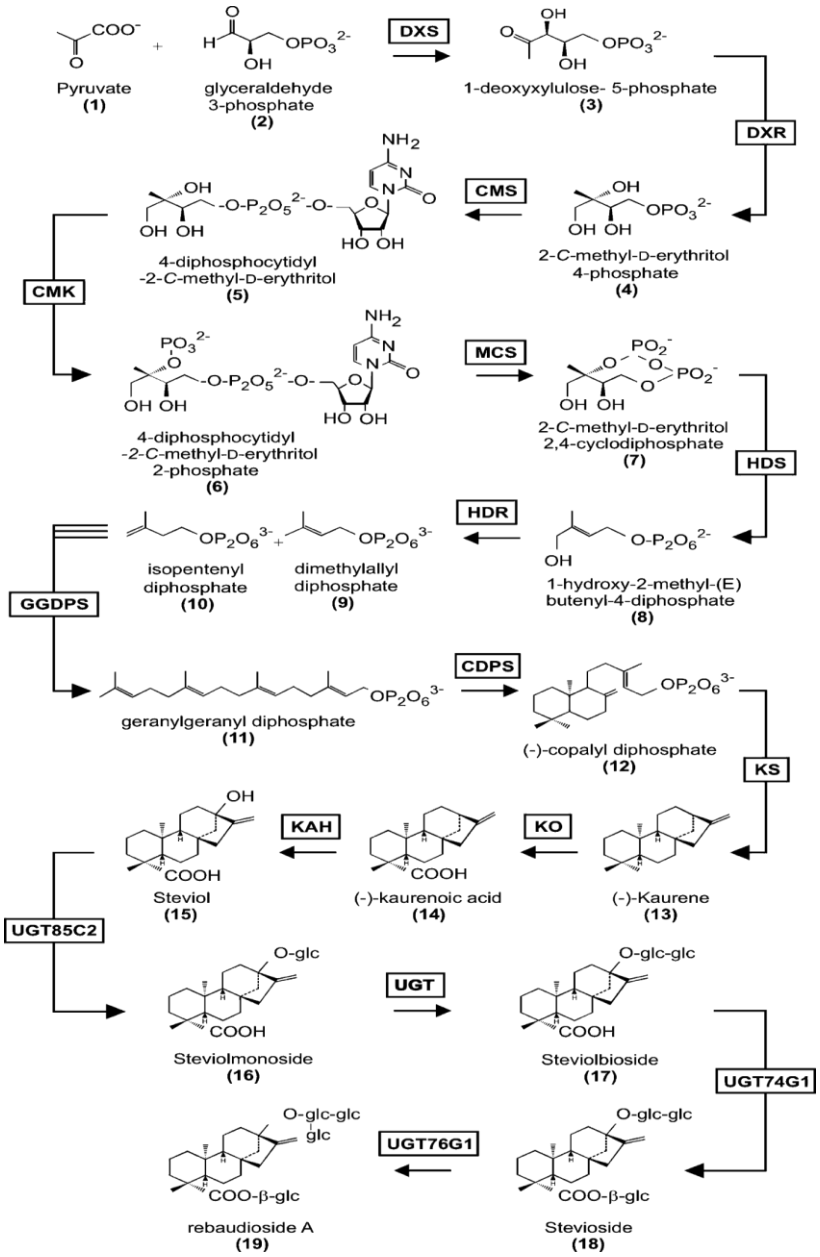

Figure 2.4 Steviol glycoside biosynthesis (Brandle and Telmer, 2007).

2.9 Other constituents found in *S. rebaudiana*

In addition to sweet chemicals, *Stevia* extract also contains other constituents such as carbohydrates, proteins, lipids, flavonoids, sterebins A and H, triterpenes, volatile oil components, pigments, cinnamic acids, coumarins, phenylpropanoids and inorganic constituents (Lemus-Mondaca *et al*., 2012; Yadav *et al*., 2011).

2.9.1 Carbohydrates, proteins and lipids

S. rebaudiana is a good source of carbohydrates, crude fibre and it reduces the risk of certain diseases (Lemus-Mondaca *et al*., 2012). The leaves and roots of *Stevia* contain natural occurring polysaccharides known as fructooligosaccharides (fructans) which may be used as dietary supplements. These are suggested to play a role in prebiotics, dietary fibre, lipid metabolism and diabetes control (Braz de Oliveira *et al*., 2011).

The sweet leaf contains sixteen amino acids comprising of essential and non-essential amino acids which match the protein requirements recommended by the World Health Organization (Lemus-Mondaca *et al*., 2012). These include glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine, methione, phenylalanine, leucine, valine, threonine and histidine.

Using methyl ester standards, Tadhani *et al*. (2007) identified 6 fatty acids in *S. rebaudiana* namely: palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids. Three of these fatty acids namely palmitic, linolenic and linoleic acid were found at high significant amounts with 27.51, 21.59 and 12.40 g 100 g^{-1} respectively. Palmitic acid is one of the most common saturated fatty acids produced during lipogenesis from which longer fatty acids can be produced. It contains mild anti-oxidant and anti-atheroscerotic properties proven in animal studies. However, high intake of this fatty acid increases the risk of cardiovascular diseases. *S. rebaudiana* leaf oil is a rich source of linolenic acid and is essential to the human diet. It belongs to the class of omega 3 fatty acids and is a nutrient essential to the formation of prostaglandins in the human body. Linoleic acid is an unsaturated fatty acid which is also considered important for the human diet as the body cannot synthesize linoleic acid from other foods. This fatty acid is mostly used in the beauty industry for its beneficial skin properties.

2.9.2 Minerals and vitamins

S. rebaudiana contains substantial amounts of both macronutrients such as sodium, magnesium, phosphorus, sulphur, chlorine, potassium, calcium and micronutrients such as chronium, manganese, iron, cobalt, copper, zinc, selenium, molybdenum and iodine which are imperative for growth, health and reproduction. They are also involved in the formation of cells, tissues and organs (Lemus-Mondaca *et al*., 2012). The plant contains both macro- and micronutrients which are required for plant physiological processes, growth and steviol glycoside productivity (Ramesh *et al*., 2006). Since the plant may be found in poor soils which have a low acid pH (Lemus-Mondaca *et al*., 2012), micronutrients such as boron, copper, iron, chloride, manganese, molybdenum and zinc are obtained in adequate amounts (Ramesh *et al*., 2006). While macronutrients are required in greater amounts as they are essential for the growth and health of the plant. A lack or reduction in the amount of macronutrients such as nitrogen, phosphorus, calcium, sulphur and potassium, results in a number of deficiencies in the plant. Extreme cases of calcium deficiency in *S. rebaudiana* result in a decline in glycoside concentrations. While a reduction in all these macronutrients excluding phosphorus, cause a decrease of stevioside concentration in the leaves of the plant. This decline in the predominant sweetener accounts for 3-8% (w/w) of dried *S. rebaudiana* leaves; resulting in fluctuations in its sweet property (Ramesh *et al*., 2006). Deficiencies in calcium, magnesium and sulphur exhibit unfavourable symptoms which eventually hinder the trade of the plant. These symptoms include apical necrosis, chlorosis, and inverted "V" shaped necrosis and pale green leaves respectively. *S. rebaudiana* contains significant amounts of these organic compounds namely: vitamin C, vitamin B2 and folic acid (Lemus-Mondaca *et al*., 2012).

2.10 Safety concerns and non mutagenic effects

The long history associated with *Stevia* use by the Guarani Indian tribes (1500 years) alludes to its safety as a sweetener (Laura *et al*., 2006; Singh and Rao, 2005; Yadav *et al*., 2011). In Japan, *Stevia* has been used since the 1970s, with no reported negative effects (Singh and Rao, 2005; Yadav *et al*., 2011). For many years, consumers in Japan and Brazil, where *Stevia* has long been approved as a food additive, have been using *Stevia* extracts as non-caloric sweeteners. It was previously reported that 40% of the artificial sweetener market in Japan is *Stevia* based and that

Stevia is commonly used in processed foods in Japan (Lester, 1999). More recent reports of consumption figures for *Stevia* reveal pronounced increases in global consumption. *Stevia* has been under scrutiny in official laboratories like that of Pomaret *et al*. (1931) since the 1930s, yet in all this time no negative evidence regarding the safety of its use has been found. It has however been reported that *Stevia* is not absorbed in the gastro-intestinal tract of the human body thus posing no threat (Brandle and Telmer, 2007; Singh and Rao, 2005; Yadav *et al*., 2011). It has been approved by the Food and Drug Administration in America and Canada as a food supplement (Khalil *et al*., 2014; Lemus-Mondaca *et al*., 2012; Razak *et al*. 2014). In order for their uses in conventional foods, dietary supplements must undergo premarket approval by the FDA as food additives or, alternatively, the ingredients must be determined to be generally recognized as safe. For high consumers, revised European exposure estimates to steviol glycosides remain above the established acceptable daily intake (ADI) of 4 mg/kg bw (steviol equivalent). From the safety perspective, there was limited data concerning the safety of *Stevia*. In the past, this included concerns on renal toxicity, effects on glucose metabolism, and fertility. As a result, its use as a sweetener in many countries like the USA by the FDA was declined and could only be used as a dietary supplement. It has only been over the last two decades that safety of steviol glycosides have been extensively investigated by employing comprehensive and modern toxicology protocols using scientifically accepted dosing regimens of purified and standardized test substances (FAO, 2007). Additional studies report that human digestive enzymes are not capable of hydrolyzing β-glycosidic bonds, and, thus, steviol glycosides are not digested in the upper gastrointestinal tract. Steviol is absorbed but is rapidly converted to glucuronides which are subsequently excreted in the urine or eliminated by the enterohepatic circulation. *Stevia* and steviol glycosides have been extensively investigated for their biological, toxicological, and clinical effects (Geuns, 2003; Huxtable, 2002). Additionally, the national and international regulatory agencies have thoroughly reviewed the safety of *S. rebaudiana* and its glycosides.

S. rebaudiana derivatives have been proved to be non-mutagenic in several mutagenicity studies done on several strains of *Salmonella typhimurium, Escherichia coli* and *Bacillus subtilis* (Geuns, 2003; Pezzuto *et al*., 1985). During the 1980's, *Stevia* extracts were banned by the FDA from health foods as insufficient information affirming safety as a food additive (Shivanna *et al*.,

2013; Singh and Rao, 2005) was available. The United States of America only used it as a dietary supplement and as a skincare product (Geuns, 2003; Lemus-Mondaca *et al*., 2012; Singh and Rao, 2005). Many have argued that the long term effects of its consumption as a sweetener on a large scale have not been well studied (Singh and Rao, 2005).

While over the years extensive research has been done on its toxicity, no direct evidence has surfaced on the toxicity of stevioside and steviols in terms of carcinogenic, mutagenic and fertility effects. Studies in the oral toxicity of stevioside revealed very low acute oral in mice, rats and hamsters (Geuns, 2003; Pezzuto *et al*., 1985). The Japanese played a great role in its commercialization and its acceptance in the food industry and ethnobotanical world (Carbonell-Capella *et al*., 2013; Ramesh *et al*., 2006).

2.11 Industrial uses

Japan has been instrumental in its commercial exploitation as researchers there developed a process of extraction and refinement of its components (Braz de Oliveira *et al*., 2011; Yadav *et al*., 2011) which could serve as a great alternative to synthetic sweeteners. It is stable in a number of different pH levels (Lemus-Mondaca *et al*., 2012; Singh and Rao, 2005; Suttajit *et al*., 1993); thus has been frequently used in Japan, Korea, Brazil and in a number of other countries to sweeten soft drinks, fruit drinks or as an alcoholic beverage enhancer. It has also been used in other foods to serve as a sugar substitute such as soy sauce, jams, pickles, yogurts, deserts, cold confectionery, jellies, sauces, delicacies, for example (Liu *et al*., 2010; Pezzuto *et al*., 1985; Singh and Rao, 2005; Tadhani *et al*., 2007). It is thermostable (enduring temperatures of up to 200 ºC without browning and caramelization (Geuns, 2003; Lemus-Mondaca *et al*., 2012)) and thus can be used to cook or bake foods like sweet corn, breads, candies, biscuits, in making chewing gum, preparing sea foods and vegetables. It is popular for use in weight control and diabetic diets, skin creams, toothpaste, mouthwashes, hypertensive treatment, as a tobacco additive and flavourant or just as a table-top sweetener (Lemus-Mondaca *et al*., 2012; Liu *et al*., 2010; Singh and Rao, 2005; Tadhani *et al*., 2007).

2.12 Artificial sweeteners

The search for alternatives to sucrose continue as high levels of sucrose are associated with many health risks (nutritional and medical) such as cardiovascular disease, weight gain, development of obesity, diabetes and dental caries (Gasmalla *et al*., 2014; Pradhan *et al*., 2014; Ventura and Mennelia, 2011). Artificial sweeteners are listed as either nutritive (mono- and disaccharides polyols) or non nutritive sweeteners depending on whether they are a source of calories (Whitehouse *et al*., 2008). These artificial sweeteners were at one point suggested to have some carcinogenic effect (Gasmalla *et al*., 2014; Weihrauch and Diehl, 2004). It was reported that large doses of artificial sweeteners greater than 1680 mg per day had a potential risk of producing bladder cancer in human (Weihrauch and Diehl, 2004). In general, acceptance of *Stevia* based chemicals is now a norm.

2.13 Aims and objectives

S. rebaudiana is a great alternative to sugar. It has become popular and this is likely to increase worldwide (www.mintel.com). The use of *S. rebaudiana* since its availability in South Africa is currently at a minor scale as products are mainly stocked by health shops and naturopathic stores as an extract. Availability of products with purified steviol glycosides is largely associated with the launch of the Canderel Green Range (http://ilovecanderel.blogspot.com) in 2012. This followed the success of these products in Europe. Brands such as Truvia which are now worth over 4 million Euros in Europe have led to a growing market at a rate of 18% every year. In 2005, it was estimated that sales of *Stevia* in the US reached 45 million dollars (The Food Institute Report, 2006). *Stevia* is forecast to grow to 275 million dollars by 2017 (www.mintel.com). However, it is not propagated on a commercial scale in southern Africa. Southern Africa has an opportunity to cultivate *S. rebaudiana* as a new crop for the agricultural sector. The skills to produce *Stevia* on a commercial scale are also available in South Africa. Tissue culture is a quick means of assessing environmental conditions required and perhaps in the future the data accumulated from this project will be used and tested in the field. The global demand presents a new opportunity for the South African economy. Thus, establishing a protocol for improving seed germination would be useful in the commercial production of *S. rebaudiana*.

The fulfillment of this project would be as a result of several key aims and objectives which included:

1. To establish a protocol to determine the best treatment for optimal seed germination using acid scarification, smoke-water, a combination of acid scarification and smoke-water and gibberellic acid.

2. To study the macronutritional requirements of *S. rebaudiana* plants utilizing nitrogen and phosphate manipulation *in vitro*.

3. To determine if *in vitro* derived plant extracts differ in metabolite profiles regarding the main bio-actives (diterpene glycosides) using a metabolomic approach that involved the application of LC-MS and GC-MS technology.

4. To determine the effects of drought and salinity stress on the growth of *S. rebaudiana* using different concentrations of (w/v) polyethylene glycol 6000 (PEG 6000) and sodium chloride (NaCl) as osmotica.

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Chapter Three

3. Effect of smoke, sulfuric acid and gibberellic acid on seed germination of *S. rebaudiana*

3.1 Introduction

Seed germination has an important role in modern agriculture. It is a necessary process for the establishment of a reproductive unit or new plant and thus a fundamental understanding of this adaptive trait is key in yielding greater crop production and for the survival of all plant species. A fast germination rate is also a desire to most agriculturalists as this assists in lowering and (at times) eliminating competition in the establishment of seedlings (Rogers *et al*., 1995). Seed germination may be defined as a process that incorporates those events that commence with the uptake of water by imbibition by the dry seed that has been in a state of quiescence or rest, followed by the expansion of the embryo (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). Seed germination is often defined as the emergence of a young root or radicle through the seed coat. Seed germination and dormancy can thus be termed as complex processes that are influenced by several environmental and genetic factors namely water, gases (oxygen), temperature and for some species light and nitrate (Abdullateef *et al*., 2015; Finch-Savage and Leubner-Metzger, 2006).

Water uptake plays a vital role in breaking coat dormancy. This triphasic process, consists of a first phase that results in the rapid initial uptake of water where imbibition by the dry seed occurs and metabolic activity resumes in the seed , followed by the plateau phase (phase II) when the seed is swollen to its maximum size. This phase is often referred to as the metabolic preparation for germination. In the third phase additional water uptake occurs as the embryonic axes expands to complete germination (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006).

Seed dormancy may be referred to as the failure of an intact viable seed to complete germination under favourable conditions (Bewley, 1997). Dormancy in many cases is observed only in the intact seed in cases where it is coat-imposed. There has been extensive work in the area of seed

dormancy and several dormancy classes were reviewed by Finch-Savage and Leubner-Metzger (2006).

Coat-imposed dormancy refers to embryos that are trapped within surrounding structures such as the endosperm, perisperm or mega-gametophyte (Bewley, 1997) which make it difficult for the radicle to emerge. Weakening of these structures (for example the endosperm) by scarification is necessary for the removal of coat-imposed dormancy. Three types of scarification methods make it possible to break coat-imposed dormancy. Mechanical scarification involves physical abrasion of the testa using various tools; like rubbing the testa with sandpaper to break this type of dormancy. Chemical scarification involves soaking of seeds for a time in some form of concentrated acid solution such as sulfuric acid, hydrochloric acid and even in bleach to break dormancy (Ibiang *et al*., 2012). Heat scarification entails the use of hot water for a certain amount of time to break coat-imposed dormancy.

Physiological dormancy is the most prevalent form of dormancy imposed by a physiological hormonal factor that is internal to the embryo. It involves the hormonal balance of abscisic acid (ABA) and gibberellins (GAs) and their metabolism (Bewley, 1997; Liu *et al*., 2015). Plant hormones play a role in the regulation of dormancy and germination. Abscisic acid and other inhibitory compounds (secondary metabolites) aid in the induction as well as maintaining seed dormancy (Bewley, 1997; Liu *et al*., 2015; Wang *et al*., 1998). Abscisic acid is important for maintaining the developmental and maturation processes of seeds such as the synthesis of storage molecules. Gibberellic acid (GA_3) has the ability to break seed dormancy of many types of seeds. It is suggested that the two hormones (ABA and GA) may have an antagonistic approach (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Wang *et al*., 1998). According to Taiz and Zeiger (2010), gibberellins induce the germination of the seed through the activation of catabolic enzymes that soften the endosperm and also decrease abscisic acid amounts. Embryo-dormancy is maintained at the genetic level through negative suppression of GA-activated gene expression by an ABA-regulated DELLA protein. The DELLA proteins belong to the superfamily of transcription factors which are peculiar to the plant kingdom, known as the GRAS family. These nuclear proteins act as growth repressors throughout the life cycle of higher palnts. The gibberellins promotes plant growth by stimulating degradation of the growth repressor protein called DELLA protein. In the presence of gibberellins, the DELLA

protein is degraded into peptide fragments by the 26S proteosome (Finch-Savage and Leubner-Metzger, 2006; Taiz and Zeiger, 2010). When DELLA repression is lifted, transcription of the gibberellin response genes and subsequent growth occurs (Taiz and Zeiger, 2010).

Apart from gibberellins, smoke has been shown to induce rapid germination of a variety of different species even those that come from environments where fire is not integral to the ecology. In fire-prone environments, plants are well adapted to germinating as a result of smoke. Smoke contains active principles made from the combustion or pyrolysis of plant material that promote the germination of some fire-prone plant species (Ghebrehiwot *et al*., 2011; Papenfus *et al*., 2014; Van Staden *et al*., 2006). It has also been shown to induce seed germination of fire-independent species as well (Ghebrehiwot *et al*., 2011). Smoke is suggested to play the role of plant growth regulators to many species including those that belong to the Asteraceae family (Van Staden *et al*., 2006). Different types of smoke extracts have been published and for instance, smoke water achieved by bubbling plant derived smoke through distilled water has been used as a smoke equivalent and has been shown to directly and indirectly increase seed germination (Papenfus *et al*., 2014; Van Staden *et al*., 2006), depending on the concentration used.

Recently, chemicals that have been isolated from smoke which are reported to be responsible for inducing seed germination are termed karrikins. Karrikins contain the compound karrikinolide (KAR_1) which is suggested to play a vital role in inducing germination in plant derived smoke (Nair *et al*., 2014). Different batches of smoke solutions can differ in the concentration of active compounds (Boucher and Meets, 2003). However, smoke is able to stimulate seed germination at very low concentrations while high concentrations tend to limit germination. Many species respond to smoke as a germination cue, for example: a number of the Everlasting species (Asteraceae family), *Syncarpha vestita*, *Edmondia sesamoides*, *Syncarpha eximia*, and *Helichrysum patulum* to name a few (Brown and Botha, 2004; Brown *et al*., 2003). All these species have horticulture potential. Smoke has also been shown to increase plant vigor, assisting with rapid seedling establishment and growth post germination.

S. rebaudiana seeds are known to possess a very low germination capacity which is a challenge for large scale propagation. Thus, the aim of the work presented in this Chapter was to investigate the viability of *S. rebaudiana* seeds and the effects of using different chemicals; namely: smoke solution, concentrated sulfuric acid, a combination of smoke and sulfuric acid and gibberellic acid were tested as a means of improving germination. Viable seed production is also a general problem and so as part of this Chapter, a viability test using tetrazolium was conducted.

3.2 Materials and methods

3.2.1 Seed material

S. rebaudiana seeds (2 000) were purchased from a commercial seed source company (Ball Straathof, Johannesburg) in July 2013. Upon arrival, these were stored in the dark at room temperature for a period of two months before germination experiments were conducted.

3.2.2 Viability test

A total of 100 *S. rebaudiana* seeds were randomly selected from the pure seed lot (2 000 seeds) and 100 seeds were subjected to a viability test using 1% (w/v) 2, 3, 5-triphenyl chloride tetrazolium salt. Each seed represented a replicate. Twenty five seeds were placed per Petri dish and four dishes were used per experiment. Petri dishes were routinely lined with moist filter paper and sealed with laboratory film (Parafilm ‘‘M’’ American National CanTM, USA). These tests were conducted three times. The seeds were first surface-decontaminated using 5% (w/v) calcium hypochlorite (10 minutes) (August 2013), followed by soaking in sterile water for 24 hours. All seeds were treated with 1% (w/v) tetrazolium chloride (TTC). The pH of the solution was adjusted to 7.0 for proper staining to occur, by dissolving 2, 3, 5 triphenyl chloride in a phosphate buffer (26.5 mM KH_2PO_4 and 40 mM $NA_2HPO_4.2H_2O$) solution. For the negative control, 25 seeds were boiled in water for five minutes in order to kill the seeds. All the samples (100 seeds) were then incubated in TTC at room temperature for 24 hours under dark conditions.

To develop the stain, the seeds were soaked in 10 mL of methyl cellosolve for 4 hours with occasional stirring till the extraction of the red coloured formazan was complete. The stained red seeds were counted using a stereomicroscope to determine those that had viable embryos. Seeds were considered viable when 90-100% of the embryo surface was stained red with TTC.

3.2.3 Germination experiment

The effect of four germination treatments; namely: 1) acid scarification, 2) smoke-water, 3) a combination of acid scarification and smoke-water; and 4) gibberellic acid were examined. Firstly, all seed surface-decontamination was achieved by using 3.5% (v/v) sodium hypochloride for 20 minutes; washed three times with distilled water (5 minutes) and then transferred aseptically to either of the four variables listed below. Thereafter, all seeds were transferred to Murashige and Skoog (1962) medium (MS) containing a tenth of the salts (no vitamins), with 0.8% (w/v) agar (agar–agar powder (Merck, Germany). The pH of all media were adjusted to 5.8 using 1 M NaOH or 1 M HCl prior to autoclaving for 20 minutes at 121 ºC at a pressure of 103 kPa. Upon cooling of the medium, 25 mL was poured under laminar flow into each Petri dish (10 cm).

