Selection and micropropagation of *Solanum nigrum* genotypes with varying calcium and iron content

by

Kimerra Goordiyal

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa

January 2018

As the candidate's supervisor I have/have not approved this dissertation for submission

Supervisor

Signed:_____ Name: Dr S. Shaik Date:_____

Co-supervisor

Signed:_____ Name: Prof. M.P Watt Date:_____

ABSTRACT

A direct organogenesis protocol was established for *Solanum nigrum* using leaf explants from seedling plants. The post acclimatisation yield of the seedling-derived leaf explants was 25 plants/explant. It included decontaminating the leaves with 1 % (v/v) sodium hypochlorite and Tween 20® (10 min), shoot multiplication on medium containing 3 mg l^{-1} benzylaminopurine (BAP) for 4 weeks, elongation on medium containing 0.1 mg l^{-1} BAP for a week, rooting on hormone-free Murashige and Skoog medium for 3 weeks and acclimatisation in pots (1 soil : 2 vermiculite [1S : 2V]) in a growth room for 2 weeks.

A population of fifty 6-week old seedlings were screened using Inductively Coupled Plasma-Optical Emission Spectrometry. They varied in leaf calcium (Ca) (331.05-916.30 mg 100 g^{-1} dry mass [DM]) and iron (Fe) (0.64-14.95 mg 100 g^{-1} DM) contents. Based on these results, genotypes for high Ca (G5 and G20), high Fe (G6 and G15), low Ca (G43 and G45) and low Fe (G35 and G50) were selected for further investigation. These were micropropagated using the established protocol to determine whether their clones maintained similar levels of Ca and/or Fe to those of their parents when grown in soil. Micropropagation influenced the Ca and Fe levels of the clones of the selected genotypes, i.e. the 6-week old clones of six (i.e. G5, G20 and G45 for Ca; and G6, G15 and G50 for Fe) out of the eight selected genotypes had either significantly higher (G45 and G50) or lower (G5, G6, G15 and G20) levels of Ca and/or Fe than their 6-week old parents when grown in soil. There were also genotypic differences regarding the *in vitro* and *ex vitro* growth responses (i.e. percentage of explants with shoots, number of shoots/explant and post acclimatisation yield) and leaf Ca and Fe levels of the clones of the selected genotypes. The Ca and Fe contents of the clones of most of the selected genotypes were not affected by substrate type, suggesting that both soil and 1S : 2V were adequate to grow the *S. nigrum* genotypes for the benefit of high Ca and Fe.

Four of the selected genotypes (*viz*. G15, G20, G35 and G43) were then chosen to investigate the effect of physiological age on their leaf Ca and Fe contents when grown in soil and 1S : 2V. Advancing age only affected the Fe levels of the G35 clones in soil and G15 clones in both the tested substrates. The significant differences in the Ca (G20>G43) and Fe (G15>G35) contents of the 6-week old parents were compared with those of their clones at 4, 6 and 8 weeks *ex vitro* in soil. A similarity (i.e. G20>G43) in the 'ranking', was only found at 4 weeks *ex vitro* for Ca.

Initial attempts to establish a minimal growth protocol for storing germplasm of the eight selected genotypes showed that *in vitro* shoots could be kept on medium containing ¼ MS + 5

g l^{-1} sucrose for 8 weeks. After this period, the *in vitro* shoots of the eight selected genotypes that were placed onto shoot multiplication medium produced 7-13 shoots/explant.

It can be concluded that micropropagation and genotype influenced both the Ca and Fe levels of the selected genotypes, while physiological age only influenced their Fe content. As the present study was a smaller component of a larger research program, further investigations need to be carried out on the selected *S. nigrum* genotypes prior to distribution to community gardens. Future work should include evaluating the effects of various environmental conditions (i.e. growth in a greenhouse, shadehouse, glasshouse and/or in the field), different light intensities, watering regimes, fertiliser treatments and soil pH on the growth, and leaf Ca and Fe levels of the clones. Regarding minimal growth storage, future work should include investigating whether the germplasm of the eight selected genotypes can be stored under the same conditions for longer than 8 weeks.

DECLARATION

I, **Kimerra Goordiyal**, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

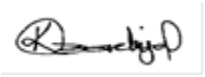
2. This dissertation has not been submitted for any degree or examination at any other tertiary institution.

3. This dissertation does not contain other person's data, graphs, pictures or other information, unless specifically acknowledged as being sourced from other persons.

4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sourced have been quoted, then:

    a. Their words have been re-written but the general information attributed to them had been referenced.
    b. Where their exact words have been used, then their writing has been placed in italics and within quotation marks, and referenced.

5. This dissertation does not contain texts, tables, graphs, figures or graphics copied and pasted from the internet, unless specifically acknowledged and the source being detailed in the dissertation and in the References sections.

Signed 

PREFACE

The experimental work described in this dissertation was conducted at the University of KwaZulu-Natal, School of Life Sciences under the supervision of Prof. M.P. Watt and Dr. S. Shaik. The research was financially supported by the National Research Foundation (NRF).