To achieve these experimental variables: 1) seeds were chemically scarified once in 10 mL 70% (v/v) H_2SO_4 for 30 seconds, washed three times with distilled water (5 minutes), blot-dried and then aseptically transferred onto germination medium;
2) for the smoke treatment, the smoke water was filter-sterilized (0.2 µm) as according to Baxter *et al*. (1994) from *Themeda triandra* Forssk. Smoke water was added to a concentration of 10^{-5} (v/v), this medium was then referred to as smoke-medium and the seeds transferred to the medium were referred to as smoked;
3) a combination of chemical scarification and smoke was tested; and
4) gibberellic acid (11.55 µM) was added to the germination medium and the seeds were allowed to grow in its presence.

For all experiments, controls were left untreated. For each treatment 10 Petri dishes, containing 5 seeds were sealed with laboratory film (Parafilm ‘‘M’’ American National CanTM, USA); and

were either transferred to a light growth chamber with standard conditions: 23 ± 2 °C, 16 hours photoperiod, irradiance of 50 mol m^{-2} s^{-1} (photosynthetic photon flux density) at culture stage provided by cool white fluorescent tubes (L75W/20X (F96712) Osram, USA); or placed in total darkness where observations were conducted under a green safe light (0.5 µmol m^{-2} s^{-1}). The experiment was conducted for 21 days. Every three days, a record of the seed germination rate was noted to obtain a repeated measure. A seed was considered to have germinated after 1 mm of radicle emergence. This experiment was repeated five times. For hardening, well developed *in vitro* seedlings were transferred into pots containing autoclaved sphagnum: perlite: sand (1:1:1, v/v) and kept in the glasshouse (Department of Botany and Zoology, Stellenbosch University).

3.2.4 Statistical analyses

For the viability test, data were subjected to one-way analysis using paired sample 'T' test, with SPSS version 16.

All data were subjected to normality testing. Percentage data were arcsine transformed prior to statistical analyses (Statistica version12). The data that were normally distributed, a Tukey's Honesty Significance Difference (HSD) was applied and for data which were not normally distributed, a Krustal-Wallis analysis was applied to separate the means. All data collected over a period of time were subjected to a repeated-measures analysis of variance (ANOVA). The overall effect of variables on the outcome was tested by conducting one-way or factorial ANOVA on the results recorded for the third week after the germination experiment initiation. To compare the smoke treatments with the viability of seeds, a Mann-Whitney test was conducted to prove that the smoke significantly enhanced germination. All statistical analyses were conducted at the 95% confidence level.

3.3 Results and discussion

3.3.1 Viability test

Stevia seeds had a very low viability percentage (Figure 3.1). Only 19% of the tested seeds stained uniformly red, while the rest were deemed to have low viability or were non-viable as they displayed yellow, brown and purple colouring (Figure 3.1) which indicates a lack of an embryo or those embryos that may have been dead (Abdullateef *et al*., 2015).

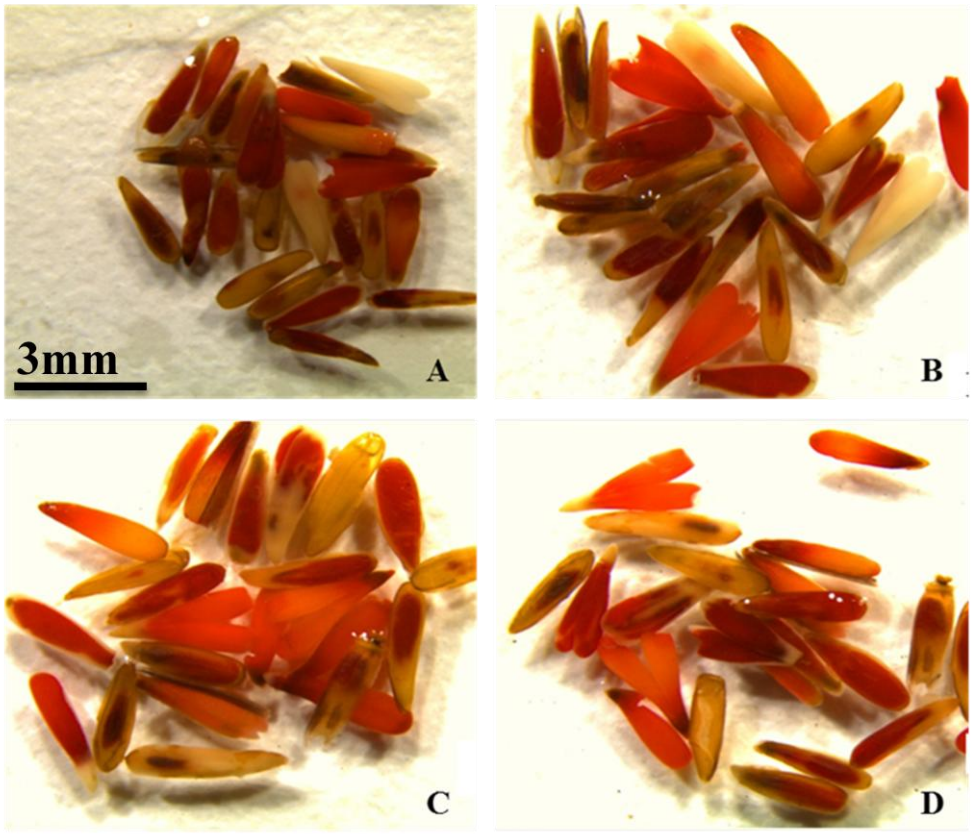

Figure 3.1 *S. rebaudiana* seeds stained with the tetrazolium solution. **A** and **B** show high numbers of brown and non-viable seeds. While **C** and **D** have a number of partially yellow and purple seeds which represents weak to non-viable seeds. **C** and **D** also show higher number of red viable seeds.

3.3.2 *S. rebaudiana*: its germination rate

All seeds started to germinate at day three. Although this plant does not come from a fire-prone environment the use of smoke treatment was highly efficacious in producing a higher germination percentage in *S. rebaudiana* seeds (Figure 3.2 B). This result is similar to many other species whose germination is improved by smoke albeit they have not evolved in environments where fire is an important factor for their ecology. Seeds that had gone through acid scarification for thirty seconds with 70% (v/v) H_2SO_4 showed the least germination compared to all the treatments (Figure 3.2 B). There was great variation in the germination rates of black *Stevia* seeds. Overall, the germination percentage was low (15%). This germination frequency is lower than that reported by Goettemoeller and Ching (1999) using black seeded *Stevia* which were recorded at 36.3%. Raina *et al*. (2013) showed that germination tests using black seeds had a higher germination percentage of 85% and tan colored seeds were completely sterile.The seed type appears to have an influence on *Stevia* germination as black seeds exhibited higher germination rate of approximately 76.7% than tan seeds which had less than a rate of 10% (Goettemoeller and Ching, 1999). Abdullateef *et al*. (2015) obtained a germination frequency using black *Stevia* seeds of 67.33% to enhance seed germination in *S. rebaudiana*. They were able to achieve such a high germination percentage acclimatized conditions. The seed germination apparatus comprised of a planting tray where the seeds germinated, peat moss, which served as the sowing medium, plastic dome to house the planting tray, light chamber affixed with two red fluorescent tubes to supply the light, watering can was used for spraying water over the peat moss, timer switch to regulate period of light exposure (red light), which was 7am to 9pm, air conditioner, to regulate the temperature at 24 ºC.

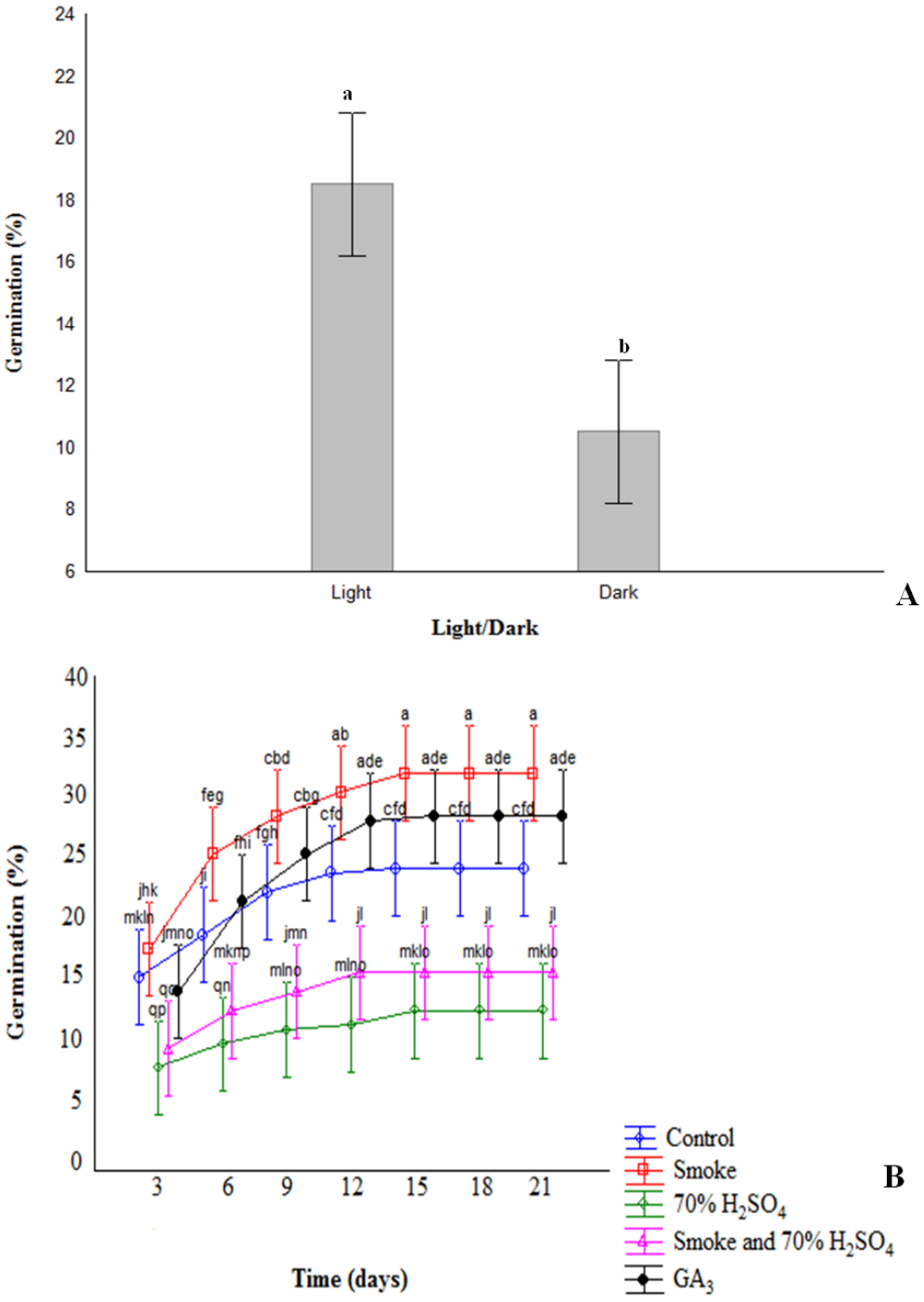

Figure 3.2 **A**. The effect of light and dark in *in vitro S. rebaudiana* seed germination on $^{1}/_{10}$ MS medium independent of time. Light treatments resulted in high levels of seed germination. **B**. The effect of treated *S. rebaudiana* seeds over a 21 day period irrespective of exposure to light or darkness. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

Light is a primary energy source for plants, it controls multiple developmental processes in the plant life cycle which includes seed germination (Li *et al*., 2011). A number of plants especially small seeds like that of a lettuce (*Grand rapids*), require light to germinate. This requirement ensures that a tiny seed germinates only if close to the surface and hinders germination when buried deep into the soil. This may be a result of insufficient food reserves to grow into the surface. The use of light to induce germination resulted in a higher germination percentage of *S. rebaudiana* seeds compared to those seeds placed in the dark (Figure 3.2 A and Figure 3.3 B). This effect was statistically significant ($P < 0.05$). *Stevia* seeds have been reported to be positively photoblastic (Brandle *et al*., 1998). The exposure to light and the amount of time required for exposure to the light are both variables needed for seed germination to occur in *S. rebaudiana* (Abdullateef *et al*., 2015; Kumar and Sharma, 2012; Ramesh *et al*., 2006; Singh and Rao, 2005; Yadav *et al*., 2011). In response to light, plants possess photoreceptors which include phytochrome which is used to detect and absorb light (Abdullateef *et al*., 2015; Li *et al*., 2011). Phytochrome responses can be distinguished by the amount of light required to induce them. Two forms of phytochrome are known namely red light (Pr) which promotes seed germination and has an absorbance ranging from 600-700 nm. While far red light (Pfr) is the active form of phytochrome that suppresses red light induction of seed germination and absorbs at 730 nm (Li *et al*., 2011). Every species has an optimal or ideal temperature at which germination is the highest. Most plants have an optimal germination between 25-30 ºC. However, Kumar and Sharma (2012) suggested that 20 ºC is the ideal temperature for seed germination of *Stevia* while low temperatures of 10 ºC hinder the germination *S. rebaudiana* seeds. Temperature and light are thus necessary for the germination of *S. rebaudiana*. Contrary to these findings, Uçar *et al*. (2016) reported that *Stevia* seeds were significantly affected by temperature and the presence of light had no significant effect in the germination of *S. rebaudiana* seeds. At 25 ºC they obtained a germination percentage of 67% in light and 71% in the dark. They were able to also report the optimum temperature for germination at both light and darkness to be 25 ºC with 20 ºC more suitable for germination in the dark. Temperatures lower than 15 ºC resulted in the lowest germination rate (31%).

3.3.3 The treatment effect on the germination of *S. rebaudiana* seeds

Seed germination of *S. rebaudiana* followed a sigmoid pattern over the 21 days after culturing (Figure 3.2 B). On the third day, signs of seed germination were observed on all the seeds both in the dark and in the light. Factorial ANOVA showed that seeds treated with smoke water extract had significantly increased germination of *S. rebaudiana* ($P < 0.05$) and germination steadily increased till day 12 were it became constant (Figure 3.2 B).

Smoke derived from plant material has been reported to have stimulatory effects on seed germination and on the growth of many plants. It is known to increase germination in both fire dependent and fire independent species (Ghebrehiwot *et al*., 2011). The active compound which is known to be a potent germination stimulant found in smoke is called butenolide (3-methyl-2*H*-furo [2, 3-C] pyran-2-one) (Van Staden *et al*., 2004). Studies report that smoke solutions and smoke derived compounds such as karrikinolide (Nair *et al*., 2014; Papenfus *et al*., 2014) and 3-methyl-2*H*-furo [2, 3-C] pyran-2-one (butenolide) (Van Staden *et al*., 2006; Van Staden *et al*., 2004) have the tendency to play the role of plant growth regulators in inducing seed germination in photoblastic and thermodormant seeds (Nair *et al*., 2014; Papenfus *et al*., 2014; Van Staden *et al*., 2006). Numerous studies done on smoke treatments have reported positive influences on seed germination and on seedling growth of various crops such as lettuce (Drewes *et al.*, 1995), tomato (Kulkarni *et al*., 2007), carrots (Merritt *et al*., 2005), celery (Thomas and Van Staden, 1995), bean (Van Staden *et al*., 2006), onion (Kulkarni *et al*., 2010) just to mention a few.

A treatment with gibberellic acid and the response to light may suggest that dormancy in *Stevia* is a physiological phenomenon. DELLA positively regulates the expression of *XERICO* which is an inducer of ABA biosynthesis (Ariizumi *et al*., 2013). GA promotes plant growth by stimulating degradation of the growth repressor DELLA protein. GAs are adversely regulated by DELLA proteins, with a C-terminal GRAS domain in their structure, which are eventually degraded by the E3 ubiquitin ligase SCF (GID2/SLY1). It is GIBBERELLINE INSENSTIVE DAWRF1 (GID1) protein which interacts with DELLA proteins resulting in their eventual degradation and can bind to the GAs which are biologically active. Through its antagonistic effects with ABA, GAs which are internal signals, are able to release seeds from dormancy. GAs enhance seed germination by inhibiting ABA activity (Ding *et al*., 2015; Miransari and Smith,

2014). Chemical scarification is a useful treatment for seeds experiencing coat-imposed dormancy. However, this treatment was ineffective in breaking dormancy (Figure 3.2 B).

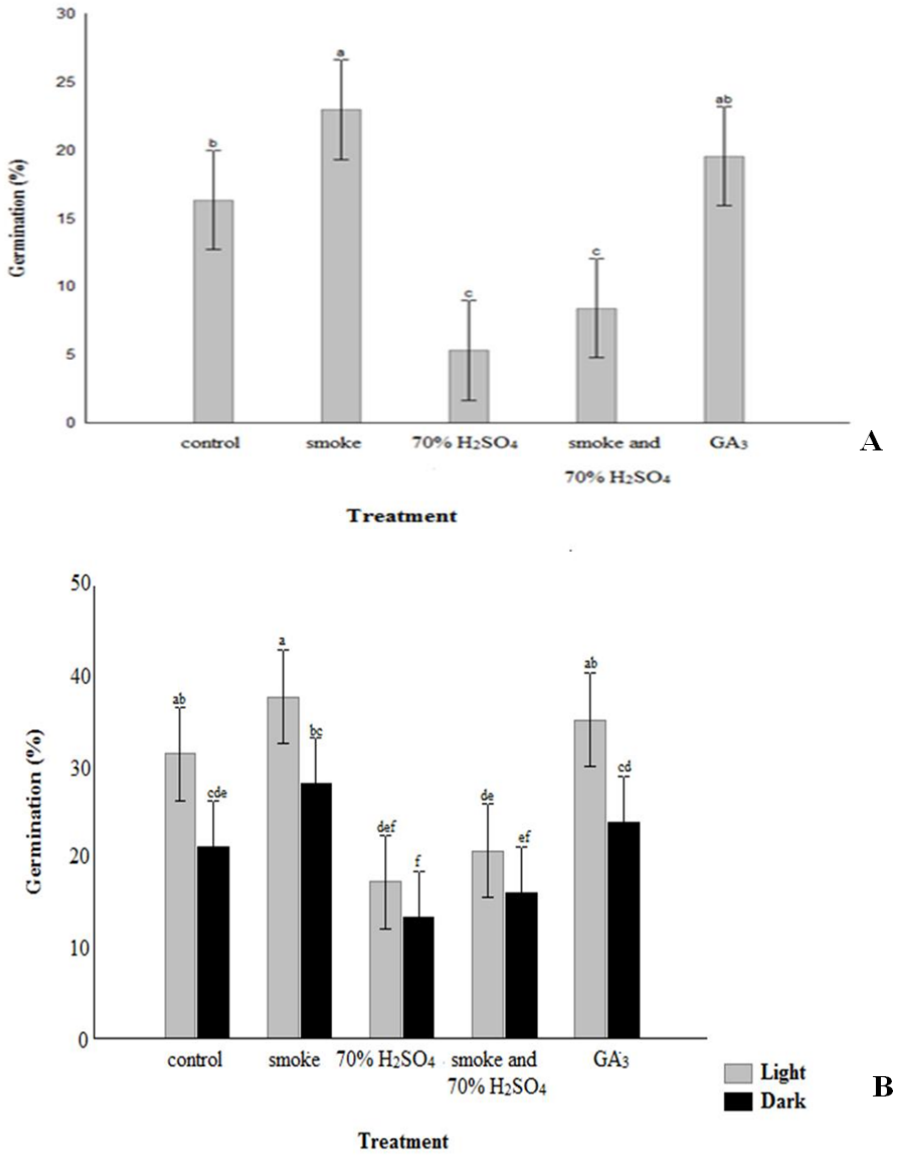

Figure 3.3 **A**. Germination of treated *S. rebaudiana* seeds (smoke, 70% sulfuric acid, combination of smoke and 70% sulfuric acid, gibberellic acid and a control with no added treatment) independent of light or dark. **B**. The grey bars indicate the total number of seeds that germinated in light after 21 days; black bars show total number of seeds that germinated in the dark in 21 days. The vertical bars denote standard error at the 95% confidence interval.

A comparison between the smoke treatment and the initial viability test further confirmed the positive effects on germination through application of the smoke treatment (P =0.02; Figure 3.4). Smoke thus has a positive effect even in instances where viability is low.

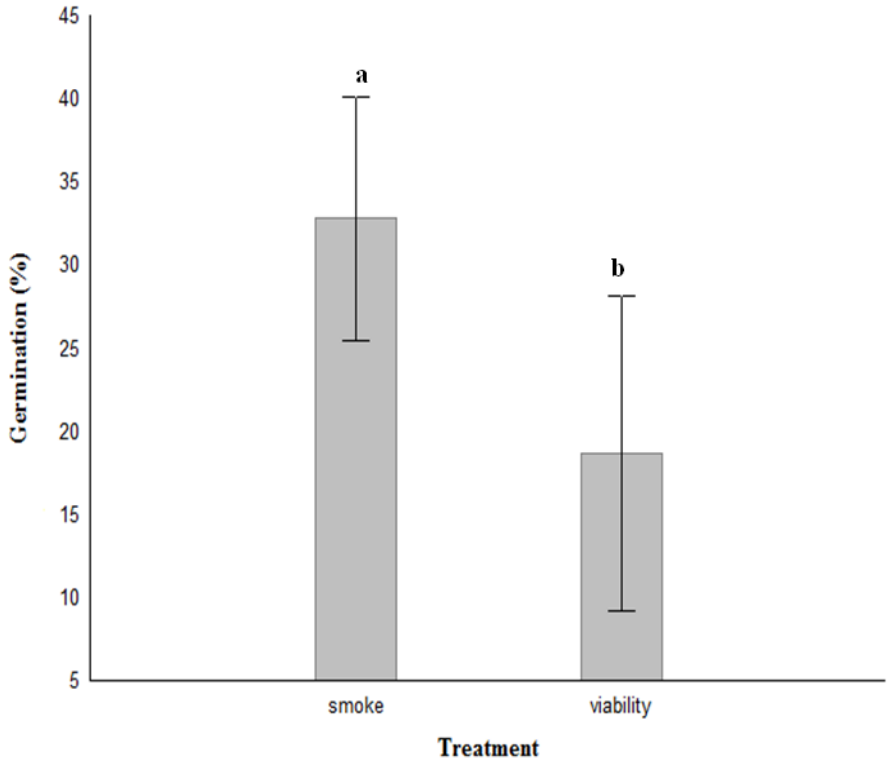

Figure 3.4 A comparison between the smoke treatment and the initial viability test. Data from this figure shows the vertical bars denote standard error at the 95% confidence interval (p=0.02).

3.4 Conclusion

Smoke as a germination cue assisted in improving germination by 36%. It appears that dormancy is controlled through a physiological process in this species that may be broken through application of smoke technology and GA. Although the mechanism remains unknown, butenolides are responsible for regulating germination in many species and this includes the seeds of the Asteraceae. Although *S. rebaudiana* does not come from a fire-prone environment, smoke treatment may be beneficial for commercial cultivation of the plant to assist with synchronized germination.

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Chapter Four

4. Effect of nitrogen and phosphate on the growth productivity and development of *S. rebaudiana*

4.1 Introduction

The influence of environmental stresses can greatly affect the growth and development of a plant and thereby altering its chemical constituents. Nutritional stress for instance may cause an increase in the concentration of secondary metabolites. Essential minerals are elements critical for a plant to complete its life cycle and are necessary for normal growth and development of the plant and have a direct or indirect role in plant metabolism (Les´niewicz *et al*., 2006; Poothong and Reed, 2014; Ramage and Williams, 2002). There are two groups of essential minerals, macro- or micronutrients which depend on the relative amount of each element required for growth. Macronutrients such as nitrogen, phosphorus, and sulfur are important components in proteins and nucleic acids, as well as constituents of many small molecules (Poothong and Reed, 2014; Ramage and Williams, 2002). They are necessary for growth, normal physiological functioning, and maintaining of life (Les´niewicz *et al*., 2006; Murashige and Skoog, 1962; Poothong and Reed, 2014; Ramage and Williams, 2002). They perform vital functions pertaining to cell metabolism and extension growth. As a supply of nitrogen, nitrate is used as a major constituent of different plant tissue culture media and it is important for many developmental processes. Phosphorus nutrition is important for both explant growth and morphogenesis (Ramage and Williams, 2002). Potassium plays a predominantly osmotic role in plants and is highly mobile throughout the plant body through selective potassium transport mechanisms (Ramage and Williams, 2002). Micronutrients are required in much smaller quantities than macronutrients and function in various roles such as enzyme cofactors or components of electron transport proteins. The essential micronutrient metals: Fe, Mn, Zn, B, Cu, Co and Mo, are components of plant cell proteins of metabolic and physiological importance. At least five of these elements are necessary for chlorophyll synthesis and chloroplast function (Les´niewicz *et al*., 2006).

For the production of steviol glycosides both macro- and micronutrients may be essential (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006). Since the plant may be found in poor soils which have a low acid pH, micronutrients such as boron, copper, iron, chloride, manganese, molybdenum and zinc are obtained in adequate amounts (Ramesh *et al*., 2006). While macronutrients are required in greater amounts as they are essential for the growth and health of the plant. A lack or reduction in the amount of macronutrients such as nitrogen, phosphorus, calcium and sulphur result in a number of deficiencies in the plant. Extreme cases of calcium deficiency in *S. rebaudiana* result in a decline in glycoside concentrations. While a reduction in all these macronutrients excluding phosphorus, cause a decrease of stevioside concentration in the leaves of the plant (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). This is important as stevioside is the most abundant steviol glycoside. This decline in the predominant sweetener accounts for 3-8% (w/w) of dried *S. rebaudiana* leaves, resulting in fluctuations in its sweet property which may hinder its commercial existence (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). Deficiencies in calcium, magnesium and sulphur exhibit unfavourable symptoms which eventually hinder the trade of the plant. These symptoms include apical necrosis, chlorosis, and inverted "V" shaped necrosis and pale green leaves respectively.

Nutritional requirements may easily be studied using *in vitro* culture systems. The production of specific secondary metabolites *in vitro* can be manipulated by adding or removing plant growth regulators or applying environmental stressors (Albrecht *et al*., 2012; Bajaj and Ishimaru, 1999). *Stevia* is nutrient-rich, containing substantial amounts of protein, calcium, phosphorous and other important nutrients and contains a range of sterols, triterpenes, flavonoids, and tannins, as well as being an extremely rich volatile oil containing lots of aromatic agents, aldehydes, monoterpenes and sesquiterpenes (Kinghorn and Soejarto, 1985).

Gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) are important metabolomic tools as they may yield an almost comprehensive report of the metabolite profile when coupled with multivariate analysis (Kopka, 2006). The analysis of organic volatile compounds and semi-volatiles is easily achieved with GC-MS provided that a derivatization step is added to sample preparation. Allwood *et al*. (2011) described LC-MS as a fundamental separation technique that can separate and allow for the quantification of non-

volatile compounds without a derivatization step. This may be particularly important for the analysis of a wide range of compounds especially those polar non-volatile compounds that may be heat-sensitive molecules. Secondary compounds such as phenolics, flavonoids, saponins, poly-amines and glucosinolates are usually most proficiently detected using LC-MS technique. This technique is well suited for discovering unknown compounds of greater polarity and is less expensive when compared to the GC-MS technique.

In this Chapter, I describe experiments that were conducted to study some of the macronutritional requirements of *S. rebaudiana* plants in tissue culture. The analysis focused on the growth and development of *Stevia* when nitrogen and phosphate manipulations of the culture medium were undertaken *in vitro*. The study aimed to investigate the effects of nitrogen and phosphate on growth. The hypothesis was that such manipulation may cause great shifts in secondary metabolism which may lead to significant changes in both chemical constituents of essential oils and non-volatiles. The manipulation of *Stevia* plants was followed by an analysis of metabolite profiles using LC-MS and GC-MS technology.

4.2 Plant material and culture

4.2.1 Plant material and culture induction

Seeds of *S. rebaudiana* seeds (2 000) were obtained from a commercial seed source company (Ball Straathof, Johannesburg) in July 2013. Upon arrival, these were stored in the dark at room temperature for a period of two months before germination experiments were conducted. Seeds were treated with smoke to induce germination (refer to Chapter 3; Section 3.2.3) (Figure 4.2.1A) and after 21 days, the surviving seedlings (Figure 4.2.1 B) were transferred onto induction medium with 1 mg/L IAA and 2 mg/L BA for two months to elongate the seedlings. Furthermore, two sets of stock plantlets were maintained in the greenhouse. One set was derived from the *in vitro* germinated *Stevia* plantlets that were left to grow further for three months on 1: 2 IAA: BA medium (mg/L) (referred to henceforth as control medium). The other set was from one year old clonal plants, generated through cuttings, provided by Ms Christina Glyn-Woods (September, 2013) purchased from the Cape Garden Market (Cape Town, South Africa). Both sets of plants were transferred to pots containing sphagnum: perlite: sand (1:1:1, v/v) in the glasshouse (Department of Botany and Zoology, Stellenbosch University) (Figure 4.2.1 C, D and E). The range of midday irradiances was 600-800 µmol m^{-2} s^{-1} and the average night-day temperatures were 15-25 °C. Plants were watered regularly every 3 days by hand (see Figure 4.2.2). The greenhouse plants were left to grow and used in a drought stress study (discussed in Chapter 5). A full Murashige and Skoog medium (1962) was used with 0.8% (w/v) agar (agar-agar powder (Merck, Germany)), 3% (w/v) sucrose and 0.1 g/L myo-inositol. This medium is regarded as the control medium throughout the study. At all times, the pH of all the media used in the study (see different media types in Section 4.2.2 and Section 5.2.1) was adjusted to 5.8 using 1 M NaOH or 1 M HCl and autoclaved for 20 minutes at 121 ºC at a pressure of 103 kPa. The seedlings (placed on the above medium) were used to obtain nodal explants (detailed below in Section 4.2.2) and maintained as a continuous *in vitro* stock of plant material. Glass bottles (110 mm × 55 mm) were used as culture vessel and these were transferred to a light growth chamber with standard conditions. Each bottle had 30 mL of the medium. These conditions were maintained by placing cultures in a room with cool white fluorescent lights (L75 W/20 9 Osram, USA; code F96T12), supplying light for 16 hours (50 µmol m^{-2} s^{-1}) at shelf-level in the growth room (25 ± 2 °C).

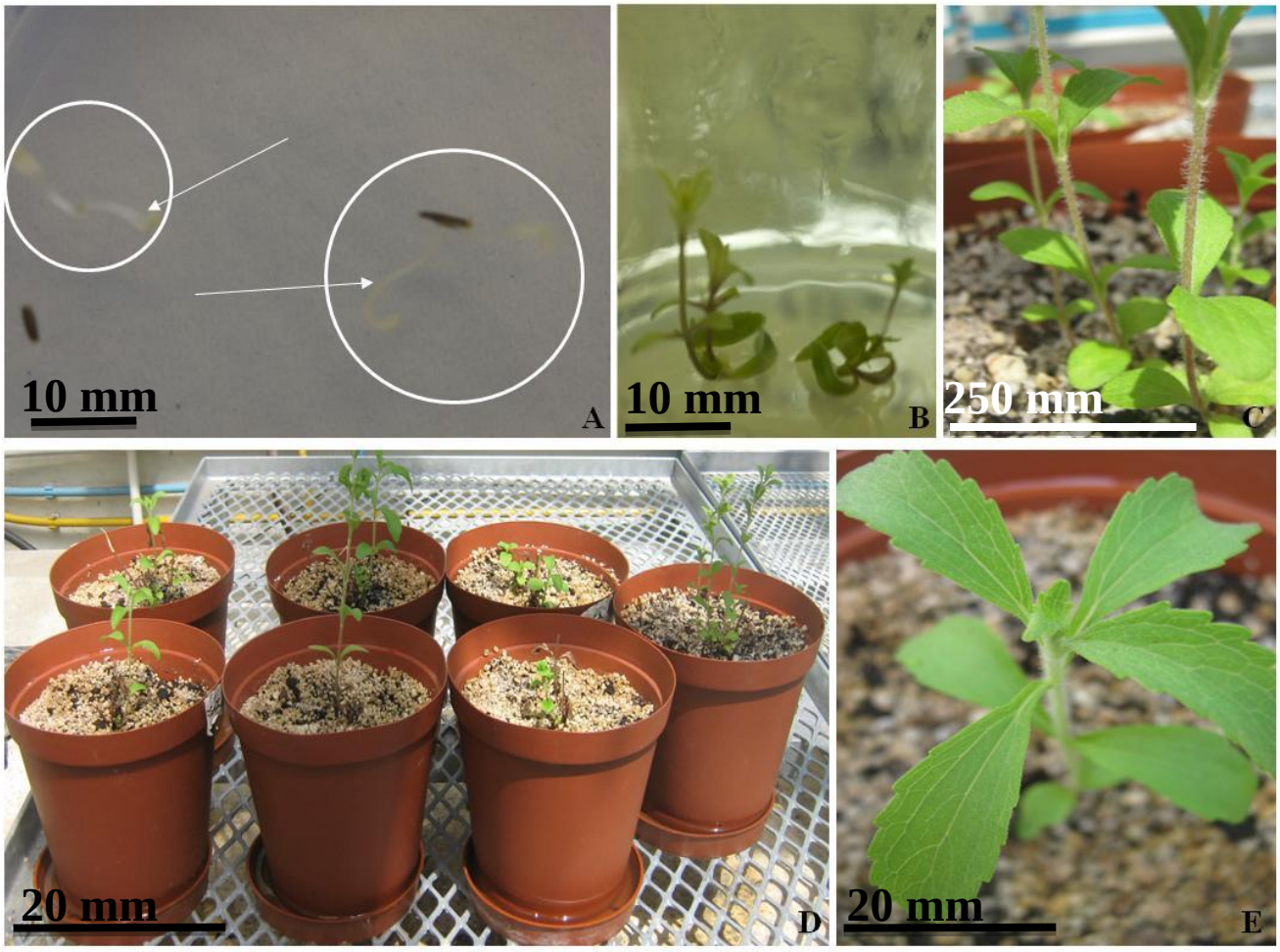

Figure 4.2.1 Stages for micropropagation of *S. rebaudiana*. **A**. *In vitro* seedlings were used as starter material for culture induction. Germinating *S. rebaudiana* seeds (indicated by arrows) growing on smoke-treated medium. **B**. Plantlets were initiated using nodal explants. *In vitro* shoot development from axillary buds produced on MS medium with 1 mg/L IAA and 2 mg/L BA. Plant growing under these conditions were used for nitrogen and phosphate treatments. **C**. After two months some of the control plantlets were transferred *ex vitro*. **D**. Purchased plants were grown as stock material in the greenhouse throughout the study. **E**. An acclimatised plantlet derived from tissue culture growing under greenhouse conditions for one year.

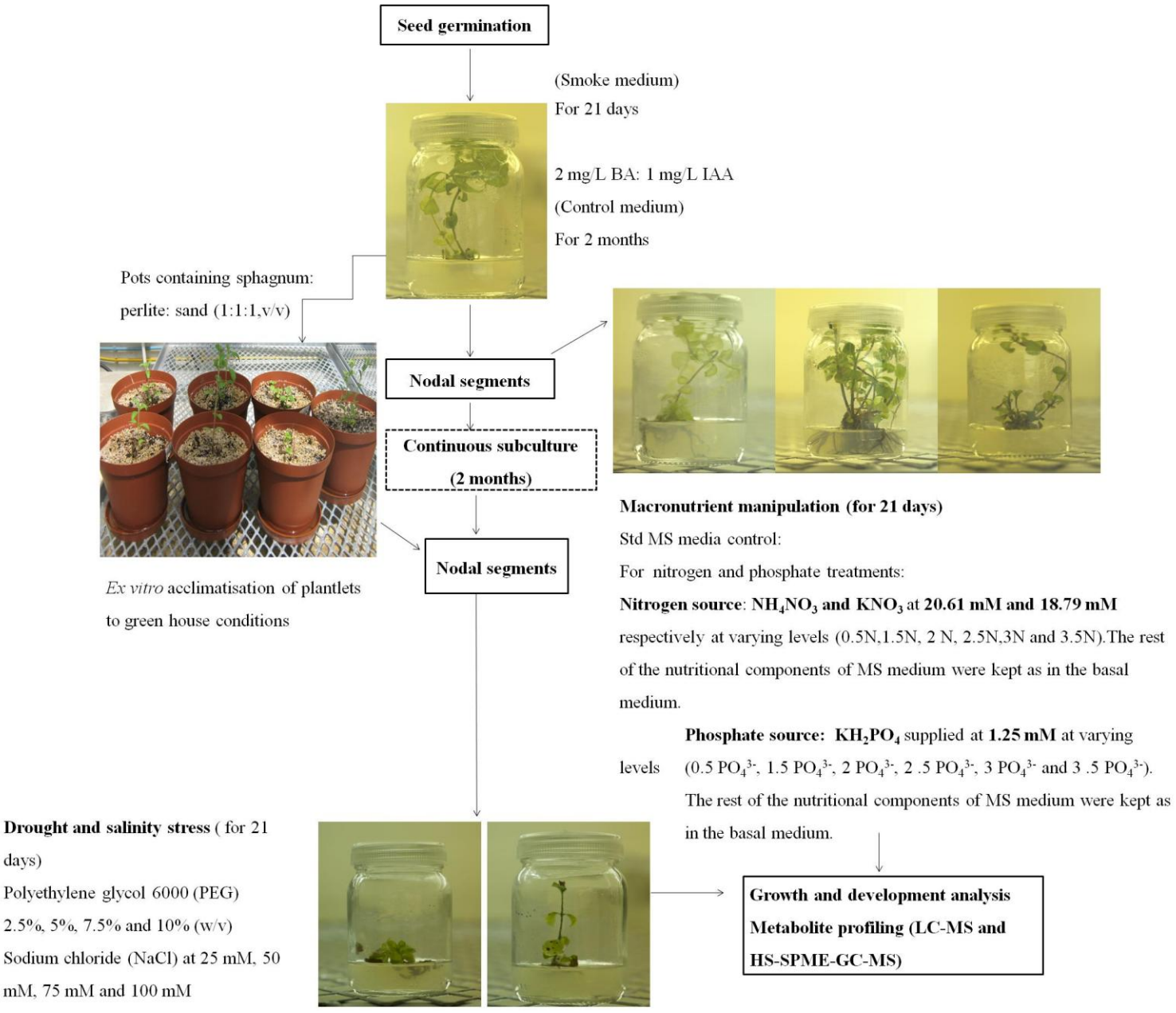

Figure 4.2.2 A general protocol for micropropagation and acclimatization of *S. rebaudiana*.

4.2.2 *In vitro* manipulation of phosphate and nitrogen

Three nodal explants (1-2 cm) were placed in each culture vessel under laminar flow conditions. For each treatment, 30 replicates were used per treatment as each explant was regarded as a replicate. Each culture vessel contained MS basal medium of 30 mL and plant growth regulator conditions were the same as in the control medium.

The level of nitrogen and phosphate supplied in the control MS medium were varied in the different media and these were regarded as the treated nodal explants. The nitrogen sources were NH_4NO_3 and KNO_3 supplied at 20.61 mM and 18.79 mM in the full basal MS medium respectively. In this set of experiments, they were either reduced to half (½ N), increased by a factor of one and a half (1 ½ N), doubled (2 N), increased by a factor of two and a half (2 ½ N), tripled (3 N), and increased by a factor of three and a half (3 ½ N) to establish low and high nitrogen conditions respectively. The concentration of phosphate levels were left as being similar to controls where KH_2PO_4 was supplied at 1.25 mM. Control experiments were those supplied with the normal compliment of nitrogen source (NH_4NO_3 at 20.61 mM and KNO_3 at 18.79 mM) and phosphate source (KH_2PO_4 at 1.25 mM). The same was done for the phosphate treatments. The treatments henceforth were denoted as ½ PO_4^{3-}, 1 ½ PO_4^{3-}, 2 PO_4^{3-}, 2 ½ PO_4^{3-}, 3 PO_4^{3-} and 3 ½ PO_4^{3-}. The rest of the nutritional components of MS medium were kept as in the basal medium. All experiments were conducted three times. After 21 days, plants treated with nitrogen or phosphate were harvested. Data were collected by recording the number of regenerated shoots, shoot length (mm), number of leaves, fresh weight (mg), and root length and root number from each explant. Three samples from each *in vitro* treatment were randomly selected to be used for metabolite profiling.

4.2.3 Extraction of plant material for LC-MS

One gram from each of the three randomly selected samples was obtained and this included the control. The fresh leaves (1 g) were ground to a fine powder using a pestle and mortar with liquid nitrogen. Extraction was carried out on the freshly ground leaves (1 g) with 10 mL of a 1:1 (v/v) water: ethanol mixture in a 20 mL glass test tube. These were then sonicated for 45 minutes (Bransonic 220, USA) before filtering with Whatman filter paper number 1. The extraction was repeated twice before 1.5 mL from each 20 mL glass test tube was poured into a microfuge tube

and then pooled extracts were dried using a speed vac (Genevac EZ-2 personal evaporator) for 4 hours. Extracts were re-suspended using 50% (v/v) acetonitrile. Samples were vortexed for 1 minute and sonicated for 30 minutes until completely re-suspended.

4.2.4. LC-MS/MS analysis

All samples were analysed using a Water Synapt G2 quadruple time-of-flight mass spectrometer (Milford, MA, USA). The instrument was connected to a Waters Acquity ultra-performance liquid chromatography (UPLC) and Acquity photo diode array (PDA) detector. Ionisation was achieved with an electrospray source using a cone voltage of 15 V and capillary voltage of 2.5 kV and only the negative mode was used. Nitrogen was used as the desolvation gas at 650 l h^{-1} and the desolvation temperature was set to 275 °C.

A Waters UPLC EH C18 column (2.1 × 100 mm, 1.7 µm particle size) was utilized and 2 µL was injected for each analysis. The gradient started with 95% using 0.1% (v/v) formic acid (solvent A) and 5% acetonitrile (solvent B). Followed by a shift in the gradient to 60% of 0.1% formic acid (solvent A) and 40% acetonitrile (solvent B) at 9 minutes, 30% solvent A and 70% solvent B over 9.1 minutes, 100% solvent B at 14 minutes, 95% solvent A and 5% solvent B at 14.01 minutes. Thereafter, conditions remained constant over 15 minutes with solvent A at 95% and solvent B at 5%.

For mass spectrometry, the chemical standards namely, steviol hydrate; stevioside hydrate and rebaudioside A (Sigma-Aldrich, Germany), were used for analysis in this study.

4.2.5 HS-SPME-GC-MS protocol

Plant material extracted from the tissue culture vessels (0.25 g) was placed inside a 20 mL headspace vials. Each container was sealed with an aluminum-coated silicone rubber septum. Headspace solid microextraction was employed for the extraction of volatiles from the leaf tissues. The vial was placed in a 50 ºC agitator. After 2 minutes as the equilibration period between the solution and the headspace, the fibre was exposed to the headspace of the capped vial to adsorb the analytes for 10 minutes.

A 50/30 µm diving/benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDSMS) solid phase micro extraction (SPME) fibre, purchased from Supelco Inc. (Bellefonte, PA, USA), was preconditioned at 250 ºC for 30 minutes in the GC injector. The fibre was selected as it is highly sensitive in the analysis of aromatic compounds and has previously been used in the analysis of *Salvia stenophylla* (Musarurwa *et al*., 2012).

Following the HS-SPME analysis, the fibre was withdrawn and introduced into the heated injector port for desorption at 240 ºC for 10 minutes. The desorbed volatile compounds using a 5:1 (v/w) split ratio were introduced for analysis on an Agilent (Palo Alto, CA) 5973 mass selective detector connected to an Agilent 6890 gas chromatograph, equipped with 60 m × 0.25 mm × 0.25 (FFAP) µm capillary column. The oven temperature was programmed from 70 ºC for 1 minute, increasing at 3 ºC/minutes to 142 ºC, and then increasing at 5ºC/minutes to 225 ºC for 3 minutes by optimizing the method used in a previous study (Musarurwa *et al*., 2012). The transfer line was heated at 250 ºC. Helium was used as a carrier gas at a linear velocity of 1.9 mL/minute. The operating conditions of the mass detector were as follows: source temperature 230 ºC; and electronic impact (EI) mode of 70 eV with a speed of 4.38 scan s^{-1} over the mass range *m/z* 30-350 amu in a 1 second cycle in a full scan mode. The identification of volatile compounds was achieved by comparing the mass spectra with the Wiley online library and National Institute of Standards and Technology (NIST) library. The relative amounts of each compound present were expressed as percentage areas of the total peak area.

4.2.6 Statistical analyses

All *in vitro* experiments were conducted three times for 21 days to generate growth data. To reiterate, ten samples were used in each treatment for growth experiments. For quantification of steviol glycoside content (rebaudioside A, stevioside and steviol) in the shoots, three replicates were used per treatment. All data were subjected to normality testing, preceding analysis of variance (ANOVA). This was followed by a Tukey's Honesty Significance Difference (HSD) for data which was normally distributed. For data which were not normally distributed, Krustal-Wallis analysis was utilized to separate the means as post-hoc test. The overall effect of variables on the outcome was tested by conducting a one-way ANOVA (Figure 4.3.1; 4.3.2 and 4.3.3) to compare the effect of phosphate versus nitrogen. All statistical analyses were conducted at the 95% confidence level.

Principal component analysis (PCA) was conducted to demonstrate distinctness between nitrogen and phosphate treated *Stevia* plantlets in terms of the metabolomic profiles for both LC-MS and GC-MS. To make a comparison between the results obtained from the nitrogen and phosphate treated *Stevia* plantlets, a two-way ANOVA was performed (Figure 4.3.7, 4.3.8 and 4.3.9). For the GC-MS data compounds that had a NIST library match of 85-100% were regarded as likely hits (Tables 4.1 and 4.2). The relative abundance of the six compounds was compared using a one-way (ANOVA) (Tables 4.1 and 4.2). All statistical analyses were conducted at the 95% confidence level. The true identity of six chemical compounds was confirmed through the use of standards and the molecular ion peaks, fragmentation patterns, and retention times.

4.3 Results and discussion

4.3.1 The effect of nitrogen and phosphate on the growth and development

Growth and yield of *Stevia* are largely influenced by the applied nutrients (Brandle *et al*., 1998). At the end of the nitrogen treatment (21 days), plants subjected to the 0.5 N treatment had significantly ($P < 0.05$) the highest height (shoot length) (61.653 mm) (Figure 4.3.1 A) and number of internodes (7.2), however, thisplants subjected to 0.5 N with regards to the number of internodes were statistically similar to the control which had 8.2 internodes (Figure 4.3.1. C). The number of leaves produced per shoot was not correlated to increasing concentrations of nitrogen. The nitrogen treated plants had significantly lower number of leaves ($P < 0.05$) (Figure 4.3.1 B) than the control (34.83) which was significantly higher than in all of the nitrogen treatments. The shoot mass (fresh weight) was not correlated to increasing concentrations of nitrogen (Figure 4.3.2 A). There was no statistical difference between the different nitrogen media except for the growth of explants on 2.5 N and 3 N media which produced the lowest fresh weight (0.100 mg and 0.119 mg respectively) which was statistically different ($P < 0.05$) from the other nitrogen treatments including the control. Roots produced in nitrogen media regardless of the concentration were typically well developed and appeared to be thick and strong. This was an expected result. Plants under nutrient stress invest in mass proliferation of roots to increase the surface area for maximum nutrient absorption (Pellny *et al*., 2008).