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, they have been acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I praise Lord Jesus Christ, through whom all things are possible, for His richest grace and mercy throughout the duration of this study.

A heartfelt thank you to my supervisors, Prof. Paula Watt and Dr. Shakira Shaik for their patience, continuous support, guidance and encouragement throughout the duration of this study.

To my parents Roshan and Romilla Goordiyal and fiance Tyren Maistry, words will not express how thankful I am for your unconditional love, understanding and moral support. Tyren, I cannot thank you enough for all that you have done for me during the course of this research.

A special thank you to Ashlin Munsamy, Karishma Singh, Philisiwe Mhlanga and Phindile Dladla for their support and assistance during the course of this study.

Finally, I would like to thank the National Research foundation (NRF) for funding this research.

LIST OF ABBREVIATIONS

%	percentage
ºC	degrees celsius
µg ml^{-1}	micrograms per millilitre
µmol m^{-2} s^{-1}	light intensity/ micro moles per square metre per second
1S : 2V	1 soil : 2 vermiculite
2,4-D	2,4-dichlorophenoxyacetic acid
ALV	African leafy vegetable
ANOVA	Analysis of Variance
BAP	benzylaminopurine
Ca	calcium
cm	centimeters
Cu	copper
Fe	iron
FELBs	frog egg-like bodies
g	grams
g l^{-1}	grams per litre
GA_3	gibberellic acid
h	hour(s)
HCl	hydrochloric acid
$HgCl_2$	mercuric chloride
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ICP-OES	inductively coupled plasma-optical emission spectrometry
K	potassium
KIN	kinetin
M	molarity
min	minute(s)
Mg	magnesium
ml l^{-1}	millilitres per litre
mg l^{-1}	milligrams per litre

mm	millimeter(s)
Mn	manganese
MS	Murashige and Skoog (1962)
Na	sodium
NAA	α-naphthaleneacetic acid
NaOCl	sodium hypochlorite
NFCS	National Food Consumption Survey
NR	not reported
P	phosphorus
PGRs	plant growth regulators
RDA	recommended daily allowance
s	second(s)
TDZ	thidiazuron,
w_0	end of rooting and start of acclimatisation
w_6	6 weeks *ex vitro* growth (which included 2 weeks acclimatisation)
Zn	zinc

1. Introduction and Rationale

Malnutrition is generally defined as the lack of proper nutrition (Labadarios, 2005) and is a consequence of chronic food shortages, poor food quality and inadequate food intake (Blossner and de Onis, 2005; Misselhorn, 2005; Govender *et al.*, 2017). In many developing countries, food consumption is mainly cereal-based and nutrient-poor, which is insufficient to meet the needs of large, mainly poor, populations (Aphane *et al*., 2003; Labadarios *et al.,* 2008; Faber *et al*., 2010; Berti *et al.*, 2014; Njume *et al.*, 2014; Ochieng *et al.*, 2016; Ngoroyemoto *et al.*, 2017). Malnutrition is prevalent in southern Africa (Food and Agriculture Organisation, 2010), with South Africa experiencing chronic malnutrition predominantly in rural areas (Faber and Wenhold, 2007; Faber *et al*., 2010; du Toit *et al.*, 2011; Govender *et al.*, 2017). At the South African national level, it has been found that many households struggle with food deficit (Labadarios, 2005; Crowther, 2008; du Toit *et al.*, 2011; Govender *et al.*, 2017). The consequences of this are negative effects on body function and increased predisposition to diseases, morbidity and mortality (Bhan *et al*., 2001; Labadarios, 2005).

Calcium (Ca) and iron (Fe) deficiencies form part of the many nutrient-based deficiencies which affect one third of the world's population and are also prevalent in South Africa (Steyn *et al.*, 2001; Zimmerman and Hurrell, 2002; Labadarios *et al.*, 2008). The 1999 National Food Consumption Survey (NFCS) report indicated an inadequate intake of Ca and Fe in infants, children and pregnant women in South Africa (Gibson, 2005; Faber and Wenhold, 2007; Steyn *et al*., 2016). It has also been found that the average consumption of Ca and Fe in South Africa is less than 67 % of the recommended daily allowance (RDA) (Labadarios *et al.*, 2000; Labadarios *et al.*, 2008; Acham *et al.*, 2012; Steyn *et al*., 2016). According to the World Health Organisation (2003), the average South African requires 400 g of fruit and vegetables per day (Gotor and Irungu, 2010) to meet their nutritional needs. To achieve this, many strategies such as micronutrient supplementation and food bio-fortification have been employed, with the more recent one being that of dietary diversification where African leafy vegetables (ALVs) can make a significant impact (Faber *et al.*, 2010; Uusiku *et al.*, 2010; Ochieng *et al.*, 2016; Ronoh *et al.*, 2017).

These ALVs are a collection of crops from which young leaves, stems and fruit are consumed as vegetables (Jansen van Rensburg *et al.*, 2007; Ochieng *et al.*, 2016). They are good sources of Ca, Fe and an array of other essential nutrients (Oniang'o *et al.*, 2004; Uusiku *et al.*, 2010;

Ebert, 2014; Achikan-Dako *et al.,* 2014; Njume *et al.,* 2014; Bvenura and Afolayan, 2015; Ochieng *et al.,* 2016; Govender *et al.,* 2017; Ngoroyemoto *et al.,* 2017). Their cultivation requires minimal input as they are commonly found growing on disturbed soils such as those from waste lands, roadsides, around houses and buildings (Edmonds and Chweya, 1997; Shukla *et al.,* 2006; Jansen van Rensburg *et al.,* 2007; Ashrafudoulla *et al.,* 2016). They also grow as weeds on cultivated fields and plantations (Edmonds and Chweya, 1997). Thus, they are easily available, highly versatile and able to withstand harsh environmental conditions (Edmonds and Chweya, 1997; Jansen van Rensburg *et al.,* 2007). In South Africa, ALVs are grown and gathered in the wild or sometimes cultivated and harvested as a source of food for certain households (Aphane *et al*., 2003; Uusiku *et al.,* 2010; Ebert, 2014; Seeiso and Materechera, 2014; Bvenura and Afolayan, 2015; Ngoroyemoto *et al.,* 2017). Their consumption depends on the localisation, preference, availability and accessibility of different species, the household status and on the degree of urbanisation (Vorster *et al*., 2002; Aphane *et al*., 2003; Ochieng *et al.,* 2016). In South Africa, researchers such as Jansen van Rensburg *et al.* (2007) and Ngoroyemoto *et al.* (2017) found that a wide range of these ALVs (such as *Amaranthus sp., Brassica rapa* subsp. *Chinensis* L., *Cleome gyandra* L., *Corchorus olirorius* L., *Corchorus tridens* L., *Cucumis melo* L., *Cucurbita pepo* L., *Momordica balsamina* L., *Solanum nigrum* L., *Solanum retroflexum* Dunal. and *Vigna inguicalata* (L.) Walp.) are consumed by communities. However, their consumption has decreased over the years due to the modernisation of South African communities and the perception as being poor people's food by the younger generation (Faber *et al*., 2010; Dweba and Mearns, 2011). Nonetheless, the consumption of ALVs are important for fulfilling the nutritional requirements of many communities (Shukla *et al.,* 2006; Vorster *et al*., 2007). In the present study, *Solanum nigrum* was of particular interest as it is one of many ALVs that are consumed by communities in SA (Steyn *et al*., 2001; Jansen van Rensburg *et al.,* 2007; Bvenura and Afolayan, 2014a, b; Ngoroyemoto *et al.,* 2017).

A major gap in ALV research is that specific ALV genotypes have not been selected for traits of interest such as micronutrient content as these species are less competitive in the market than commercial vegetables (Genc *et al.,* 2005; Smith and Eyzaguirre, 2007; Stamp *et al.,* 2012). Apart from that, there is extensive variation in the micronutrient contents of a plant population. According to Genc *et al.* (2005), the genetic variability of ALVs need to be invesitgated in order to obtain plants with high levels of micronutrients. In this regard, Dubois (2009), Tadele and Assefa (2012) and Cheema *et al.* (2017) suggested micropropagation of ALVs for upscale

production and delivery of large quantities of superior (high nutrient content) cloned cultivars with desirable characteristics but an extensive search of the literature revealed that no such studies have been previously undertaken.

The aim of this study was, therefore, to micropropagate selected *S. nigrum* genotypes with high or low levels of Ca and Fe, and determine whether the cloned genotypes maintained similar levels of Ca and/or Fe to those of their parents. The overall aim of the broader research programme, of which this study was only a component, is to produce a natural breeding population of *S. nigrum* with high Ca and Fe content for distribution to community gardens. Towards these aims, the objectives of the present study were to:

1) **To screen a population of seed-germinated *Solanum nigrum* and select genotypes with high or low Ca and Fe content.**

   A population of fifty 6-week old seedling plants was screened using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) to determine their leaf Ca and Fe levels on a dry mass (DM) basis. Then, some genotypes with high or low concentrations of Ca and Fe were selected for micropropagation.

2) **To establish a direct organogenesis protocol for micropropagation of the selected genotypes.**

   The effectiveness of published *in vitro* propagation protocols on the post-acclimatisation yield (plants/explant) of leaf and nodal explants from different sources (seedling and field plants) was investigated. The effect of three rooting media, containing low/no plant growth regulators (PGRs), on root production was also evaluated.

3) **To assess *ex vitro* growth responses of the selected cloned genotypes.**

   After 2 weeks of acclimatisation, clones of the selected genotypes were maintained in soil or 1 soil : 2 vermiculite in a greenhouse and harvested 4 weeks later (6 weeks *ex vitro* growth). The number of leaves, the number of branches, shoot and root lengths and fresh and dry masses of shoots and roots were measured at the start of acclimatisation and after 6 weeks *ex vitro*.