However, it was interesting to note that reducing nitrogen by half gave the highest number of roots (38.5) (Figure 4.3.2 B) and the highest root length (80.533 mm) (Figure 4.3.2 C).

Growth is impaired if the concentration in the medium of either essential or non essential elements exceeds a certain level. In general, macronutrients are much less toxic than micronutrients and their concentration can be raised considerably above the optimum level without significantly affecting growth (Pal *et al.,* 2013). However, in this case raising the nitrogen level lowered the above mentioned parameters which pose a risk in the yield of *Stevia* plants. The control medium was best for the production of shoots as compared to those media with various levels of nitrogen (Figure 4.3.1 B). This is consistent with the results obtained by Ibrahim *et al*. (2008) who studied the influence of plant MS medium and its components at different levels on the *Stevia* plant. They obtained the best nutrient medium using MS at full salt strength or modified MS major elements at various levels (NH_4NO_3 at 1237.5 mg/L, KNO_3 at

950 mg/L, $MgSO_4$ at 185 mg/L, $CaCl_2$. $2H_2O$ at 440 mg/L and KH_2PO_4 at 85 mg/L), whereas, the manipulation of the MS medium at different strengths reduced the leaf number. There is no congruency in terms of the nutritional requirements for producing high biomass for *Stevia*. In a field study, Pal *et al*. (2013) concluded on the foliar application of KNO_3 and Ca $(NO_3)_2$ as a means to increase the dry leaf yield of *Stevia* without risking the quality of the yield. Allam *et al*. (2001) showed a significant effect of nitrogen to enhance the concentration of stevioside in the *Stevia* leaf. Yücesan *et al*. (2016) developed a micropropagation protocol for *S. rebaudiana* using MS medium, that could possibly produce over 120 000 plantlet within a period of 9 months from a single nodal segment. They were also able to show that clonal propagation using nodal explants gave the best quality *S. rebaudiana* production with a high rebaudioside A content of 11.7% (v/v). The 0.5 N was the best treatment for producing plants that were elongated. This treatment was also effective in generating plants that had a high number of internodes similarly to controls, in this study.

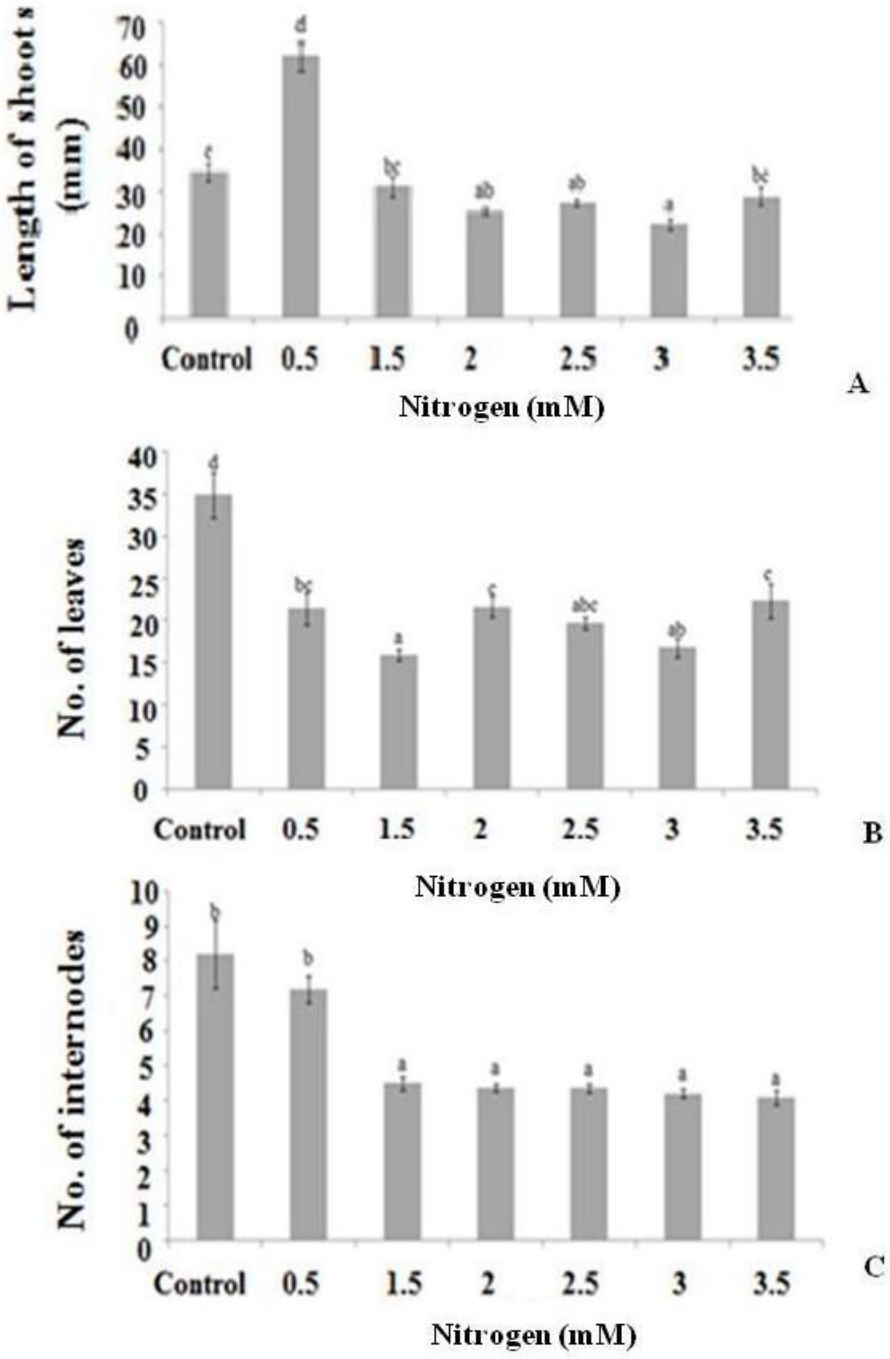

Figure 4.3.1 *S. rebaudiana in vitro* growth after 21 days in varying levels of nitrogen treatments. **A**. Length of shoots (mm). **B**. Number of leaves. **C**. Number of internodes. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

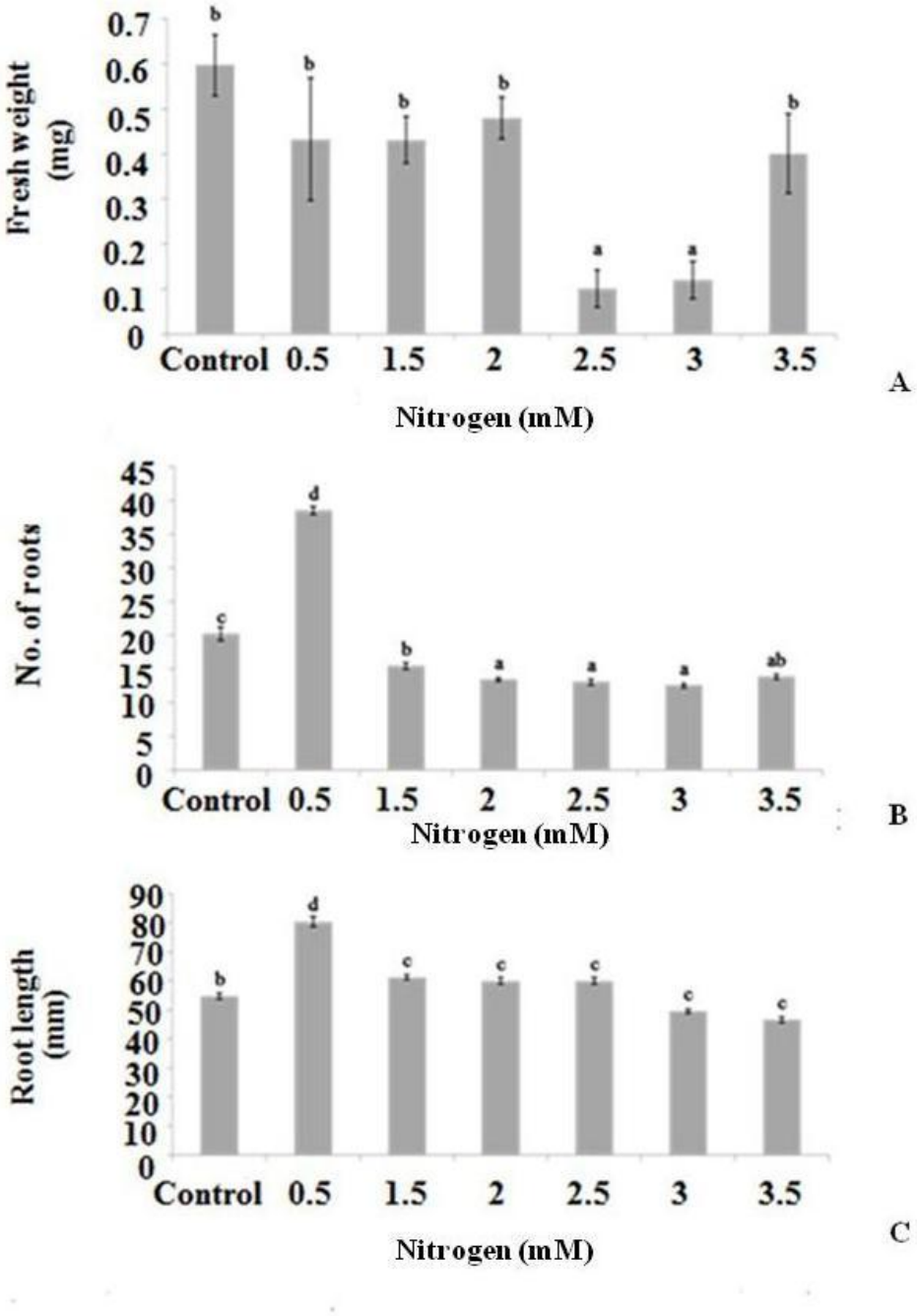

Figure 4.3.2 *S. rebaudiana in vitro* growth after 21 days in varying levels of nitrogen treatments. **A**. Fresh weight measured in milligrams (mg). **B**. Number of roots. **C**. Length of roots measured in millimetres (mm). Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

Figure 4.3.3 Plants of *S. rebaudiana* grown with varying levels of nitrogen for a period of 21 days. **A**. Plantlets grown on basal MS medium (Standard MS media containing the nitrogen source NH_4NO_3 and KNO_3 at 20.61 mM and 18.79 mM respectively and phosphate source as KH_2PO_4 supplied at 1.25 mM., see Section 4.2.2) represent the control. **B**. Plantlets grown on medium with reduced (0.5 N) nitrogen levels. **C**. 1.5 N, **D**. 2 N, **E**. 2.5 N, **F**. 3 N and **G**. 3.5 N. All plantlets were grown on MS medium with 2 mg/L BA and 1 mg/L IAA.

The medium containing half of the phosphate concentration, termed 0.5 PO_4^{3-} significantly promoted the elongation of the shoots (58. 903 mm) (Figure 4.3.4 A) but the control plants had the highest number of leaves (Figure 4.3.4 B) and internodes (Figure 4.3.4 C). Increasing levels of phosphate in the medium were linked with a reduction of internodes. Although fresh weight is not the best measure for biomass production, doubling the phosphate supply produced the highest fresh weight (0.816 mg) (Figure 4.3.5 A). Lowered phosphate levels were important for significantly increasing the root number ($P < 0.05$) (Figure 4.3.5 B) and root length (Figure 4.3.5 C).

There was a reduction of root growth accompanied by an increase in nitrogen supply (Figure 4.3.2 B). Most plants obtain the bulk of the mineral elements they require through the roots. There is generally a close relationship between root surface area and ion uptake. Plants grown on low salt medium possess a greater capacity for ion uptake than those grown on normal or high salt medium. This effect is partly attributable to the high sugar content of low salt tissues. Plants in nutrient limiting environments invest in mass proliferation of roots so as to increase the surface area for maximum nutrient absorption (Pellny *et al*., 2008).

The sensitivity of different parts of the plant to high concentrations of individual elements varies greatly. Nitrogen is highly mobile in the plant and moves progressively into younger leaves during growth so that it is the older leaves which show deficiency symptoms first and this is also true for phosphorus deficiency (George *et al*., 2008). A high concentration of one element may lead to a deficiency of another by interfering with uptake in which case the toxicity symptoms of one element may resemble those for deficiency of another. This may explain the lowered growth observed with *Stevia* plants under increasing mineral levels. Depending on the plant species, and the development stage and organ, the nitrogen content required for optimal growth varies.

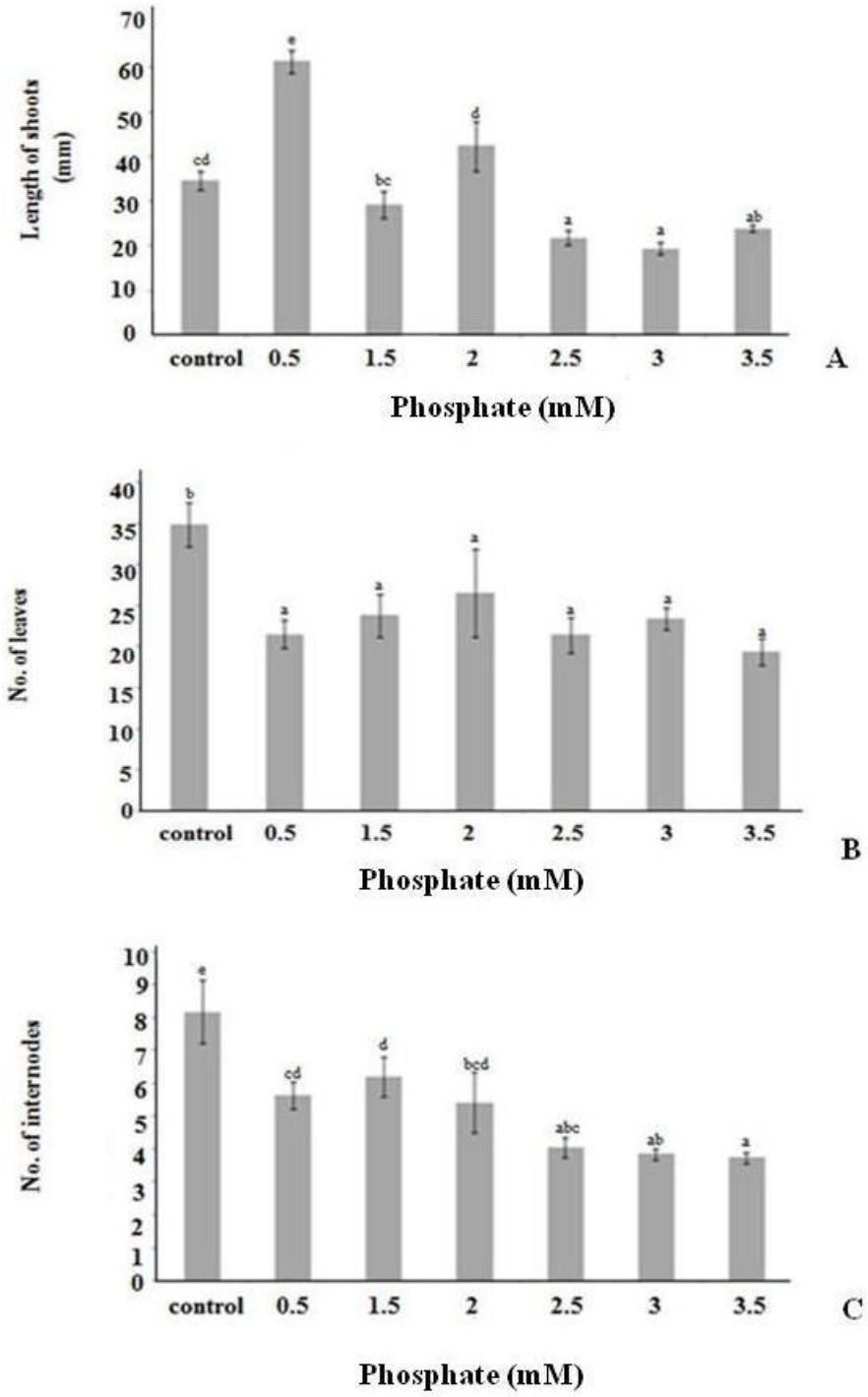

Figure 4.3.4 *S. rebaudiana in vitro* growth after 21 days in varying levels of phosphate treatments. **A**. Length of shoots (mm). **B**. Number of leaves. **C**. Number of internodes. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

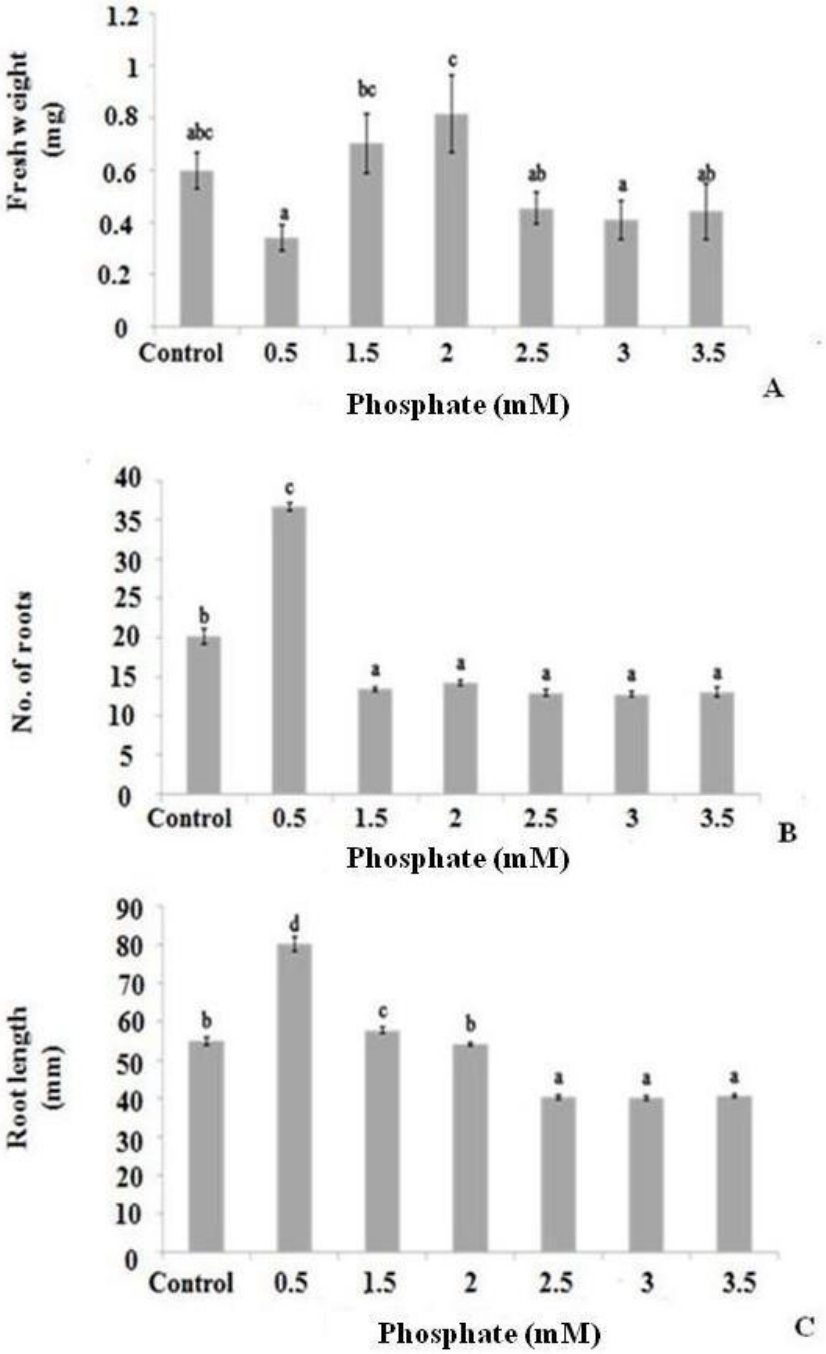

Figure 4.3.5 *S. rebaudiana in vitro* growth after 21 days in varying levels of phosphate treatments. **A**. Fresh weight (mg). **B**. Number of roots. **C**. Length of roots (mm). Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

Figure 4.3.6 Plants of *S. rebaudiana* grown with varying levels of phosphate for a period of 21 days. **A**. Plantlets grown on basal MS medium (std MS media containing the nitrogen source NH_4NO_3 and KNO_3 at 20.61 mM and 18.79 mM respectively and phosphate source as KH_2PO_4 supplied at 1.25 mM, see Section 4.2.2) represented the control. **B**. Plantlets grown on medium with reduced (0.5 PO_4^{3-}) phosphate levels. **C**. 1.5 PO_4^{3-} **D**. 2 PO_4^{3-}, **E**. 2.5 PO_4^{3-}, **F**. 3 PO_4^{3-} **G**. 3.5 PO_4^{3-}. All plantlets were grown on MS medium with 2 mg/L BA and 1 mg/L IAA.

Table 4.1 Volatile metabolite accumulation in response to changes in nitrogen concentrations in MS media.

Compound	Retention time	Kovats index	Control	Relative abundance (%)					
				0.5	**1.5**	**2**	**2.5**	**3**	**3.5**
α-Pinene	5.80	1007	4.36 ±1.25 a	4.73±0.36 ab	7.11±0.08 d	5.10±0.55 abc	6.43±0.07 bcd	6.41±0.43 bcd	6.50±0.39 cd
Butanoic acid	6.20	1617	4.71±2.30 b	3.08±1.08 a	_	_	_	_	_
β-Pinene	7.34	1138	49.74±16.85 ab	30.85±1.21 a	62.46±15.70 ab	50.43±8.08 ab	67.04±0.74 b	77.20±2.82 b	53.82±13.18 ab
Sabinene	7.62	1147	3.30±1.07 c	1.07±0.27 a	2.33±0.21 b	1.18±0.22 a	1.70±0.13 ab	2.52±0.38 bc	_

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

Table 4.2 Volatile metabolite accumulation in response to changes in phosphate concentrations in MS media.

Compound	Retention time	Kovats index	Control	Relative abundance (%)					
				0.5	**1.5**	**2**	**2.5**	**3**	**3.5**
α-Pinene	5.80	1007	4.36 ±1.25 a	3.00±0.85 a	4.51±0.71 a	3.11±1.36 a	4.69±0.71 a	4.99±1.73 a	3.56±1.42 a
Butanoic acid	6.20	1617	4.71±2.30 a	11.86±5.85 a	_	_	_	_	_
β-Pinene	7.34	1138	49.74±16.85 a	36.40±12.03a	45.99±11.74 a	31.13±12.06 a	51.96±12.40 a	48.83±17.99 a	46.95±19.07 a
Sabinene	7.62	1147	3.30±0.37 b	1.54±0.52 a	2.55±0.32ab	1.44±0.44 a	1.85±0.28 a	3.36±0.57 b	_
Limonene	9.82	1229	0.33±0.06 a	0.44±0.03 ab	1.12±0.20 b	0.98±0.35ab	_	_	_
Trans-β.-farnesene	28.21	1711	4.73±0.36 a	22.73±9.95 a	7.89±4.38 a	_	_	_	_

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

4.3.2 Effect of nitrogen and phosphate on essential oil components

The impact of mineral components highly influences secondary metabolism causing variation in the chemical profile both quantitatively and qualitatively. With this idea in mind, it was important to ascertain the effects of changing nitrogen and phosphate on the overall chemical constituents of *Stevia* plants *in vitro*. I focused on the major chemicals and only those with a 85% match or above are reported on Tables 4.1 and 4.2. Siddique *et al*. (2012) characterised the steam distillate of field grown *Stevia* plants and identified α-cadinol (2.98%), caryophyllene oxide (1.23%), (−)-spathulenol (2.21%) and β-guaiene (0.32%) as some of the key chemicals. In this study, six volatile compounds were detected in propagated plants of *S. rebaudiana* (Tables 4.1 and 4.2). High levels of nitrogen in the growth medium resulted in plants that had no butonoic acid. The highly lowered levels of this chemical may have been difficult to detect with the GC-MS. A similar trend was noted with the phosphate treatment that resulted in several essential oil chemicals being undetectable (Table 4.2).