4) **To compare the Ca and Fe contents of the clones of the selected genotypes, maintained in the two substrates, to each other and to those of their parents.**

The leaves of 6-week old cloned genotypes, grown in soil or 1 soil : 2 vermiculite, were manually digested and screened for their levels of Ca and Fe (mg $100g^{-1}$ DM) using ICP-OES. The Ca and Fe content of the selected cloned genotypes from the two substrates were compared to each other and to those of their respective 6-week old seedling parent genotype.

5) **To evaluate the effect of physiological age and substrate on the levels of Ca and Fe of the clones of the selected genotypes.**

The selected cloned genotypes were maintained in soil or 1 soil : 2 vermiculite in a greenhouse and the number of leaves, shoot and root fresh and dry masses and, leaf Ca and Fe levels (mg $100g^{-1}$ DM) were determined at 4, 6 and 8 weeks *ex vitro*.

6) **To compare the response of *S. nigrum in vitro* plantlets between two minimal growth storage media.**

Minimal growth storage of *S. nigrum in vitro* plantlets was investigated by changing the composition of the growth medium. The Murashige and Skoog (MS) (1962) basal salt medium with vitamins was reduced from full strength to one third or a quarter strength and the sucrose concentration was reduced from 30 g l^{-1} to 5 or 10 g l^{-1}. *In vitro* plantlets of each genotype were maintained on media consisting of either ⅓ MS with 10 g l^{-1} sucrose or ¼ MS with 5 g l^{-1} sucrose for 8 weeks. The ability of *S. nigrum in vitro* plantlets to multiply after minimal growth was assessed by placing these plantlets onto the established multiplication medium. Rooting and acclimatisation were also evaluated.

2. Literature Review

2.1 Background and importance of *Solanum nigrum*

Solanum nigrum is a herbaceous annual plant which belongs to one of the largest and most diverse genera of the family Solanaceae (Yousaf *et al.*, 2010; Onyango *et al.*, 2016; Ronoh *et al.*, 2017). *Solanum nigrum* is commonly known as the Black Nightshade and is found mainly in tropical and temperate regions, within centres of diversity in the southern hemisphere (D' Arcy, 1991; Edmonds and Chweya, 1997; Weese and Bohs, 2007; Kandimalla *et al.*, 2015). *Solanum nigrum* tends to thrive on disturbed soils such as those from wastelands and roadsides (Jagatheeswari *et al.*, 2013; Gogoi and Islam, 2014; Das and Borah, 2015). This plant also grows as a weed on cultivated fields, flower gardens and in vegetable gardens (Basset and Munro, 1985; Edmonds and Chweya, 1997; Sridhar and Naidu, 2011a). Its 'weedy' nature makes *S. nigrum* easy to cultivate, thus providing a source of food to many households (Basset and Munro, 1985; Knapp 2008; Taab and Anderson, 2009; Bvenura and Afolayan, 2014a, b).

The tender shoots and leaves of *S. nigrum* are most commonly used for preparing meals (Chweya, 1997; Jansen van Rensburg *et al.*, 2007; Matasyoh and Bosire, 2016; Ngoroyemoto *et al.*, 2017). The most preferred method of preparation is by boiling or stewing the shoots and leaves and using them as a relish or consuming them as one would do with spinach (Edmonds and Chweya, 1997; Akubugwo *et al.*, 2007; Akubugwo *et al.*, 2008), and they are frequently incorporated into soups and sauces (Akubugwo *et al.*, 2008; Sridhar and Naidu, 2011a). The unripe berries of *S. nigrum* tend to be poisonous but the ripe ones are considered to have culinary value, as they are most often eaten raw, used in pies or preserves as a substitute for raisins (Edmonds and Chweya, 1997; Jacoby *et al.*, 2003; Matasyoh and Bosire, 2016).

Apart from its culinary usage, *S. nigrum* has been found to be a valuable medicinal plant, with its use recorded since ancient times (Edmonds and Chweya, 1997; Matasyoh and Bosire, 2016; Nyeem *et al.*, 2017). A clarified juice prepared from *S. nigrum* has been found to have a soothing effect on inflammation of the throat and eye, shingles, ringworm, gout, ear-ache and ulcers (Edmonds and Chweya, 1997; Matasyoh and Bosire, 2016). This plant has also been found to have many antiseptic, anti-dysenteric, anti-inflammatory, anti-spasmodic, diuretic, purgative and sedative properties (Edmonds and Chweya, 1997; Pandhure *et al.*, 2010; Sundari *et al.*, 2010; Sridhar and Naidu, 2011a; Khattak *et al.*, 2012; Rathore and Gupta, 2013; Nyeem *et al.*, 2017).

2.2 Taxonomy, growth and genetics of *Solanum nigrum*

Taxonomy

Solanum nigrum belongs to the section *Solanum* from the genus *Solanum* in the family Solanaceae, in the order Solanales in the kingdom Plantae (Nandhini and Paramaguru, 2014; Kandimalla *et al.*, 2015; Matasyoh and Bosire, 2016; Ronoh *et al.*, 2017). The Solanaceae family is of 'cosmopolitan' distribution and found throughout the world, and is composed of 90 genera and 3000 species (Edmonds and Chweya, 1997; Yousaf *et al.*, 2010; Poczai and Hyvoenen, 2011; Das and Borah, 2015). The family comprises of many essential fruits, vegetables and ornamental plants, as well as the economically indispensable, tobacco (*Nicotiana sp.*) (Edmonds and Chweya, 1997). The genus *Solanum* is one of the largest and most diverse genera in the family and encompasses approximately 1500 species globally (Jacoby *et al.*, 2003; Weese and Bohs, 2007; Knapp, 2008; Yousaf *et al.*, 2010; Oyekunle *et al*., 2014; Kandimalla *et al.*, 2015). It is also economically important, as it includes many familiar plants such as potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.) and many minor food plants (Soria and Heiser, 1961; Jacoby *et al.*, 2003; Poczai and Hyvoenen, 2011; Das and Borah, 2015; Kazi, 2015).

The section *Solanum* has previously been referred to as *Morella*, but is now considered to be the *Solanum nigrum* complex (Soria and Heiser, 1961; Edmonds, 1977; Dehmer and Hammer, 2004). This complex includes the species *Solanum nigrum* L. (commonly known as Black Nightshade) and is made up of many morphogenetically distinct taxa (Stebbins and Paddock, 1949; Edmonds and Chweya, 1997; Jacoby *et al.*, 2003). Apart from *Solanum nigrum* L., other members of the complex include *Solanum americanum* Mill., *Solanum villosum* Mill., *Solanum chenopodioides* Lam., *Solanum retroflexum* Dunal., *Solanum surattense* Burm. F. and *Solanum physalifolium* Rusby. (Weese and Bohs, 2007; Nandhini and Paramaguru, 2014; Kandimalla *et al.*, 2015; Matasyoh and Bosire, 2016; Ronoh *et al.*, 2017).

Taxa from the *Solanum nigrum* complex have been at the centre of many taxonomic studies for decades, as their morphologically variable nature has made taxonomic classification, based on morphological characters challenging (Stebbins and Paddock, 1949; Venkateswarlu and Rao, 1972; Yousaf *et al.*, 2010; Oyekunle *et al.*, 2014; Ronoh *et al.*, 2017). Since the genus *Solanum* L. was first described by Linnaeus in 1753, these taxa have been reclassified innumerable times as the earlier studies did not focus on their cytomorphological

characteristics (Bukenya and Hall, 1988; Edmonds and Chweya, 1997; Nandhini and Paramaguru, 2014; Oyekunle *et al.*, 2014). Many species from the complex have, therefore, been identified as *S. nigrum*, due to morphological markers such as plant habit, pubescence and leaf characteristics proving to be insufficient for proper taxonomic classification (Bukenya and Hall, 1988; Yousaf *et al.*, 2010).

The taxonomic complexity within the genus *Solanum* is attributed to various factors, such as phenotypic plasticity, genetic variation, polyploidy and natural hybridisation (Stebbins and Paddock, 1949; Edmonds and Chweya, 1997; Ronoh *et al.*, 2017). Phenotypic plasticity and genotypic variation are most likely to occur in an individual plant as it grows, or in different populations of a particular species (Ogg *et al.*, 1981, Nandhini and Paramaguru, 2014). The present study investigated the potential for such plasticity with regard to leaf Ca and Fe contents.

Growth

Phenotypic plasticity is the consequence of a species developing several ways of adaptation to various environmental conditions and exhibits itself as variation (Barrett, 1982; Krstic' *et al.*, 2002; Matasyoh and Bosire, 2016). In *S. nigrum*, phenotypic plasticity is mainly exhibited in the vegetative parts of the plant (Table 1) such as habit, size and form of the leaves, and stem winging (Edmonds and Chweya, 1997).

Solanum nigrum grows as either an annual or a short-lived perennial (Table 1). It has a height that ranges from 0.25 to 1.2 m tall (Stebbins and Paddock, 1949; Rogers and Ogg, 1981; Basset and Munro, 1984; Bukenya and Hall, 1988; Edmonds and Chweya, 1997; Jansen van Rensburg *et al.*, 2007; Sridhar and Naidu, 2011a; Chauhan *et al.*, 2012; Khattak *et al.*, 2012; Matasyoh *et al.*, 2015; Matasyoh and Bosire, 2016). The inflorescence type is a racemose, or racemose variations such as sub-racemose or lateral racemes (Stebbins and Paddock, 1949; Rogers and Ogg, 1981; Symon, 1981; Basset and Munro, 1984; Jagatheeswari *et al.*, 2013). However, variations of cymes and umbel-like inflorescences have also been reported in the literature (Bukenya and Hall, 1988; Edmonds and Chweya, 1997; Mwai *et al.*, 2007; Chauhan *et al.*, 2012; Wahua and Olaleye, 2013; Matasyoh and Bosire, 2016).

Table 1: Summary of the reported phenotypic variation exhibited in the height, habit, leaf shape and margin, and inflorescence type of *Solanum nigrum*.