Essential oil compounds are important as chemical defence against pathogens. *In vitro* plants of *Stevia* may thus have a lowered capacity for synthesis of these chemicals due to the nature of the tissue culture environment being largely sterile. Although the essential oil chemistry of *Stevia* is interesting, the non-volatile fraction of this plant is the most important from a commercial perspective. As stevioside, rebaudioside A and steviol glycosides are important for sweetness and using an LCMS-based approach, these were quantified. High levels of nitrogen caused reductions in the amount of glycosides obtained whereas increasing the concentration of phosphates in the growth medium to 3.5 had a similar effect on steviol production as those plants growing in the 1.5 PO_4^{3-} treatment (Figure 4.3.7, Figure 4.3.8 and Figure 4.3.9). Similar trends were observed with the rebaudioside A and stevioside chemicals (Figure 4.3.7 and Figure 4.3.8 respectively) where those plants growing on the 1.5 PO_4^{3-} medium had reduced levels.

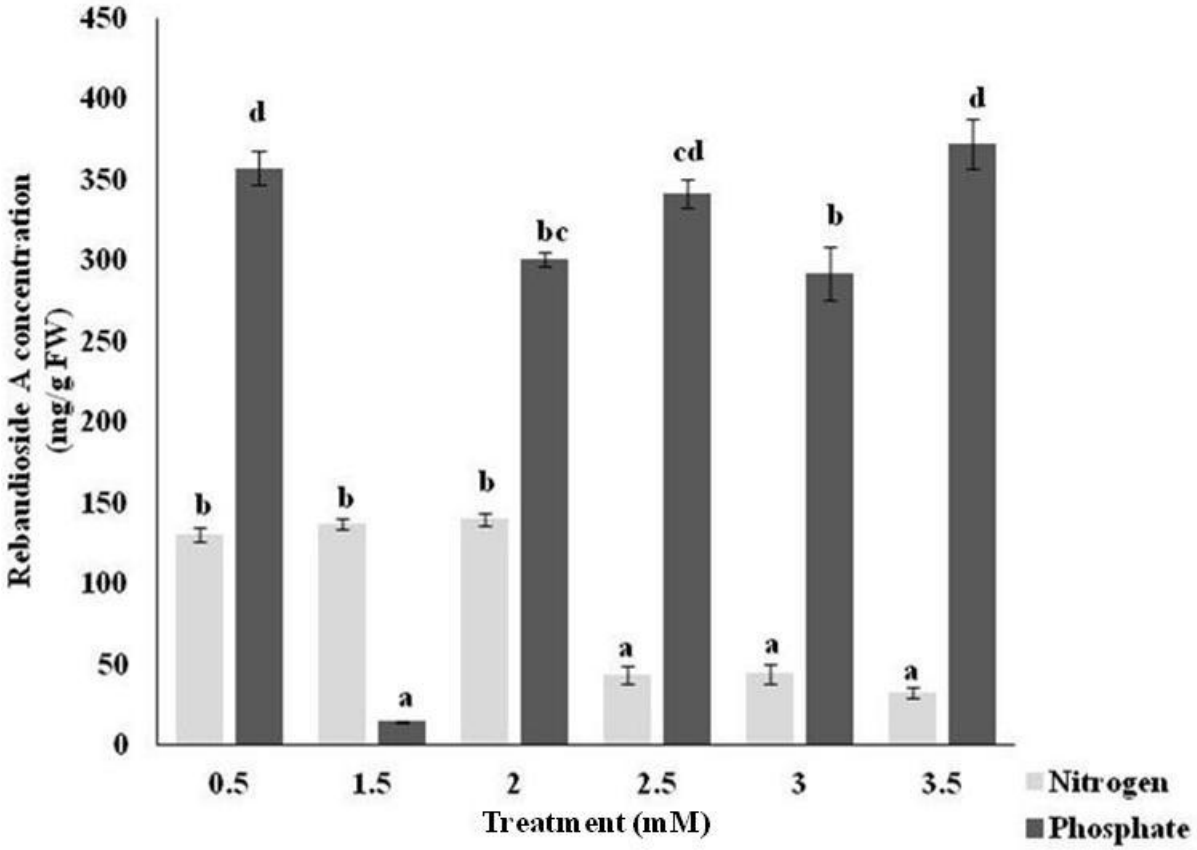

Figure 4.3.7 Effect of nitrogen and phosphate treatments on rebaudioside A production in *S. rebaudiana*. Plantlets grown under *in vitro* conditions for 21 days in varying levels of nitrogen and phosphate. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval (p=0.0000).

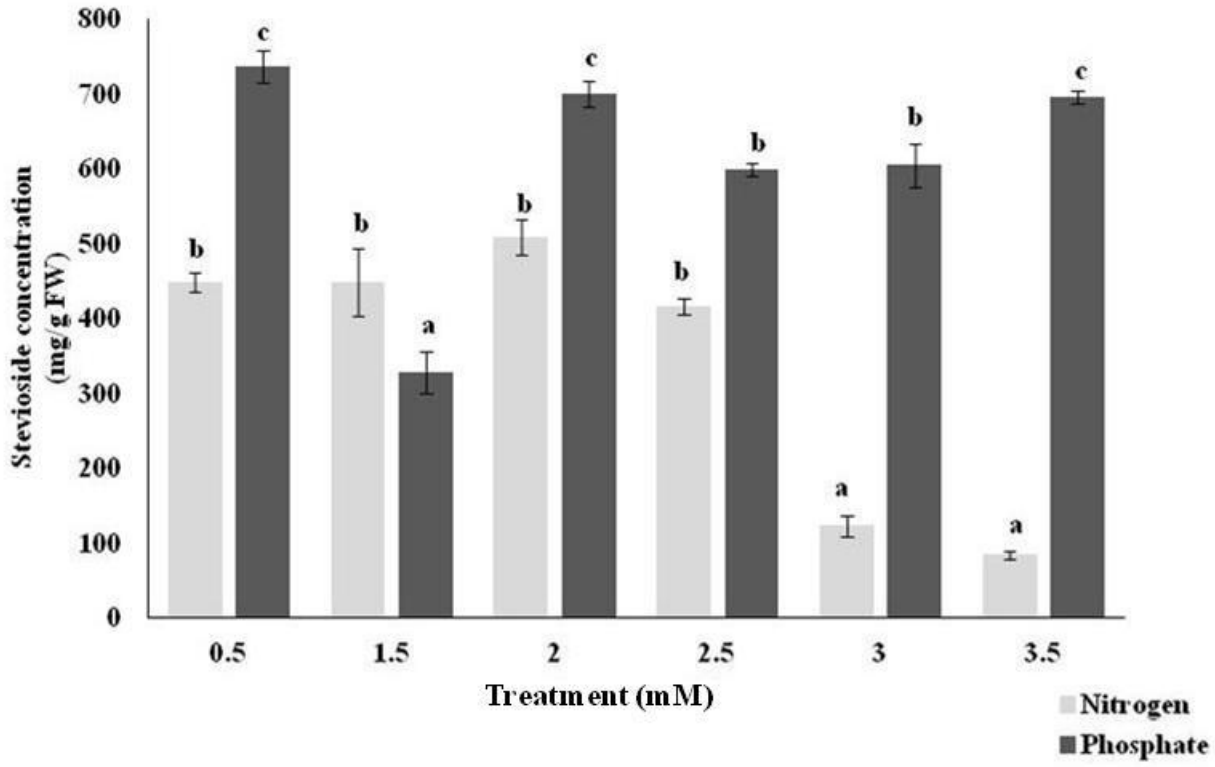

Figure 4.3.8 Effect of nitrogen and phosphate treatments on stevioside production in *S. rebaudiana*. Plantlets grown under *in vitro* conditions for 21 days in varying levels of nitrogen and phosphate. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval (p=0.0000).

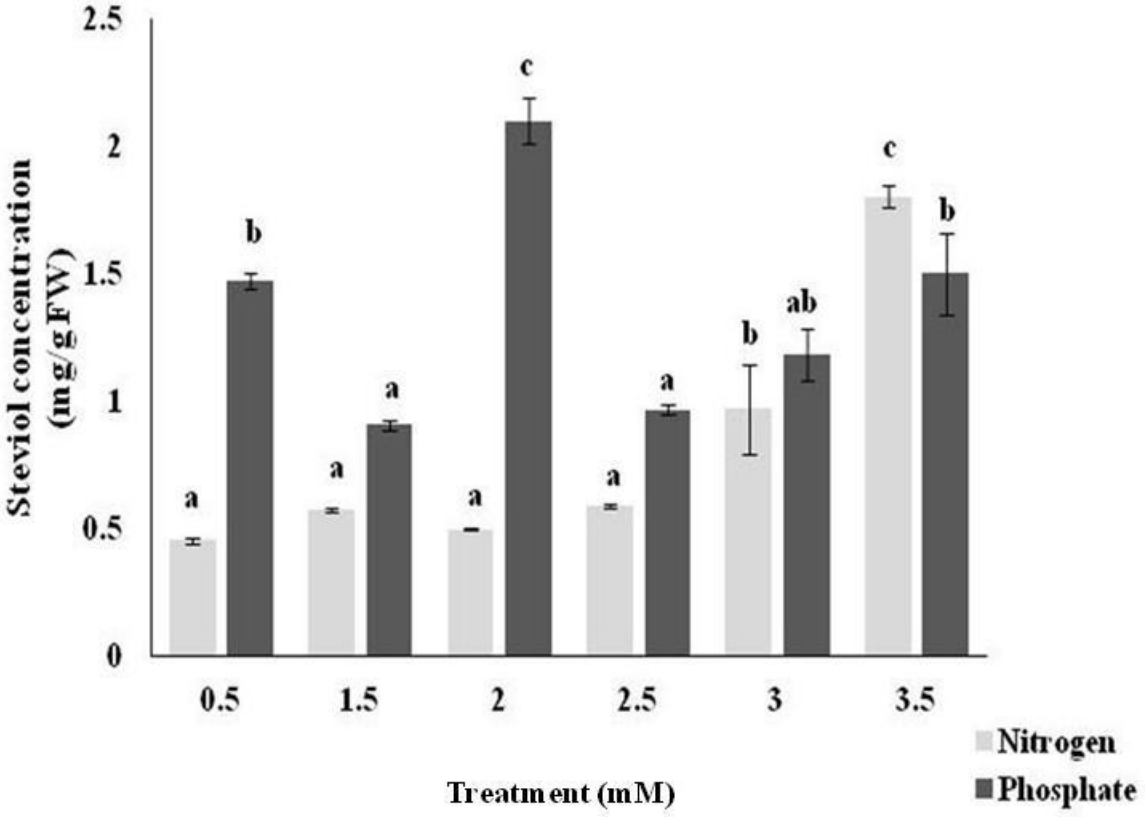

Figure 4.3.9 Effect of nitrogen and phosphate treatments on steviol production in *S. rebaudiana*. Plantlets grown under *in vitro* conditions for 21 days in varying levels of nitrogen and phosphate. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval (p=0.0000).

The production of steviol was directly correlated with increasing nitrogen supply (Figure 4.3.9) but not phosphate. In order to understand the influence of phosphate and nitrogen overall on secondary metabolism, a principal component analysis was used. Two separate clusters were observed (those of nitrogen and phosphate treatments). The phosphate cluster is located on the positive side of principal component 1 (Figure 4.3.10) which indicates its positive influence on the steviol glycosides presented in this study. Circles in the score plot of orthogonal partial least squares discriminant analysis of the LC-MS spectra of nitrogen (red) and phosphate (black) are assigned to unknown compounds that separate the nitrogen and phosphate from each other (Figure 4.3.11). The compounds responsible for separating the nitrogen and phosphate treated *Stevia* plantlets were tentatively identified according to their molecular weight, retention time and fragmentation. For nitrogen treated plants the compound responsible for this separation in *Stevia* plants is reported to have a molecular formula of C_4H_{62} O_{21} and forms the same fragment ion as stevioside and is speculated to be related to stevioside (Figure 4.3.12). Whilst compounds responsible for the separation of phosphate *S. rebaudiana* plants have been tentatively identified as Chlologenic acids, under these phenolic compounds the compound containing a mass to charge ratio *(m/z*) of 353.0809 and a molecular formular of $C_{16}H_{18}$ O_9 has been identified as caffeoylquinic acid and the compound with a *m/z* of 515.1188 and a molecular formular of $C_{25}H_{23}$ O_{12} was tentatively identified as a dicaffeoylquinic acid (Figure 4.3.13). The ion at m/z 707.1815 which is also responsible for the phosphate separation in *Stevia* plants is the dimer [2M-H] of the chlorogenic acid ion and is thus the same compound. Chlorogenic acids are found in many plant materials. They have many health benefits (anti-viral, hypoglycemic, hepatoprotective and immunoprotective activities) due to their high anti-oxidant activity (Perrone *et al*., 2008).

The compounds responsible for separating the nitrogen and phosphate treated *Stevia* plantlets are important to note as this suggests that phosphate and nitrogen act on different biochemical pathways, resulting in the production of different metabolites. At the highest concentration of nitrogen, applied in the current study, the 3.5 N treatment could also be distinguished from the other treatments. This pattern was different from the phosphate treatments as all treatments congregated in one zone. Furthermore, the 0.5 N and 1.5 N treated plants could be distinguished from the others as they formed a subgrouping lying on the third quadrant. This suggests similarities in terms of the chemical makeup. All other nitrogen treatments were located in the

fourth quadrant with the 2 N and 3 N plants clustering in closer proximity to the plants exposed to 3.5 N.

Production of steviosides is linked to terpenoid biosynthesis. Availability of nitrogen or phosphate may affect different enzymes linked to this biochemical pathway. Within the nitrogen source in MS media, both nitrate (NO_3^-) and ammonium (NH_4^+) are likely to have different effects in the plant. Although most plants grow rapidly on nutrient solutions that contain both nitrate and ammonium ions, than on solutions or media containing only one of the two nitrogen sources (George *et al*., 2008). Steviol precursors accumulate in the plastid and are linked to the MEP pathway (Brandle and Telmer, 2007). The chloroplast is thus central to the production of steviol glycosides and treatments that may influence chloroplastic function may alter steviol glycoside metabolism. Although not measured in this particular study, it would be interesting to understand the impacts of nitrogen and phosphate at the molecular level in terms of gene expression of the key regulatory genes linked to steviol biosynthesis and production of other terpenes.

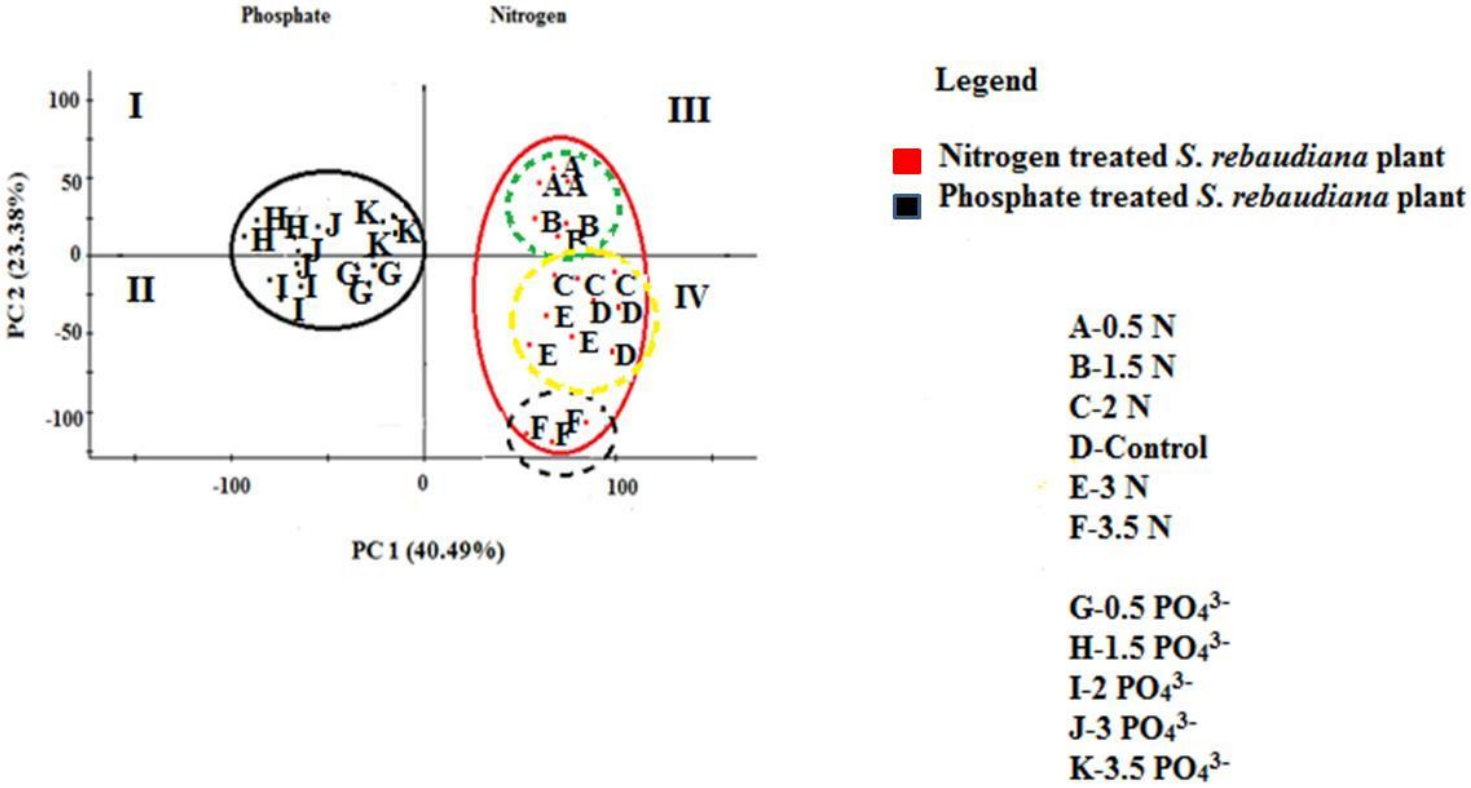

Figure 4.3.10 Score plot of principal component analysis based on LC-MS spectra of nitrogen and phosphate treated *S. rebaudiana* plantlets. Three replicates were represented for each sample. The black circle represents the phosphate treatments and the red circle represents the nitrogen treatments with three subgrouping (dashed circles). Green represents the 0.5 and 1.5 N, yellow represents the 2 N, control and 3 N and dashed black the 3.5 N treatments.

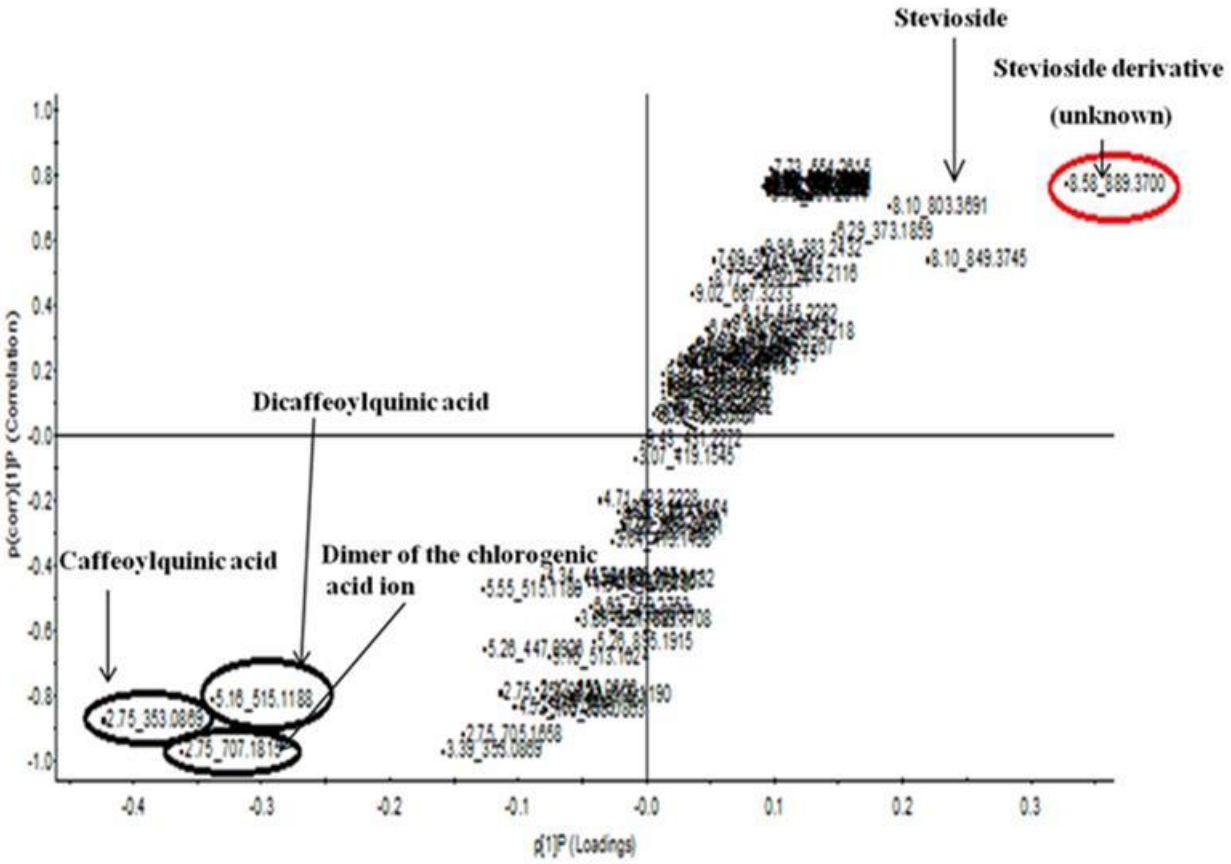

Figure 4.3.11 Score plot of orthogonal partial least squares discriminant analysis of LC-MS spectra of *S. rebaudiana* treated with nitrogen (red circles) and phosphate (black circles). The arrow (no circle) represents the precence of stevioside. Those chemicals surrounded by the circles were responsible for the groupings. The red circle represents the stevioside derivative and the black circles represent caffeoylquinic acid, dicaffeoylquinic acid and the dimer of the chlorogenic acid.