Character					Reference
Height (m)	**Habit**	**Leaves**		**Inflorescence type**	
		Shape	**Margin**		
-	Annual	Variable	Entire Slightly serrate	Sub-racemose	Stebbins and Paddock (1949)
0.5 to 1.0	Erect annual to short lived Perennial herb/ subshrub	Variable	Variable	Racemose	Rogers and Ogg (1981)
-	Herb Soft wooded shrub	Ovate	Entire Bluntly toothed	Short raceme	Symon (1981)
0.5	Erect, subglabrous, annual herb with sparsely branched stem	Elliptic to ovate	Entire Wavy	Racemose	Basset and Munro (1984)
1.0	Subglabrous herb	Elliptic	Entire Toothed	Umbellate cymes	Bukenya and Hall (1988)

Table 1 continued

Character					
		Leaves			
Height (m)	**Habit**	**Shape**	**Margin**	**Inflorescence type**	**Reference**
0.7	Erect, subglabrous, annual herbs Sometimes shrubs and epiphytes	Ovate Ovate to lanceolate Ovate to rhomboid	Entire to sinuate Dentate	Simple Lax Extended cymes	Edmonds and Chweya (1997)
0.75	Erect annual/ Biannual herb	-	-	Umbel-like	Jansen van Rensburg *et al.* (2007)
-	Herb	Ovate to lanceolate	Entire Sinuate	Simple raceme like cymes	Manako (2007)
1.0	-	Ovate Lanceolate	Entire/ Sinuate Sinuate to dentate	Mixture of cymes	Mwai *et al.* (2007)
1.0	Erect herbaceous annual	Ovate	Irregularly toothed Wavy	-	Sridhar and Naidu (2011a)
0.25-1.0	Erect annual herb	Ovate	-	Axillary umbels	Chauhan *et al.* (2012)

Table 1 continued

Character					
		Leaves			
Height (m)	**Habit**	**Shape**	**Margin**	**Inflorescence type**	**Reference**
0.3- 1.2	Herb Short-living perennial herb	Ovate Lanceolate Lanceolate to diamond	Wavy Large toothed Slightly dentate Entire	-	Khattak *et al.* (2012)
-	Branched prickly herb	Ovate	Lobed	Lateral racemes	Jagatheeswari *et al.* (2013)
-	Erect herbs	Ovate	Lobed	Umbellate cyme	Wahua and Olaleye (2013)
0.3- 1.2	Herb Short-living perennial herb	Ovate Lanceolate Lanceolate to diamond	Wavy Large toothed Slightly dentate Entire	-	Matasyoh *et al.* (2015)
1.0	Branched herb	Ovate	Smooth	Mixture of cymes and forked cymes	Matasyoh and Bosire (2016)

The leaves exhibit the most plasticity as they are more easily influenced by changes in the environment than other vegetative organs (Krstic' *et al.*, 2002; Jacoby *et al.*, 2003). In earlier studies by Stebbins and Paddock (1949) and Rogers and Ogg (1981) it was found that the leaf shape of *S. nigrum* was variable (Table 1). Some studies have shown the leaf shape as being ovate, and others described variations of the leaf shape which ranged from elliptic to rhomboid, even lanceolate (Symon, 1981; Basset and Munro, 1984; Bukenya and Hall, 1988; Edmonds and Chweya, 1997; Manako, 2007; Mwai *et al.*, 2007; Sridhar and Naidu, 2011a; Chauhan *et al.*, 2012; Khattak *et al.*, 2012; Wahua and Olaleye, 2013; Jagatheeswari *et al*., 2013; Matasyoh *et al.*, 2015; Matasyoh and Bosire, 2016). The leaf margin, on the other hand, ranges from entire to dentate or serrate (Table 1). According to Edmonds and Chweya (1997) these features (Table 1) are dependent on environmental conditions.

Genetics

Genetic variation may occur within or between *S. nigrum* populations and over different geographic locations (Ogg *et al.*, 1981; Nandhini and Paramaguru, 2014). There are many factors that influence and, therefore, determine the level of genetic variation in a population, such as polyploidy and hybridisation (Barrett, 1982).

Polyploidy occurs in plants that contain three or more complete chromosome sets and is the basis for genetic variation in plant taxa (Barrett, 1982; Kazi, 2015). *Solanum nigrum* forms part of a series of polyploid plants with the other related species in the *Solanum nigrum* complex (Edmonds and Chweya, 1997; Kazi, 2015). These polyploids are often either diploid ($2n=2x=24$; e.g. *S. americanum* and *S. chenopodioides*), tetraploid ($2n=4x=48$; e.g. *S. retroflexum* and *S. villosum*) or hexaploid ($2n=6x=72$, e.g. *S. nigrum*) in nature and they play an important role in the evolution of plants, as they promote changes in gene expression and divergence, thereby making them valuable sources of genes for research (Edmonds and Chweya, 1997; Nandhini and Paramaguru, 2014; Kazi, 2015).

According to Barrett (1982) and Ojiewo *et al.* (2007), polyploid plants are capable of naturally hybridising with related plant species. Hence, due to the polyploid nature of *S. nigrum* and other plants in the *S. nigrum* complex, natural hybridisation is highly favoured and occurs frequently (Nandhini and Paramaguru, 2014). The product of natural hybridisation is extremely variable and can be one of: a) novel and more versatile genotypes that are capable of reproduction; b) genotypes that are vegetatively versatile but are sterile; c) hybrid swarms that are capable of

interbreeding with other hybrids and backcrossing to parent type plants; or d) new species (Barrett, 1982; Edmonds and Chweya, 1997).

2.3 Calcium and iron content in *Solanum nigrum*

The nutritional composition of the various plant parts in *S. nigrum* has been studied extensively (Tables 2 and 3) because of the suitability of this leafy vegetable as a food source (Edmonds and Chweya, 1997). The mineral content found in the various plant parts of African leafy vegetables (ALVs) such as *S. nigrum* has been highlighted in many studies as they contain appreciable amounts of micronutrients that are important to human health (Potawale *et al.*, 2008; Ngoroyemoto *et al.*, 2017). They include calcium (Ca) (Table 2) and iron (Fe) (Table 3), with reported Ca contents ranging from 3.63 to 4421 mg $100g^{-1}$ DM in leaves, 12 to 73 mg $100g^{-1}$ DM in seeds and 171 to 1870 mg 100 g^{-1} DM in the fruit (berries) (Table 2). The reported Fe contents, on the other hand, ranged from 2.5 to 85 mg $100g^{-1}$ DM in the leaves, 3.8 to 13 mg $100g^{-1}$ DM in the seeds and approximately 52 mg $100g^{-1}$ DM in the fruit (Table 3). Apart from the leaves it is, therefore, evident that Ca and Fe also occur in their greatest concentration in the fruit of *S. nigrum* (Tables 2 and 3, respectively), even though most studies focus on the leaves, as this is the most frequently consumed part (Jansen van Rensburg *et al.*, 2007). The leaves have also been found to be a good source of fibre, carbohydrates, protein and vitamins (Edmonds and Chweya, 1997; Afolayan and Jimoh, 2008; Jimoh *et al.*, 2010; Uusiku *et al.*, 2010; Acipa *et al.*, 2013; Bwembya *et al.*, 2014).

2.4 Factors that affect micronutrient content in ALVs

The nutritional quality of a plant is determined by the amount of micronutrients contained in its edible parts. As presented before (Tables 2 and 3), a lot of variation exists amongst different plants regarding micronutrient content. There are many factors that influence the micronutrient content of plants (Table 4) and the degree to which their synergistic effects influence micronutrient content may vary (Oelberg, 1956; Baligar *et al.*, 2001). The most important influencing factors include genotype, stage of plant maturity, edaphic and environmental effects (Oelberg, 1956; Kilcher, 1981; Clark, 1983; Nicolle *et al.*, 2004, Bouis and Welch, 2010). However, in contrast to the economically important leafy vegetables, information on the effect of plant age and environmental conditions on nutritional content in ALVs is scarce (Modi, 2007). Jansen van Rensburg *et al.* (2004) speculated that the influence of these factors was

Table 2: Examples of calcium content (mg $100g^{-1}$ DM) in various plant parts of *Solanum nigrum* derived from different locations.

Plant part	Ca content	Locality and origin		Reference
Leaves	2000	Nigeria	Field	Aletor and Adeogun (1994)
Fruits	171	Ghana	Field	Tayie and Asibey-Berko (2001)
Leaves	204	India	Field	Sheela *et al.* (2004)
Leaves	2380	Ghana	Farm	Glew *et al.* (2005)
Leaves Seeds	17* 12*	Nigeria	Market	Akubugwo *et al.* (2007)
Leaves	2067	South Africa	Field	Odhav *et al.* (2007)
Leaves	4421	South Africa	Field	Afolayan and Jimoh (2008)
Leaves	18	Nigeria	Farm	Akubugwo *et al.* (2008)
Leaves	4.4	South Africa	Field	Jimoh *et al.* (2010)
Seeds	73	India	Field	Sarma and Sarma (2011)
Leaves	12.9	Nigeria	Market	Oduse *et al.* (2012)
Leaves	873	Nigeria	Market	Achikanu *et al.* (2013)
Leaves	447	Uganda	Field	Acipa *et al.* (2013)
Leaves	3.63	Nigeria	Market	Nupo *et al.* (2013)
Leaves	1370	Uganda	Field	Tumwet *et al.* (2013)
Leaves Fruit Roots	1890 and 1930** 1840 and 1870** 1200 and 1490**	India	Field	Vats and Nagpal (2013)
Leaves	161	Swaziland	Field	Bwembya *et al.* (2014)
Leaves	22	India	Market	Padmashree *et al.* (2014)

*Variety *virginicum*
**Values from two locations

Table 3: Examples of iron content (mg $100g^{-1}$ DM) in various plant parts of *Solanum nigrum* derived from different locations.