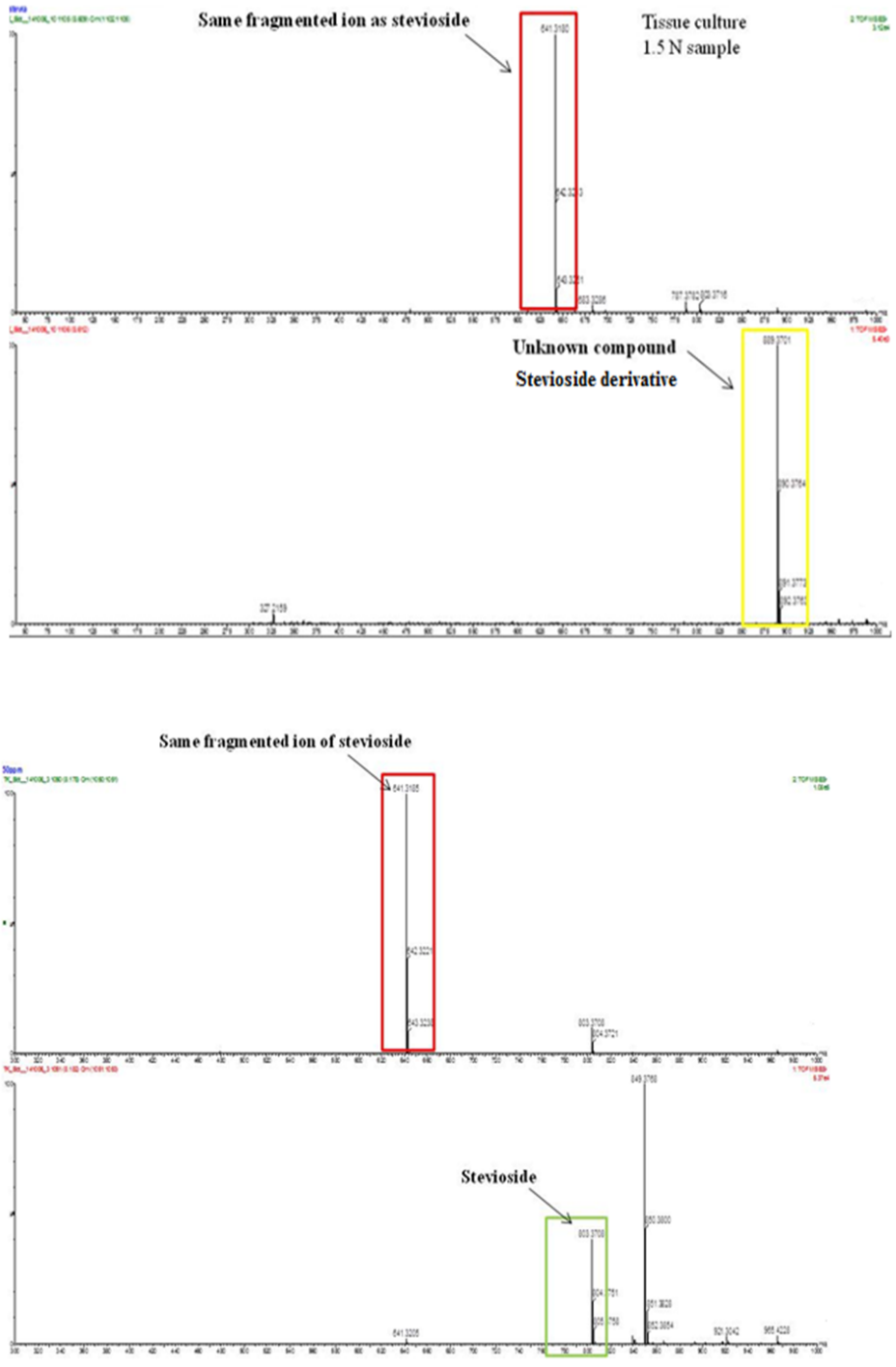

Figure 4.3.12 MS^E spectra of compound obtained using LC-MS operating in negative mode. **A**. *S. rebaudiana* sample treated with 1.5 N, the red rectangle marking shows the same fragmented ion present in stevioside. **B**. Unfragmented ion as the stevioside derivative (unknown compound) (yellow rectangle marking). **C**. Fragmented ion of stevioside (red rectangle marking). **D**. Stevioside (green rectangle marking).

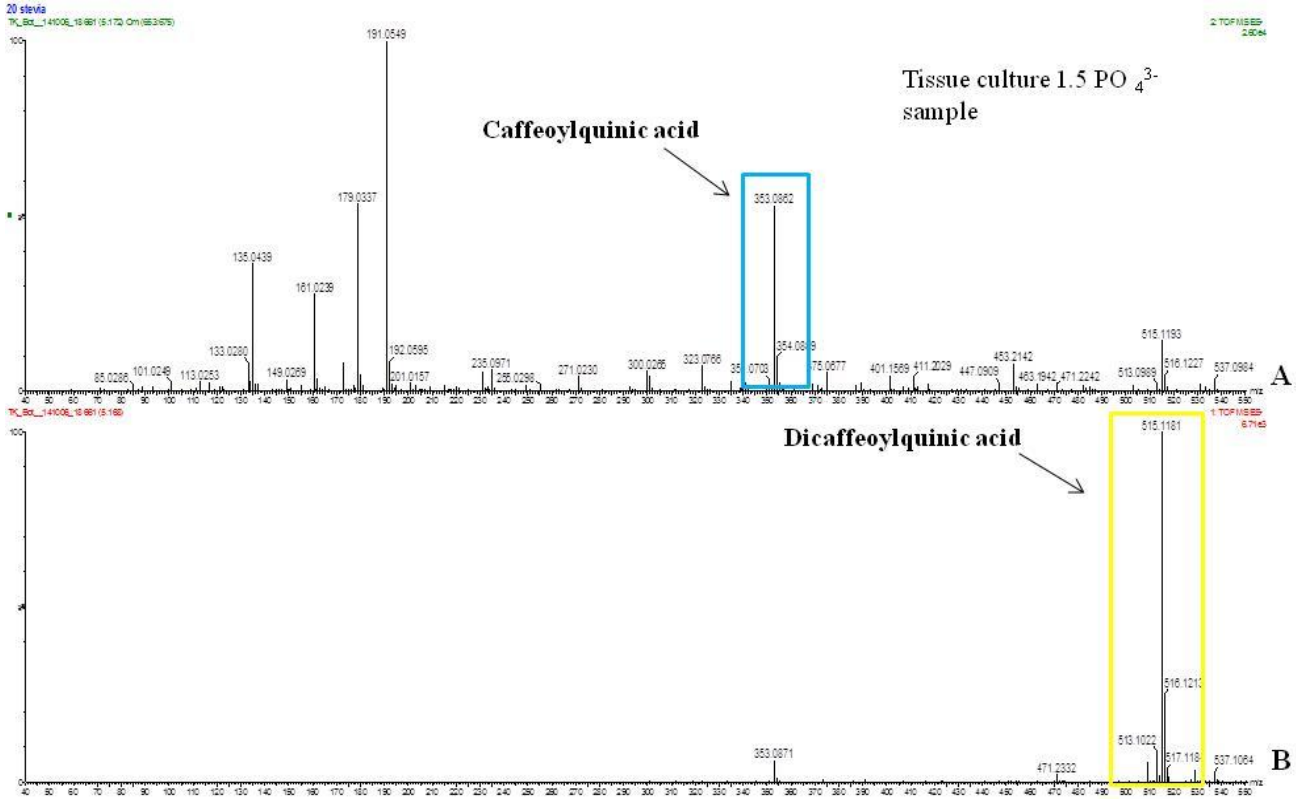

Figure 4.3.13 MS^E spectra of compound obtained using LC-MS operating in negative mode. **A**. Caffeoylquinic acid (blue rectangle marking). **B**. Dicaffeoylquinic acid (yellow rectangle marking).

4.4 Conclusion

In conclusion, growth of *Stevia* plants isinfluenced by nitrogen and phosphate levels with lowering of nitrogen in the medium to 0.5 N being linked to the tallest plants. Fewer roots were observed with higher levels of nitrogen and phosphate. Although speculative, clustering of phosphate treatments as a group distinct from the nitrogen treatments may indicate different influences at the enzyme and gene level on secondary metabolism resulting in different chemical profiles that distinguish these treatments. Furthermore, increasing the nitrogen levels in the medium causes chemical differences that result in chemotypic variation leading to distinct subgroups. This effect was not obvious with those plants growing on phosphate media. This suggests that the phosphate treatment may cause minor to negligible changes to the chemistry whereas the nitrogen treatment has a greater qualito-quantitative influence in *Stevia*. These treatments need to be tested and adopted in the field.

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Chapter Five

5. The influence of abiotic stresses on growth and development and differences in metabolite profiles regarding the main bio-actives (diterpene glycosides) of *S. rebaudiana*

5.1 Introduction

High salinity and drought conditions are the major abiotic stresses that prevail in many parts of the world. Soil salinity, however, is said to be the most limiting growth factor experienced by most plants (FAO, 2007). It is known to be one of the most severe abiotic stresses and as a result it has a very negative effect on agricultural productivity where almost 20% of irrigated land all over the world and one-third of the world arable soil are affected by this stress (Pérez-López *et al*., 2013; Rhoades *et al*., 1990). There is a relation between growth and yield of crops and salinity that is well established in the scientific literature, usually the higher the salinity level results in less growth and yield of the crop (Ityel *et al*., 2012; Jamil *et al*., 2012; Shannon and Grieve, 1999). It is important to also note that over 40% of the world's arable lands does not have enough rainfall to support economically viable agriculture (Tanwar, 2003). Some plants implement strategies of resisting these stresses by using mechanisms such as osmotic adjustment which helps preserve water in the plants tissues when facing soil water stress (Chen and Jiang, 2010).

According to literature, *S. rebaudiana* is prone to drought and salinity stress (Cony and Trione, 1998; Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). This plant naturally grows in mountainous areas, open forests, dry valleys, borders of rivers and is commonly found on the outskirts of the marshland of sandy, infertile (poor soils), acid soils of Paraguay but for an economic production, crop irrigation is required (Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). *S. rebaudiana* originates from a semi-humid area (Paraguay and Brazil) with a yearly average rainfall of 1500 mm. Drought stress limits the appearance of leaves and hence affects steviol glycoside accumulation. *Stevia* can be cultivated as an irrigated summer crop in Europe, being suitable to be cultivated in semi-arid climates and

coastal areas, like the Mediterranean region, which are characterized by the low quality of the irrigation water. It has also been suggested to grow under different conditions regarding climate and soils (Hajar *et al*., 2014; Lemus-Mondaca *et al*., 2012). Reis *et al*. (2015) concluded that *Stevia* is suitable to be grown in semi-arid regions, if well irrigated, even with a relatively high salinity, but only one harvest could be possible. Two harvests can be obtained if only low salinity irrigation water is used, positively due to plant regeneration problems related to the increased soil solution salinity. Although *Stevia* is affected by salinity, according to the experimental results, it was demonstrated that *Stevia* tolerance (2 dS m^{-1}) to salinity was higher than sugar cane (1.1 dS m^{-1}), and crop sensitivity was lower in *Stevia* than in conventional sugar crops such as sugar beet (4.7 dS m^{-1}). Hajihashemi and Ehsanpour (2013) used *in vitro* culture conditions and applied polyethylene glycol to stimulate drought stress on *Stevia*. Their results reported that fresh and dry weight, water content, chlorophylls, carotenoids and anthocyanins were negatively affected. Yet, antioxidant activity and enzymatic defense systems (catalase, ascorbate peroxidase, polyphenol oxidase and peroxidase) increased (Hajihashemi and Ehsanpour, 2014). Lavini *et al.* (2010) reported that the harvest index and water use efficiency in *Stevia* decreased with the increase in irrigation regime (irrigation with 33, 66 and 100% restitution of water consumption), while the stevioside and the rebaudioside A contents were unaffected by irrigation regimes.

The accumulation of secondary compounds in plants is influenced by both biotic and abiotic factors (Colling *et al*., 2010; Musarurwa *et al*., 2012; Selma and Kleinwächter, 2013). Under drought stress, the same amounts of natural products are synthesized and accumulated as under well-watered conditions but due to the reduction in biomass their concentration simply is enhanced (Selma and Kleinwächter, 2013). The application of drought stress for better secondary accumulation is reported in several plants; *Salvia miltiorrhiza* (Liu *et al*., 2011), *Senecio jacobaea* (Kirk *et al*., 2010), *Petroselium crispum* (Petropoulos *et al*., 2008), *Hypercicum brasliense* (De Abreu and Mazzafera, 2005), *Picea abies* (Turtola *et al*., 2003) to name a few but this occurrence has not been sufficiently investigated in *Stevia*.

In this Chapter, the effect of water and salinity stress were studied *in vitro*. Thereafter, the metabolite content was analysed to determine the effects of these *in vitro* conditions.

5.2 Materials and methods

5.2.1 Plant material

The plant material was prepared similarly to the previous chapter (refer to Chapter 4; Section 4.2.1).

5.2.2 *In vitro* manipulation of polyethylene glycol 6000 (PEG) and sodium chloride (NaCl)

To stimulate water stress, four treatments using polyethylene glycol 6000 (PEG) were established to represent low and high water stress 2.5%, 5%, 7.5% and 10% (w/v) were added to the MS medium. To induce salt stress, nodal explants were placed in MS containing different concentrations of sodium chloride (NaCl) namely 25 mM, 50 mM, 75 mM and 100 mM. Control experiments were those supplied with the no addition of PEG or NaCl on the MS medium. One nodal explant was placed in each glass test tube (90 mm × 50 mm) containing 10 mL medium. For each treatment ten replicates were used. The experiment was repeated three times. Nodal explants were cultured for 21 days. After this time, both PEG 6000 and NaCl treated *Stevia* plants were harvested. Data were collected by recording the number of regenerated shoots, shoot length (mm), number of leaves, fresh weight (mg), root length (mm) and root number from each explant. Three samples from each treatment were randomly selected for metabolomic profiling.

5.2.3 Extraction of plant material for LC-MS

The same extraction process was carried as previously documented (see Chapter 4; Section 4.2.3). The gradient started with 95% using 0.1% (v/v) formic acid (solvent A) and 5% acetonitrile (solvent B). Followed by a change in the gradient to 60% of 0.1% formic acid (solvent A) and 40% acetonitrile (solvent B) at 9 minutes, 30% solvent A and 70% solvent B over 9.1 minutes, 100% solvent B at 14 minutes, 95% solvent A and 5% solvent B at 14.01 minutes. Thereafter, this remained constant over 16 minutes with solvent A at 95% and solvent B at 5%. Similar approaches were then followed as in Chapter 4 (Section 4.2.4).

5.2.4 HS-SPME-GC-MS protocol

For Headspace Solid-Phase Micro Extraction (HS-SPME) and Gas Chromatography-Mass Spectrometry (GC-MS) analysis refer to Chapter 4 (Section 4.2.5).

5.2.5 Statistical analyses

Arcsine transformations of percentage data were conducted. After normality testing, data were analyzed via ANOVA and a Tukey's HSD test or Krustal Wallis post hoc analysis was applied to the data at the 95% confidence level to differentiate the means. The chemical data were subjected to PCA analysis to reveal relationships linked to metabolite profiles after LC-MS analysis for both NaCl- and PEG-treated plants. For the GC-MS data, only those identified compounds with a library match of 85% to 100% are shown in Table 5.1 and Table 5.2. The NIST library was used as a reference to guide the identification of metabolites. Data are represented as relative percentages and where possible, chemical reference standards were used to determine the identity together with molecular ion peaks, MS fragmentation, and retention times.

5.3 Results and discussion

5.3.1 The effect of salt stress on the growth and development of *S. rebaudiana*

This study examined the effects of four different NaCl concentrations (25, 50, 75 and 100 mM) on the growth, development and steviol glycoside composition of *S. rebaudiana* for 3 weeks. The results showed that the total fresh weight decreased by 71.1% at 100 mM NaCl. The control (0.724 ± 0.181 mg) and treatment 25 mM treatment (0.677 ± 0.079 mg) were the highest in producing fresh weight which was statistically similar (Figure 5.3.2 A). It was interesting to note that there was no statistical difference with the addition of NaCl from 50 to a 100 mM with regards to the fresh weight. Increasing salt concentrations correlated to negative effects linked to growth and development. Addition of 50-100 mM NaCl generated shorter plants than the control (Figure 5.3.1 A). This effect was the same for other parameters measured (Figure 5.3.2). The same pattern was observed for the number of leaves (Figure 5.3.1 B), internodes (Figure 5.3.1 C), roots (Figure 5.3.2 B) and root length (Figure 5.3.2 C). For instance, no roots were found on *Stevia* plants treated with 75 and 100 mM NaCl.

Zeng *et al*. (2013) looked at three different NaCl concentrations (60, 90, and 120 mM) using a modified Hoagland's solution for 4 weeks. This solution was modifeied by adding iron with a chelating agent known as ethylenediaminetetraacetic acid (EDTA) to stop precipitation.They also observed that an increase of the salt treatment in *S. rebaudiana* significantly reduced the growth of the plant. However, no statistical difference was established with those NaCl treatments respectively. The leaves are the main plant organ accountable for light absorption, for the manufacture of food through the process of photosynthesis providing energy and nutrients. Zheng *et al*. (2013) suggested that stunted growth may be caused by salt stress mainly through the damage to leaf function. This could explain the decrease in leaf number and size with the increase in NaCl in the present study.

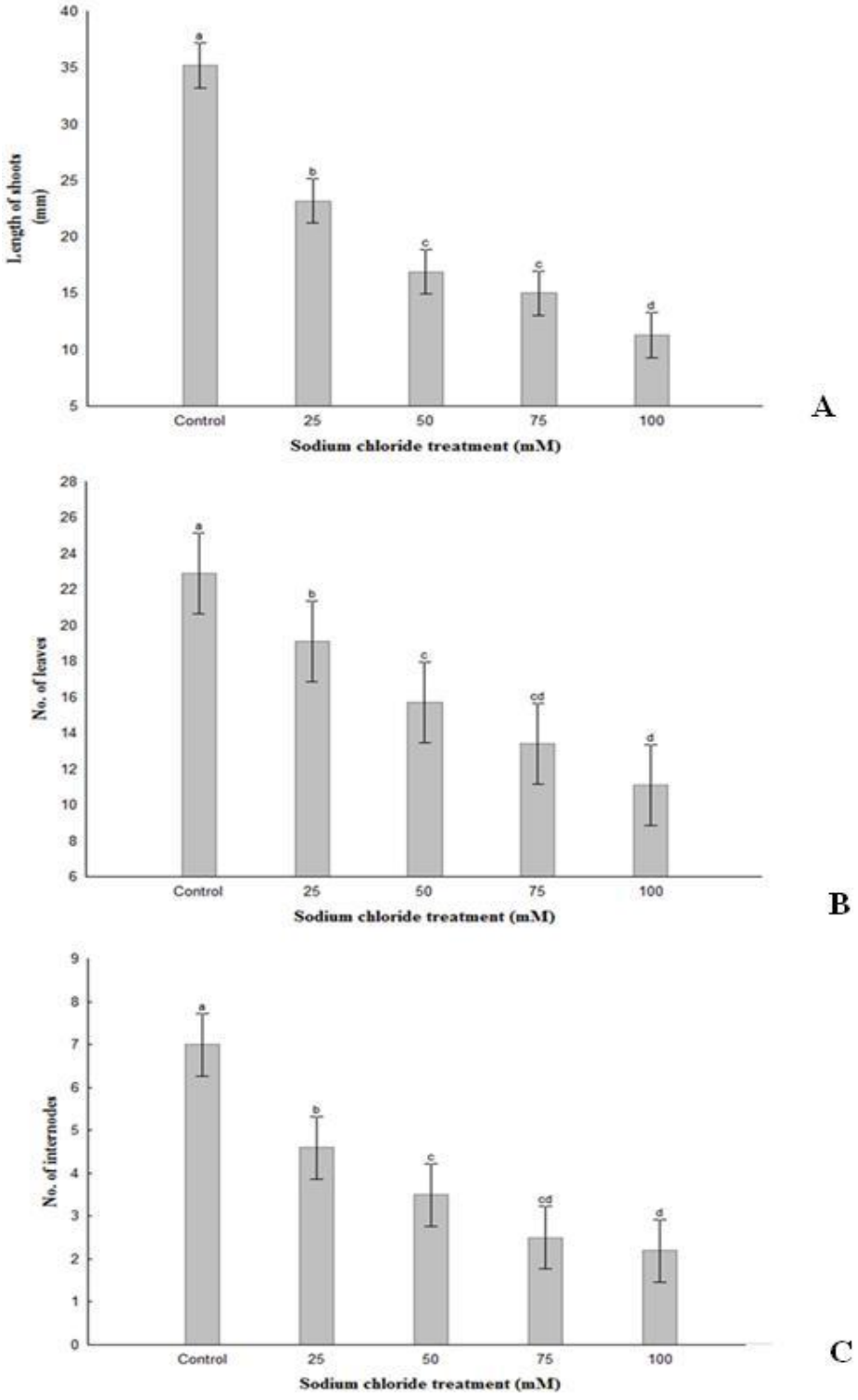


Figure 5.3.1 *S. rebaudiana in vitro* growth after 21 days in varying levels of sodium chloride treatments. **A**. Length of shoots (mm). **B**. Number of leaves. **C**. Number of internodes. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

Salinity reduces the ability of plants to take up water, and this quickly causes reductions in the growth rate. Sheng and Xiuling (1997) reported that soil salinity may not necessarily decrease the amount of crop yield considerably until a threshold level is excelled. The growth reduction due to salinity stress also depends on the period of time over which the plant has grown in such conditions. Plants exposed for short periods of time also experience a significant decrease in the growth rate (Munns, 2002). Leaf growth is often more reduced than root growth by salinity (Munns, 2002), however, in this study the roots decreased with an increase in saline conditions (Figure 5.3.2 B and C). At 75 and 100 mM NaCl, no roots were formed. Higher Na^+ content in leaves may inhibit photosynthesis (Munns, 2002; Sheng and Xiuling 1997). In salt-sensitive species, salt build up in leaves becomes toxic. This results in a progressive loss of the older leaves with time. However, if old leaves die faster than new leaves are produced, then the proportion of leaves that are injured starts to increase, and the number of green and healthy leaves will ultimately decline.

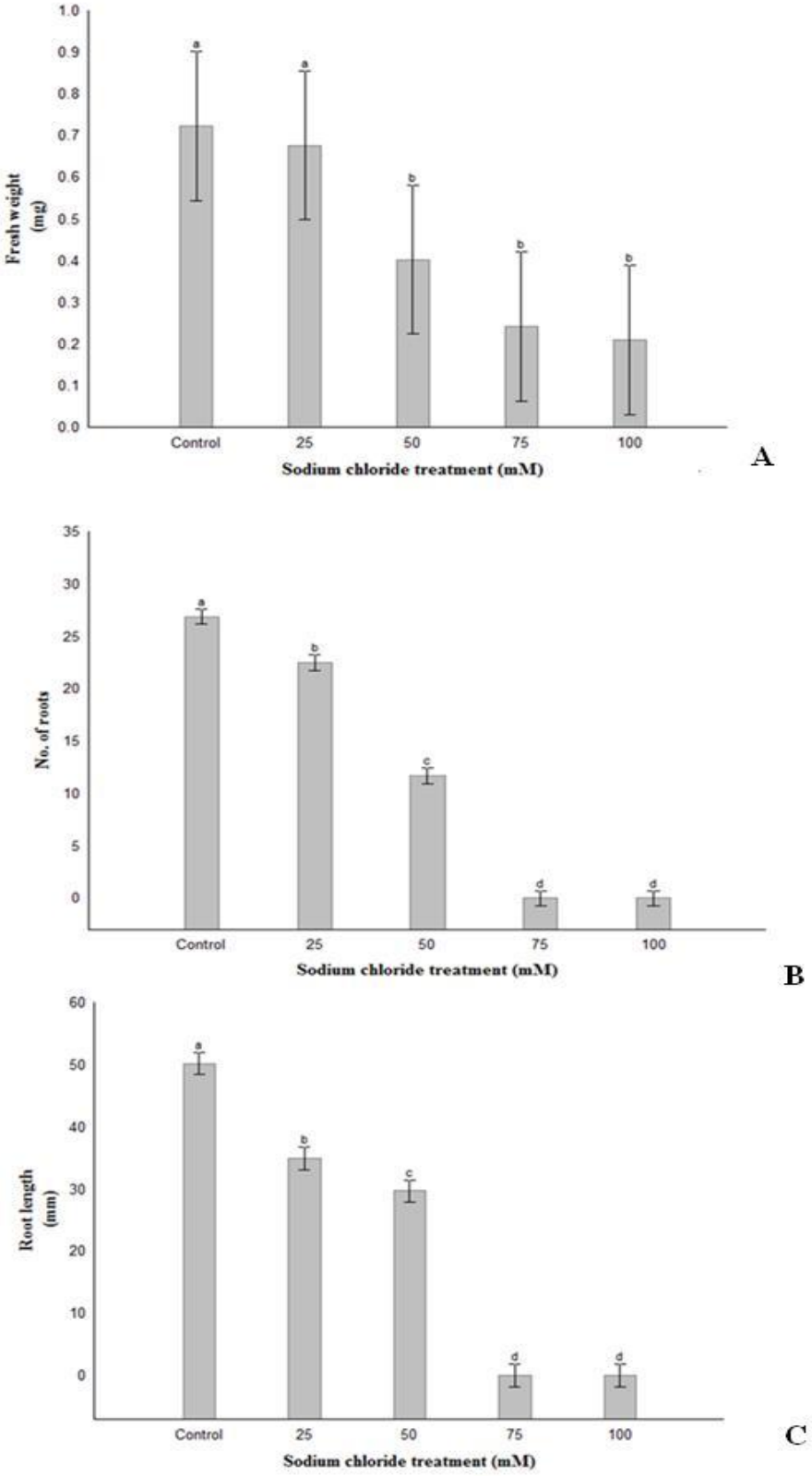


Figure 5.3.2 *S. rebaudiana in vitro* growth after 21 days in varying levels of sodium chloride treatments. **A**. Fresh weight (mg). **B**. Number of roots. **C**. Root length (mm). Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

Figure 5.3.3 *S. rebaudiana in vitro* growth after 21 days in varying levels of sodium chloride treatments. **A**. Control (with no addition of NaCl). **B**. 25 mM NaCl. **C**. 50 mM NaCl. **D**. 75 mM NaCl and **E**. 100 mM NaCl.

5.3.2 The effect of drought stress on the growth and development of *S. rebaudiana*

Four treatments using PEG were used to stimulate water stress, to represent low and high water stress 2.5%, 5%, 7.5% and 10% (w/v) were added to the MS medium on the growth, development and steviol glycoside composition of *S. rebaudiana* for 21 days. The results showed that increase in drought stress had a negative impact on all the parameters measured in this study (Section 5.2.2). There was a close correlation with the increase of drought stress imposed by PEG 6000 with growth of *S. rebaudiana* (Figure 5.3.4 and Figure 5.3.5). At 7.5% PEG 6000 showed the lowest growth of *Stevia* plant. This indicates that *Stevia* can take moderate drought stress. Drought stress is known to limit photosynthesis in plants as it alters carbon allocation between roots and shoots and changes nutrient uptake ratios and nutrient circulation (Turtola *et al*., 2003). Hajihashemi *et al*. (2013) showed that PEG treatments could significantly reduce steviol glycoside accumulation in *S. rebaudiana*. They investigated a possible correlation between transcription of some genes involved in steviol glycoside biosynthesis and steviol glycoside contents in *S. rebaudiana* treated with PEG in combination with paclobutrazol (PBZ) and gibberellic acid (GA). Lavini *et al*. (2010) reported that the harvest index and water use efficiency in *Stevia* decreased with the increase in irrigation regime (irrigation with 33, 66 and 100% restitution of water consumption), while the stevioside and the rebaudioside A contents were unaffected by irrigation regimes.

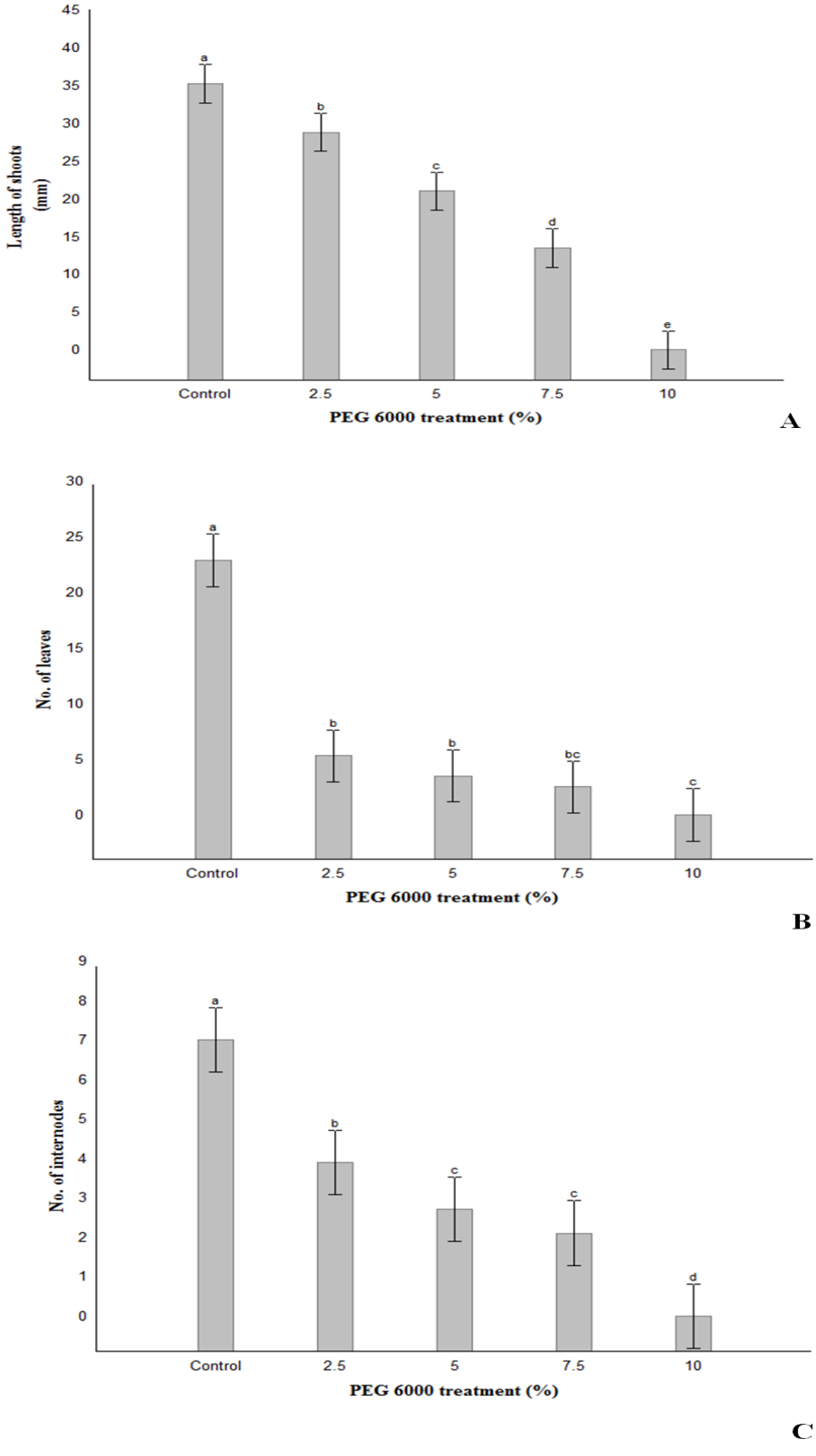


Figure 5.3.4 *S. rebaudiana in vitro* growth after 21 days in varying levels of polyethylene glycol 6000 (PEG 6000) treatments. **A**. Length of shoots (mm). **B**. Number of leaves. **C**. Number of internodes. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

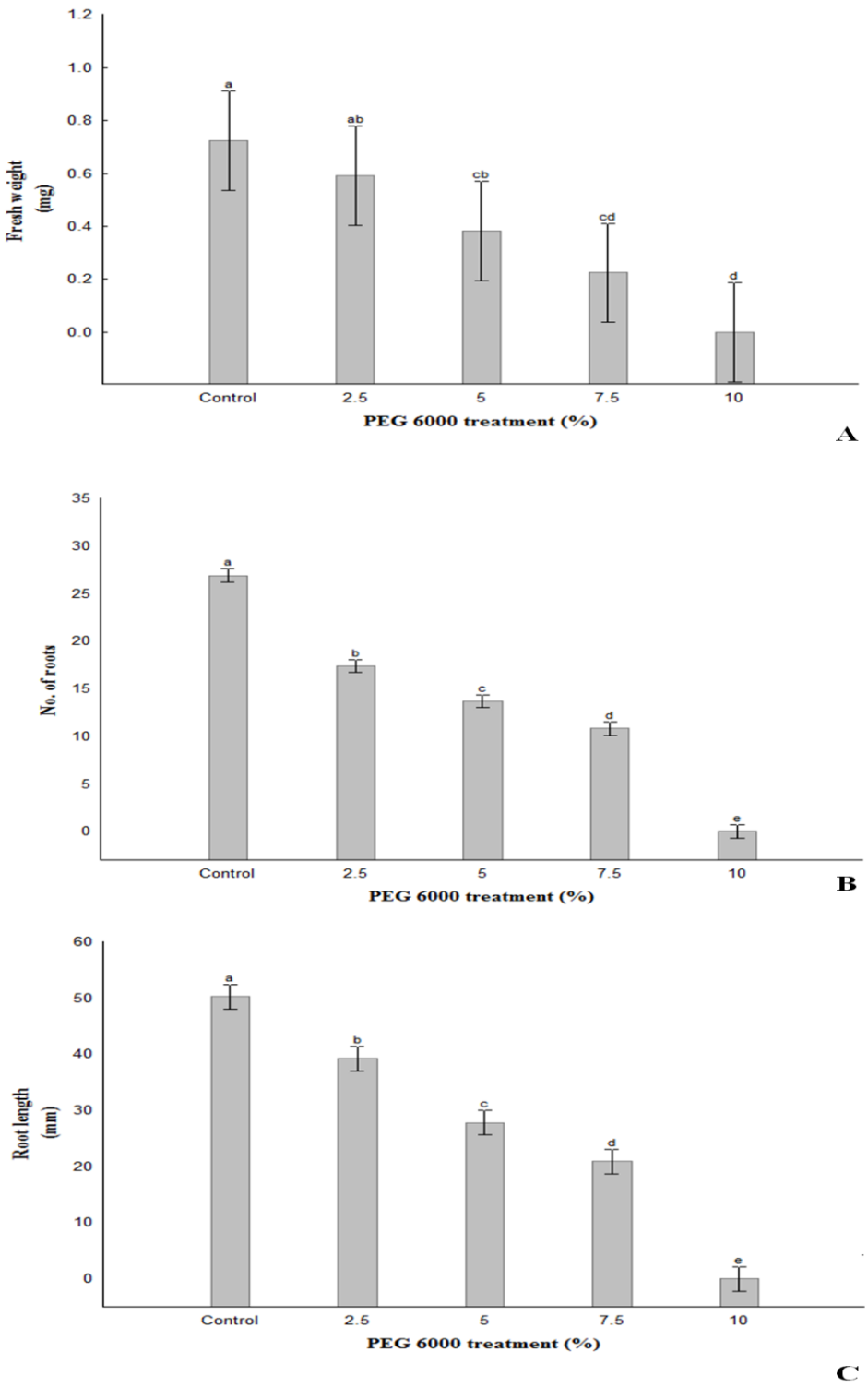


Figure 5.3.5 *S. rebaudiana in vitro* growth after 21 days in varying levels of polyethylene glycol 6000 (PEG 6000) treatments. **A**. Fresh weight (mg). **B**. Number of roots. **C**. Root length (mm). Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

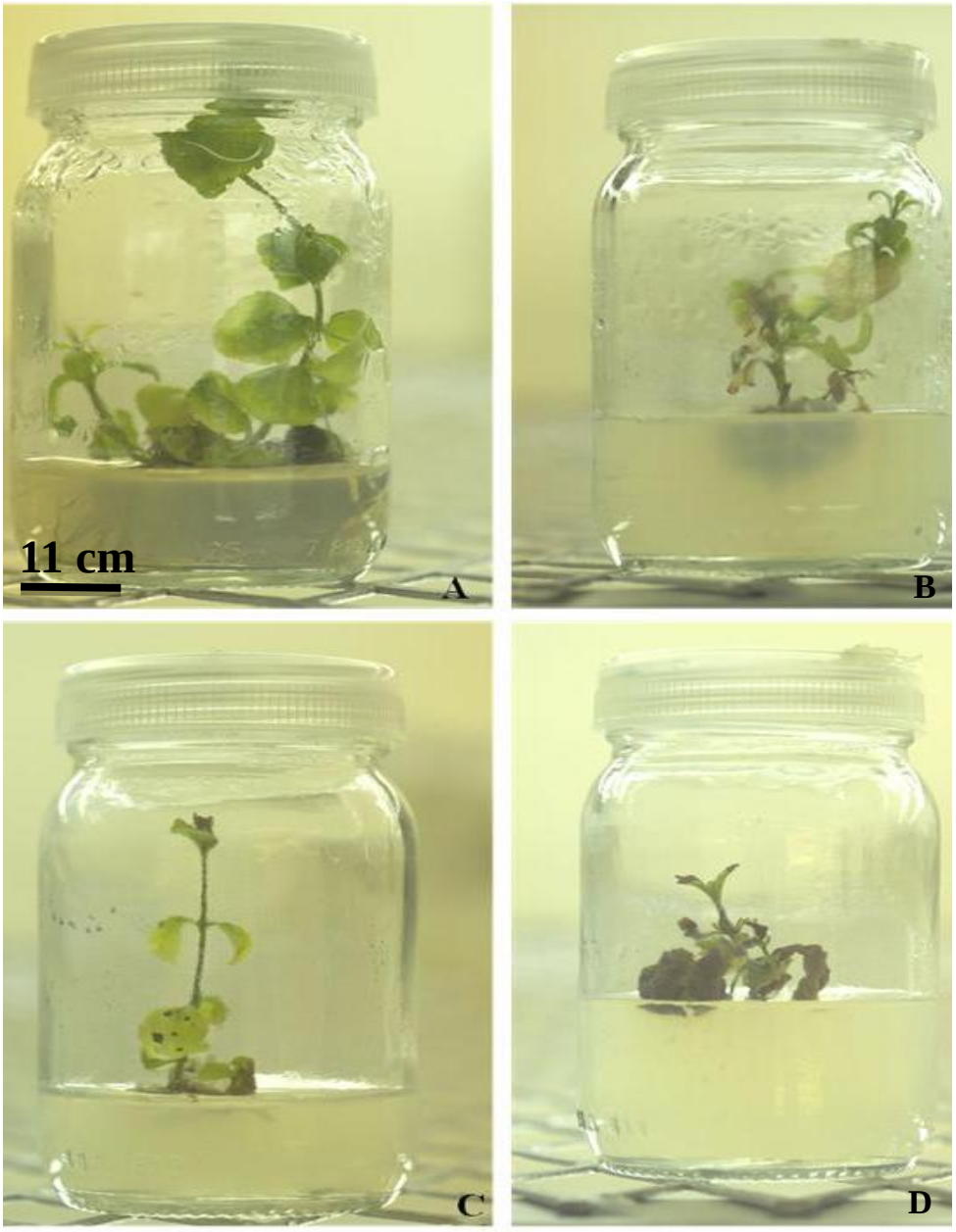


Figure 5.3.6 *S. rebaudiana in vitro* growth after 21 days in varying levels of polyethylene glycol 6000 (PEG 6000) treatments. **A**. Control (with no addition of PEG 6000). **B**. 2.5% (w/v) PEG 6000. **C**. 5% (w/v) PEG 6000. **D**. 7.5% (w/v) PEG 6000. *Stevia* plantlets showing signs of growth reduction with increase of PEG 6000.

Table 5.1 Volatile metabolite accumulation in response to changes in NaCl concentrations in MS media.

Compound	Retention time	Kovats index	Control	Relative abundance (%)			
				25 mM	50 mM	75 mM	100 mM
α-Pinene	5.80	1007	6.076 ± 0.699 a	5.709 ± 0.321 a	6.051 ± 0.622 a	3.193 ± 0.524 b	3.823 ± 0.318 b
β-Pinene	7.34	1138	62.939 ± 4.2118 a	60.733 ± 4.405 a	46.933 ± 14.424 b	41.376 ± 0.793 c	40.589 ± 2.612 c
Sabinene	7.62	1147	3.093 ± 0.197 a	2.786 ± 0.273 ab	2.219 ± 0.335 b	2.107 ± 0.119 b	1.309 ± 0.122 c

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

Table 5.2 Volatile metabolite accumulation in response to changes in PEG 6000 percentages in MS media.

Compound	Retention time	Kovats index	Control	Relative abundance (%)			
				2.5%	5%	7.5%	10%
α-Pinene	5.80	1007	6.076 ± 0.699 a	6.072 ± 0.259 a	6.012 ± 0.718 a	0 b	0
β-Pinene	7.34	1138	62.939 ± 4.2118 a	57.435 ± 2.611 a	41.901 ± 4.519 b	21.725 ± 3.684 c	0
Sabinene	7.62	1147	3.093 ± 0.197 a	2.526 ± 0.254 a	1.884 ± 0.174 b	0 c	0

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

5.3.3 Effects of NaCl and PEG 6000 on essential oil components found in *S. rebaudiana*

I paid attention to the major chemicals that had an 85-100% library match (Table 5.1 and 5.2). In this study, three volatile compounds were detected in propagated plants of *S. rebaudiana* (Tables 5.1 and 5.2). High levels of PEG 6000 in the growth medium resulted in plants that had none of the above compounds (Tables 5.2).

As essential oils are expensive phytochemicals produced and extracted from specific species, great attention has to be given to such valuable natural resources. It is known that *S. rebaudiana* is prone to both salinity and drought stress (Cony and Trione, 1998; Lemus-Mondaca *et al*., 2012; Ramesh *et al*., 2006; Yadav *et al*., 2011). I was able to show that at high concentrations of 10% (w/v) PEG 6000, essential oil compounds were not detected. This is different to the report given by Selma and Kleinwächter (2013) using sage (*Salvia officinalis*) under drought stress. The same amounts of natural products were synthesized and accumulated as under well-watered conditions but due to the reduction in biomass their concentration simply was enhanced. When the plants had been cultivated under moderate drought stress, the overall content as well as the concentration of total monoterpenes was markedly higher than in the corresponding, well-watered control plants. In other species, this stress-induced enhancement of natural products could be explained by the fact that when stomata close as a result of lack incipient water, the uptake of carbon dioxide (CO_2) also lowers. Although speculative, this results in the consumption of reduction equivalents ($NADP^+H$) for the CO_2^- fixation via Calvin cycle which declines considerably, generating a massive oversupply of $NADP^+H$ (Selma and Kleinwächter, 2013). The outcome of this is that the metabolic processes are pushed toward the synthesis of highly reduced compounds such as secondary metabolites (Bajaj and Ishimaru, 1999; Selma and Kleinwächter, 2013). Gupta *et al*. (2016) looked at four chemicals namely: NaCl, Na_2CO_3, Proline and PEG, investigating their influence on growth parameters and steviol glycoside production in *S. rebaudiana* shoots cultured *in vitro*. From these chemicals tested, they were able to report that the stressed *S. rebaudiana* plants were able to give enhanced steviol glycoside production almost three times more than the control. This was an interesting result as we did not obtain the same data except in the case of *S. rebaudiana* plants treated with 25 mM NaCl that seemed to increase steviol concentration significantly (Figure 5.3.7 C). No suggestions could be made for this increase.

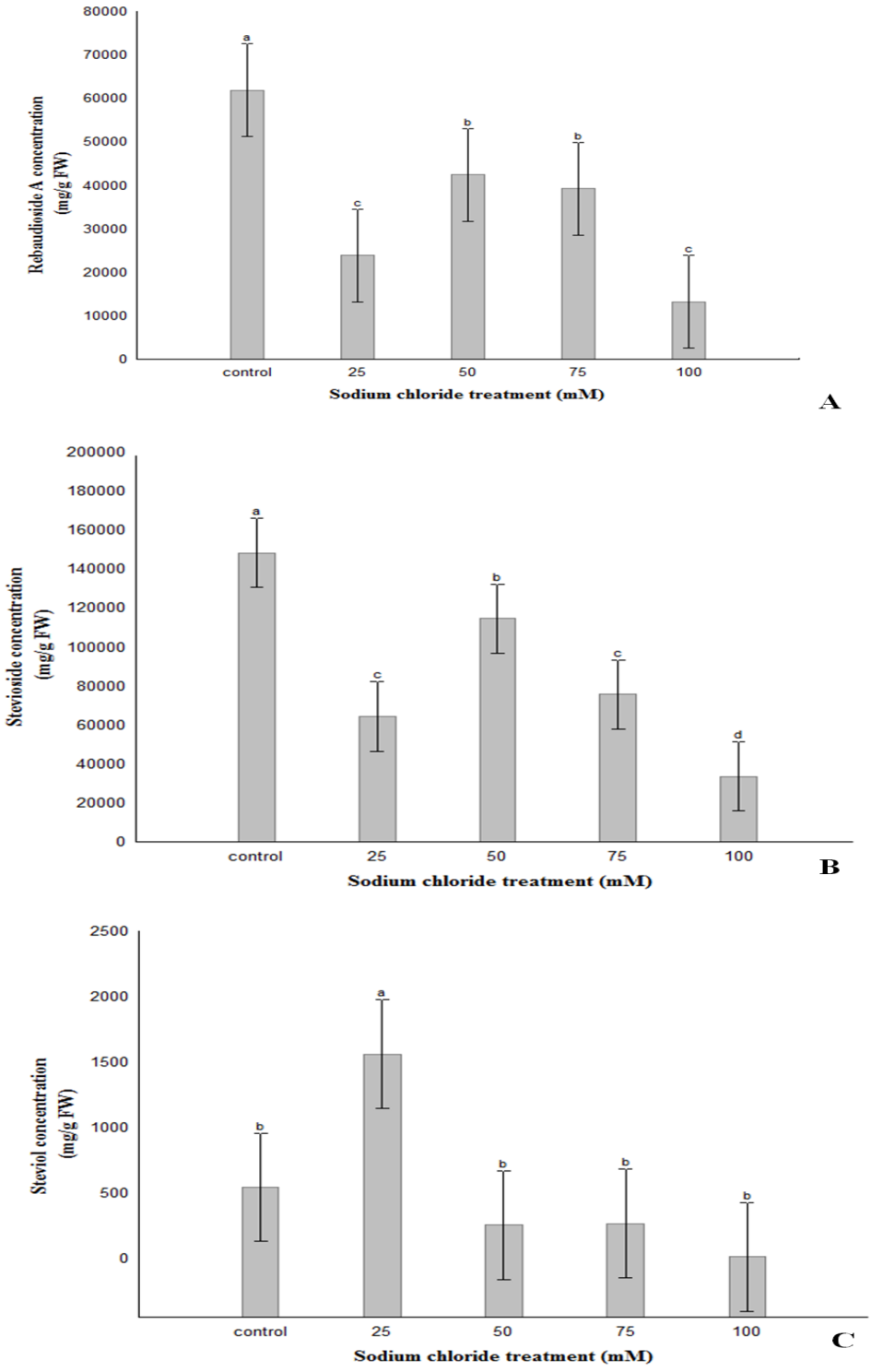


Figure 5.3.7 *S. rebaudiana in vitro* growth after 21 days in varying levels of sodium chloride treatments. **A**. Rebaudioside A content. **B**. Stevioside content. **C**. Steviol content. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

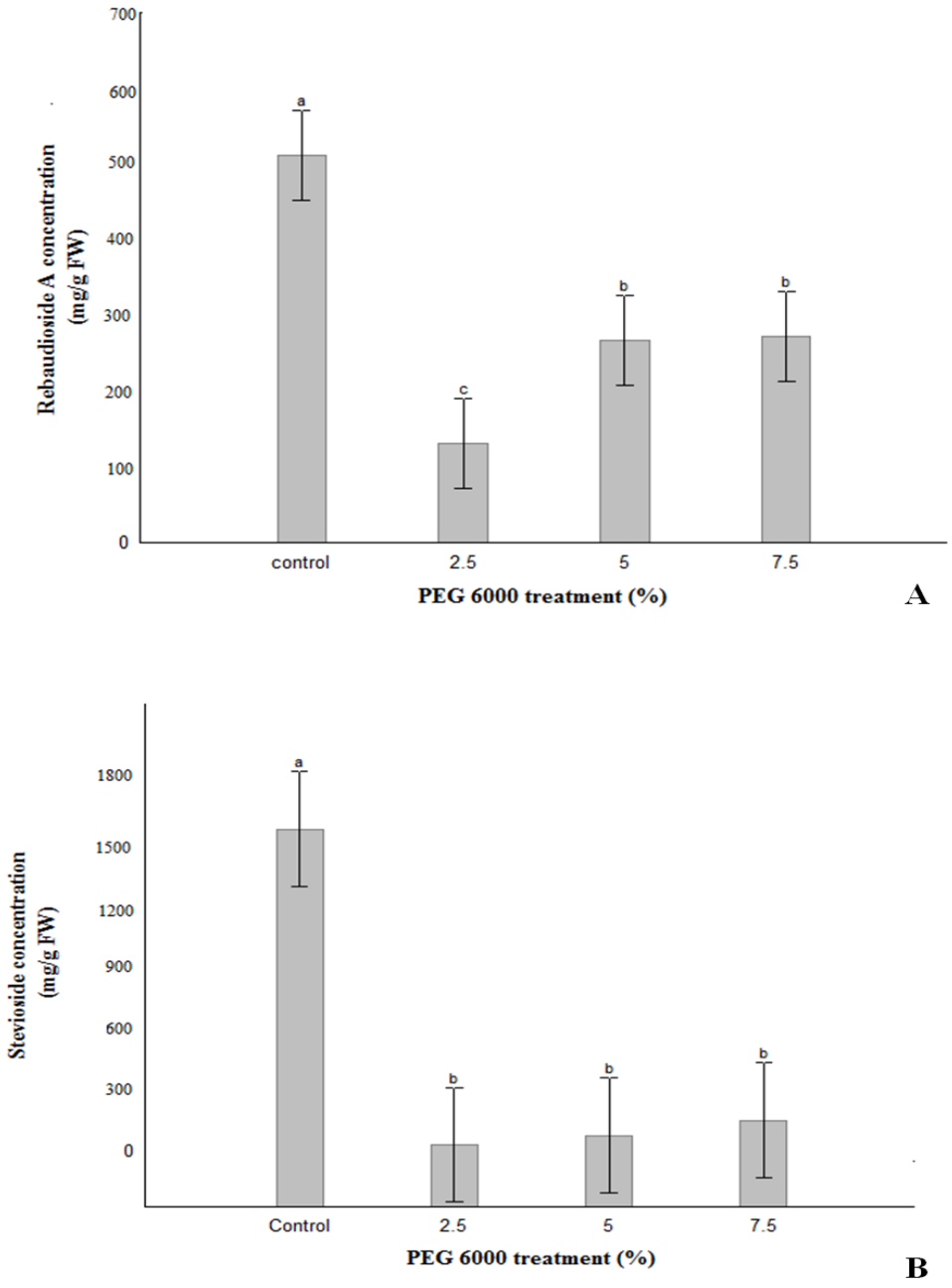
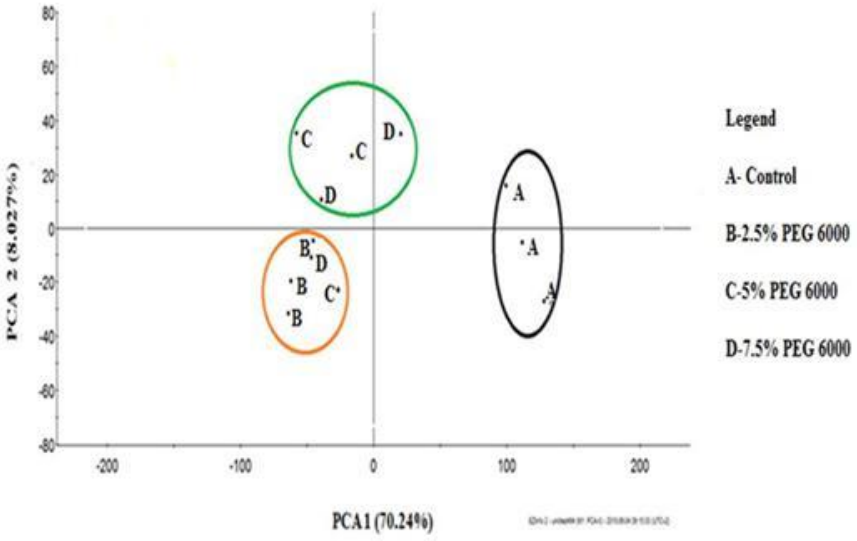


Figure 5.3.8 *S. rebaudiana in vitro* growth after 21 days in varying levels of polyethylene glycol 6000 (PEG 6000) treatments. **A**. Rebaudioside content. **B**. Stevioside content. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95% confidence interval.

Moreover, a reduction in the steviol glycosides namely rebaudioside A and stevioside was noted with the increase of NaCl and PEG 6000 (Figure 5.3.7 and Figure 5.3.8 respectively). In the case of 25 mM NaCl there was a significant increase (1560.339 ± 396.297 mg/g FW) of steviol (Figure 5.3.7 C). The addition of NaCl to 50 mM resulted in the reduction of steviol (254.595 ± 54.527 mg/g FW) until it remained constant with any salt increase (Figure 5.3.7 C). The addition of PEG 6000 caused drastic declines of steviol and this chemical could not be detected. It is also important to report that the addition of PEG 6000 left both rebaudioside A and stevioside at significantly lower levels than the control (Figure 5.3.8 A and Figure 5.3.8 B respectively).

A similar result was reported by Hajihashemi *et al*. (2013) where stevioside and rebaudioside A content both noticeably decreased in PBZ and PEG treatments. It can be concluded that the change in rebaudioside A content is due to its precursor and UGT76G1 transcription. Overexpression of some glycosyltransferase genes led to a significant increase in their respective glucosides. The results indicated that inhibition of growth by both PBZ and/or PEG treatments resulted in the same negative effect on gene transcription and steviol glycoside accumulation. In order to understand the influence of the different percentages of PEG 6000 on the secondary metabolism of the *Stevia* plantlet, a principal component analysis was used (Figure 5.3.9 A). There was a clear separation of the treated *Stevia* plantlets from the control. The control was located on the positive side of PC1 (Figure 5.3.9 A). Circles in the loading of principal component analysis based on LC-MS spectra of treated *Stevia* extracts reveal unknown compounds (Figure 5.3.9 B), whereas, the arrows indicate rebaudioside A and stevioside.



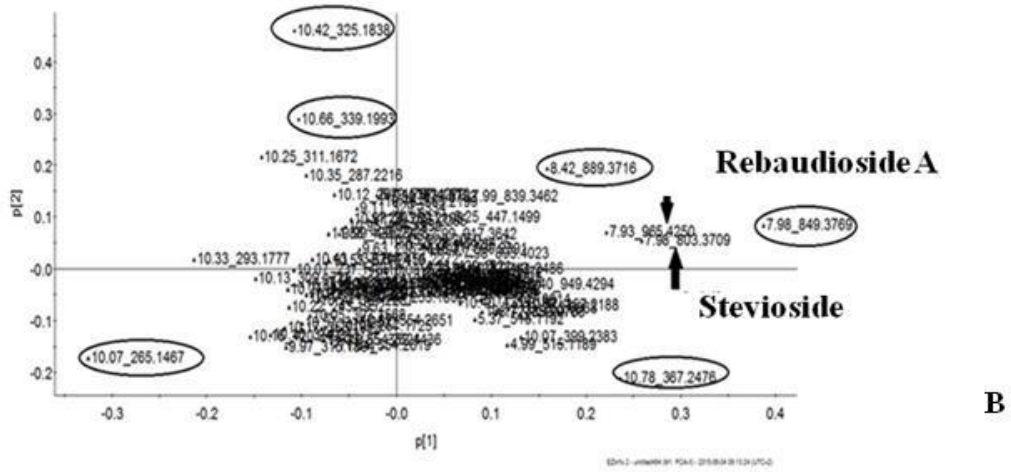


Figure 5.3.9 **A**. Score plot of principal component analysis based on LC-MS spectra of different percentages (2.5, 5 and 7.5 %) of PEG 6000 treated *S. rebaudiana* extracts. Three replicates were represented for each sample. **B**. Loading of principal component analysis. Arrows indicate rebaudioside A and stevioside content. Circles are assigned to unknown compounds.

5.4 Conclusion

In conclusion, both salinity and drought stress affected most of the growth parameters measured in the *Stevia* plantlets. High concentrations of salt, at 75 and 100 mM, adversely affected growth. Addition of polyethylene glycol 6000 reduced the concentrations of rebaudioside A and stevioside significantly. Interestingly, the addition of 25 mM NaCl resulted in a significant increase of steviol. Addition of 10% PEG 6000 to MS, completely hampered growth and the explants lost their regeneration potential. Further studies are required to conclude on transcriptomic and proteomic changes in order to learn the effects at the molecular level.

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Chapter Six

6. General conclusion and future prospects

The interest in *S. rebaudiana* is a result of its great economic and scientific value around the world due to its sweetness and reported therapeutic properties. Due to its popularity it is cultivated in many part of the world except in southern Africa. Commercial cultivation of *Stevia* in southern Africa would thus add another crop of economic value to the existing agricultural sector. *S. rebaudiana* seeds possess a very low germination capacity which is a challenge for large scale propagation. In order to accomplish the aim of improved germination, in **Chapter 3** a tissue culture based protocol to investigate optimal seed germination was utilised. The smoke treatment that includes smoke water (1000 µm) was added to 1000 mL of germination medium. This was the best method for increasing the germination frequency illustrating the importance of butenolides in regulating germination in this particular species. Smoke treatment can be used as a very effective tool for enhanced seed germination, and hence crop production.

Chapter 4 explored the effects of macronutritional needs of *S. rebaudiana* through phosphate and nitrogen applications at different concentrations. The tallest plants were obtained with the 0.5 N treatment and high concentrations of either macronutrient resulted in the reduction of roots. At the metabolomic level, two distinct clusters were revealed after PCA, separating the phosphate and nitrogen treatments. This may suggest that different enzymatic and genetic pathways of secondary metabolism are regulated by nitrogen and phosphate leading to distinguishable chemical profiles. This study aimed to improve the total metabolite content of *S. rebaudiana* either by increasing growth or metabolite levels (steviol glycosides) using *in vitro* studies. Manipulation of nitrogen and phosphate at different concentrations was 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. This study thus contributed to the knowledge of the metabolites found in *S. rebaudiana* and methods on how to

successfully promote the production of some of the important metabolites found in this sweet medicinal plant. The aim to increase metabolite levels and growth of *S. rebaudiana* plant was achieved. However, improvements on this study can be made by increasing the period of time which in this study was 21 days to grow *Stevia* plants in a controlled environment to atleast 8 weeks. This would allow for further investigation on the alterations made by the different levels of chemicals applied at different concentrations.

Additionally to these findings, **Chapter 5** reported on different concentrations of sodium chloride and polyethylene glycol 6000 to assess salinity and water stress *in vitro* on growth, organ development and metabolite profile of *S. rebaudiana* respectively. At high concentrations of 75-100 mM NaCl and addition of 10% PEG 6000, root growth and other organ development was negatively affected. A reduction in secondary metabolites was correlated to an increase of the two stresses. For example, the inclusion of PEG 6000 in the growth media resulted in lowered rebaudioside A and stevioside levels. Contrary to this, a 25 mM NaCl-treatment resulted in a significant increase of steviol.

This study used an *in vitro* approach to better understand the microenvironmental conditions that alter growth and plantlet production of *S. rebaudiana*. This information provided here may thus be tested and adopted in in-field production of *Stevia* plants. Using other biotechnological approaches, drought resistance through genetic modification may be an interesting avenue to explore in the future due to the low growth tolerance of this species in water-stressed environments. To obtain a deeper understanding of the mechanisms at play regulating responses to nitrogen and phosphate, effect on gene expression of key regulatory enzymes is proposed. Broad scale transcriptomic and proteomic analysis may be useful in revealing genomic-metabolic pathways regulating production of stevioside and its derivatives when plants are subjected to different levels of phosphate and nitrogen. This study has formed a useful platform to assist with a deeper molecular interrogation of the role of the microenvironment in the genetic and biochemical regulation of stevioside metabolism in the future. Such a study may improve our overall physiological understanding and further aims of domestication of this plant for the South African agricultural sector.

Appendix A

Abstracts from conference contributions obtained from this thesis

A. Indigenous Plant Use Forum 18th Annual Conference 2015, Clanwilliam, South Africa

Poster Presentation

MAGANGANA T.P. & MAKUNGA N.P. (2015). Phytochemical and molecular analyses of *Stevia rebaudiana* extracts generated from different cultivation methods

Stevia rebaudiana Bertoni a perennial herb native to Paraguay contains sweet zero-calorie *ent*-kaurene diterpene glycosides found in the leaves. There is potential to establish this plant as a crop leading to a new agricultural industry which may be of benefit to South Africa's economy. Using tissue culture as a scientific study tool the germination of *Stevia* seeds were tested after a tetrazolium test showed a low viability of 23%. Furthermore, seeds were germinated *in vitro* on one-tenth Murashige and Skoog medium (MS) (1962). Seeds were subjected to four variables; scarification using 70% (v/v) sulphuric acid, placed on media with smoke solution, placed a combination of both treatments or in gibberellic acid. Seed germination was best when seeds were treated with smoke solution and gibberellic acid induced germination (final mean of 0.21 and 0.19 respectively) but other treatments showed very poor germination. An efficient tissue culture system for *S. rebaudiana* was established using the seedlings. Nodal explants showed better growth than the leaf explants. To study the macro- and micronutritional requirements of *S. rebaudiana* plants, the effects of nitrogen and phosphate were studied on the *S. rebaudiana* micro-plant system. Phosphates affect the growth production and regeneration *in vitro* but higher levels of steviosides are accumulated depending on higher nitrate-medium. Distinct clusters based on the metabolomic profile are due to nitrogen and phosphate treatment affecting stevioside metabolism and flavonoid production respectively.

B. South African Association of Botanists Conference 2016

Hosted at the Business School Complex on the campus of the University of the Free State, Bloemfontein, South Africa

Poster

MAGANGANA T.P. & MAKUNGA N.P. (2016). The effect of various factors on seed germination and the influence of abiotic stresses on growth productivity, physiology and differences in metabolite profiles (diterpene glycosides) of *Stevia rebaudiana* Bertoni

Stevia rebaudiana Bertoni is a plant native to South America. Due to its popularity it is cultivated commercially worldwide. This however excludes southern Africa. South Africa has an opportunity to cultivate *S. rebaudiana* as a new crop for the SA agricultural sector. *Stevia rebaudiana* seeds possess a very low germination capacity which is a challenge for large scale propagation. To investigate the germination of *S. rebaudiana* seeds, the effects of smoke solution, concentrated sulfuric acid, a combination of smoke and sulfuric acid and gibberellic acid were tested as a means of improving germination using tissue culture system in total darkness and light for 21 days. The smoke treatment was highly efficacious in producing a significant germination percentage. This suggests that dormancy in *S. rebaudiana* is not coat-imposed, requiring some form of cell wall abrasion to break dormancy but a physiological dormancy. To assess the effects of drought and salinity stress on the growth and chemistry of *S. rebaudiana*, polyethylene glycol 6000 (PEG) and sodium chloride (NaCl) were used respectively as osmotica on nodal segments of *S. rebaudiana* via tissue culture for 21 days. Headspace solidphase microextraction gas chromatography spectrometry technology revealed an abundance of α-pinene, β-pinene and sabinene in all treated plants while liquid chromatography mass spectrometry showed very low amounts of *ent*-kaurene diterpene glycosides found on the leaves of the plant; which are responsible for this plant's sweetness and therapeutic properties.

C. South African Association of Botanists 42nd Annual Conference 2016

Hosted at the Business School Complex on the campus of the University of the Free State, Bloemfontein, South Africa

Oral

MAGANGANA T.P. & MAKUNGA N.P. (2016). Effect of nitrogen and phosphate on the growth productivity and biochemicals of *Stevia rebaudiana*

Stevia rebaudiana Bertoni is a perennial herb from South America; it produces *ent*-kaurene diterpene glycosides on its leaves responsible for its sweetness. This natural, low calorie sweetener is more than 300 times sweeter than sucrose. This study aimed at profiling the phytochemistry of *S. rebaudiana* through liquid chromatography mass spectrometry (LC–MS) and Headspace solidphase microextraction gas chromatography spectrometry (HS-SPME-GC-MS) technology. Nodal segments of *Stevia rebaudiana* were either treated with phosphate or nitrogen at different concentrations for 21 days to establish low and high macronutrient conditions. All other 85 nutritional elements were kept similar to the control which contained similar concentrations as MS medium with both nitrogen (NH_4NO_3 at 25.76 M and KNO_3 at 18.79 M) and phosphate (KH_2PO_4 at 1.25 M). Four distinct clusters were revealed after principal component analysis (PCA) of the metabolite profiles. To further get the data separated and interpreted, orthogonal partial least squares discriminant analysis (OPLS-DA) was applied. This program allowed the organization of the clusters into two distinct groups. The occurrence or absence of *ent*-kaurene diterpene glycosides namely steviol hydrate, stevioside hydrate and rebaudioside A contributed significantly to the separation of phosphate treated plants from the nitrogen treated plants. Nitrogen resulted in higher levels of stevioside accumulation. With aims to increase stevioside yields, a regimen that leads to higher nitrogen accessibility is preferred. Terpene metabolism is linked to amino acid metabolism via the shikimate pathway. This may explain the influence of nitrogen on key bioactive accumulation.

Appendix B

Medium preparation

Components	Quantity (mg/L)	Concentration (Molar)
$MgSO_4 7H_2O$	180.7	1.5 mM
KH_2PO_4	**170**	**1.25 mM**
KNO_3	**1900**	**18.79 mM**
NH_4NO_3	**1650**	**20.61 mM**
$CaCl_2$	332.2	2.99 µM
Micronutrients		
H_3BO_3	6.2	100 µM
$MnSO_4 4H_2O$	16.9	100 µM
$ZnSO_4 7H_2O$	8.6	29.91 µM
$CoCl_2 6H_2O$ cobalt	0.025	0.11 µM
KI	0.83	5 µM
$FeSO_4$7H2O ferrous	27.8	100 µM
NaEDTA2H_2O	37.26	100 µM
Organic supplements		
myo-inositol	100	0.56 µM

Table 6.1 Murashige and Skoog, 1962

Dissolve in distilled water and adjust the pH of all media to 5.8 using 1M NaOH or 1M HCl.

Add BA/IAA prior to changing the pH.

Appendix C

Principal component analysis of *S. rebaudiana* solvent extracts

Figure 6.1 Hotelling's T2 range score measuring the distance between factor 1 and factor 2 of the PCA analysis. Scores above the green and red horizontal lines shows the probability that they are similar to each other is less than 5% and 1% respectively.

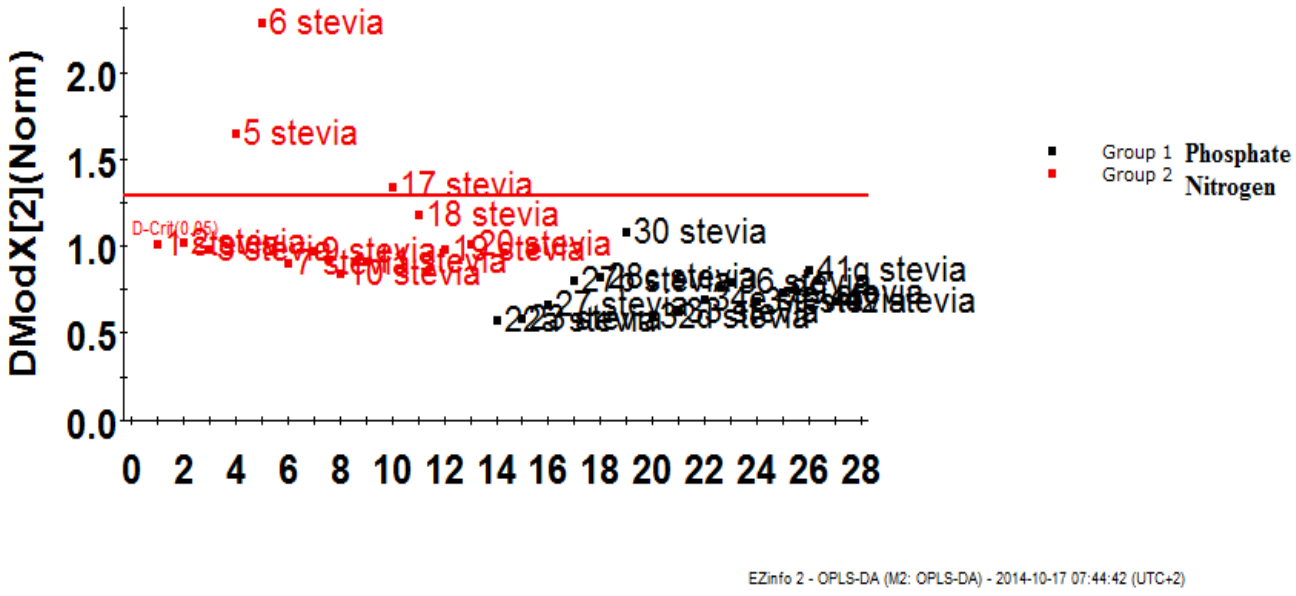

Figure 6.2 Distance model summarizing the noise of observation during the PCA analysis. The larger the noise the less similar is the observation to the others and plots above the red line are significantly dissimilar.

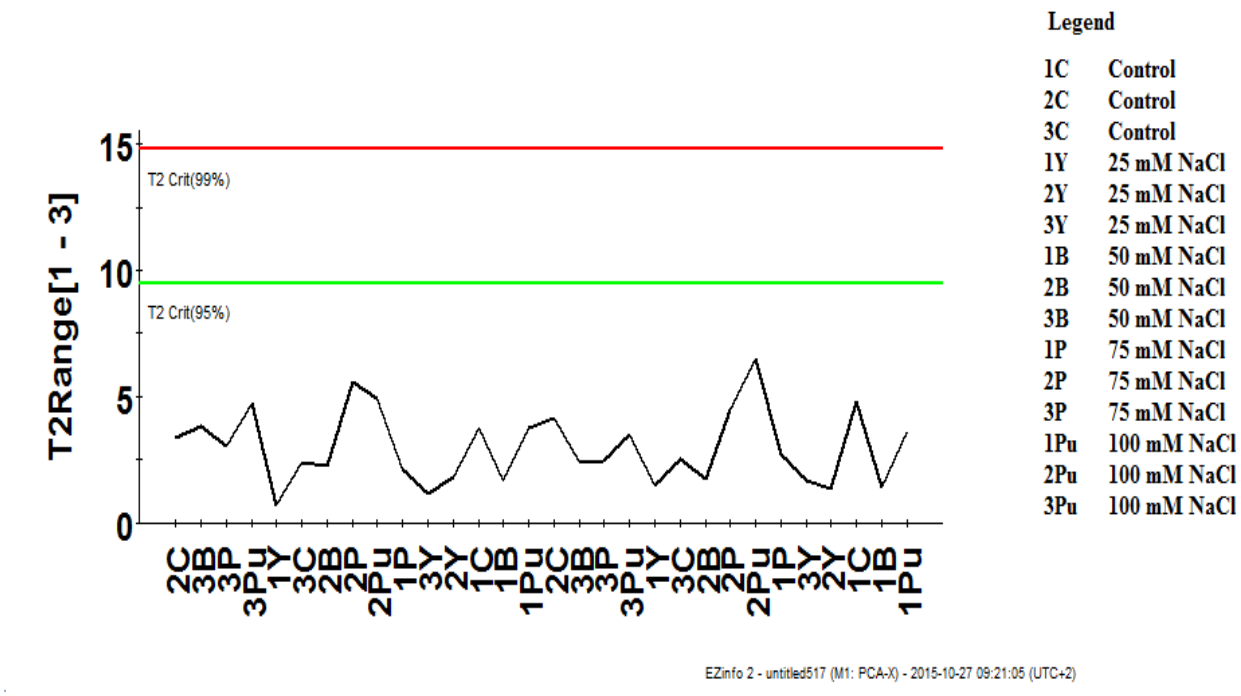

Figure 6.5 Hotelling's T2 range score measuring the distance between factor 1 and factor 3 of the PCA analysis. Scores above the green and red horizontal lines shows the probability that they are similar to each other is less than 5% and 1% respectively.

Appendix D

LC-and GC-MS total ion chromatograms for *S. rebaudiana*