Plant part	Fe content	Locality and origin		Reference
Leaves	57	Nigeria	Field	Aletor and Adeogun (1994)
Fruits	52	Ghana	Field	Tayie and Asibey-Berko (2001)
Leaves	33	Ghana	Farm	Glew *et al.* (2005)
Leaves	13*	Nigeria	Market	Akubugwo *et al.* (2007)
Seeds	13*			
Leaves	85	South Africa	Field	Odhav *et al.* (2007)
Leaves	49	South Africa	Field	Afolayan and Jimoh (2008)
Leaves	13.5	Nigeria	Farm	Akubugwo *et al.* (2008)
Leaves	49	South Africa	Field	Jimoh *et al.* (2010)
Seed	3.8	India	Field	Sarma and Sarma (2011)
Leaves	2.5	Nigeria	Market	Oduse *et al.* (2012)
Leaves	31	India	Field	Subramanian *et al.* (2012)
Leaves	64	Nigeria	Market	Achikanu *et al.* (2013)
Leaves	42	Uganda	Field	Tumwet *et al.* (2013)
Leaves	18	Swaziland	Field	Bwembya *et al.* (2014)
Leaves	4.2	Pakistan	Field	Ghani *et al.* (2014)
Leaves	14	India	Market	Padmashree *et al.* (2014)

*Variety *virginicum*

Table 4: Summary of studies on the effect of various factors on micronutrient content of annual and biennial leafy vegetables.

Species	Micronutrient	Influencing factor	Reference
Amaranthus sp.	Ca, Fe	Stage of maturity Genotype Temperature	Modi (2007)
Amaranthus tricolor	Ca, Mg, Zn, Fe, Mn	Genotype	Shukla *et al.* (2006)
Brassica oleracea	Fe, Ca, Mg, Zn	Genotype Season	Kopsell *et al.* (2005)
Carrot cultivars	Ca, Fe, Mg	Genotype	Nicolle *et al.* (2004)
Corchorus olitorius	Fe, Ca, Mg, Zn	Nitrogen fertiliser	Musa *et al.* (2010)
Corchorus olitorius	Fe, Ca, Mg, Zn	Leaf age/ position	Musa (2012)
Cleome gynandra	Fe, Ca, Mg	Season	Agbo *et al.* (2014)
Cynara cardunculus var. *scolymus* (L.)	Ca, Fe, Cu, Zn	Genotype Location Season	Pandino *et al.* (2011)
Lactuca sativa var. *capitata L.*	Fe, Mn, Zn	Nitrogen forms Soil type	Safaa and Abd El Fattah (2007)
Lycopersicum esculentum	Ca, Mg	Poultry manure	Ewulo *et al.* (2008)
Lycopersicum esculentum	Ca, Mg	Poultry manure NPK fertiliser	Ayeni *et al.* (2010)
Solanum nigrum	Ca, Fe	Stage of maturity Stage of maturity	Chweya (1997)
Solanum nigrum	Cu, Fe, Mn, Zn	Stage of maturity Fertiliser treatment	Bvenura and Afolayan (2014a)
Solanum nigrum	Ca, Mg, P, K, Na	Stage of maturity Fertiliser treatment	Bvenura and Afolayan (2014b)
Telfairia occidentalis	Fe, Ca, Mg, Zn	Leaf age/ position	Musa and Ogbadoyi (2012)

likely to cause extensive variation on the micronutrient content of leafy vegetables during plant growth. This is consistent with the findings of other studies on various ALVs as outlined below.

In *S. nigrum*, the effect of the stage of plant maturity on Ca and Fe content was investigated in plants harvested at 3, 5, 7, 9 and 11 weeks, after transplanting 6-week old seedlings (Chweya, 1997). The Ca levels increased over the 11 weeks of growth and morphogenesis, while Fe content slightly decreased from week 3 to 7, before significantly increasing from week 9 to 11 (Chweya, 1997). Similarly, Modi (2007) observed that Ca and Fe content increased with advancing age in different genotypes of *Amaranthus sp.* and at different maintenance temperatures. In contrast, Ayeni *et al.* (2010) found that Ca and magnesium (Mg) content in *Lycopersicum esculentum* decreased with increasing plant maturity. Musa and Ogbadoyi (2012) observed that the micronutrient content differed with leaf position (i.e. age) in *Telfairia occidentalis* as Ca, Fe, Mg and Zinc (Zn) content decreased from the basal to the upper leaves. However, in a similar study on *Corchorus olitorius*, the levels of Ca, Fe, Mg and Zn fluctuated from the basal to the middle and upper leaves (Musa, 2012).

According to Oelberg (1956) and Fong *et al.* (2015), the genetic control of micronutrient acquisition in leafy plants is such that some plants which are maintained in low Ca soil may exhibit high amounts of Ca in their plant parts and *vice versa.* Modi (2007) found that Ca and Fe content of *Amaranthus sp.* were higher at warmer (27/21 ºC) than at cooler (21/15 ºC) (day/night) temperature regimes. *Brassica oleracea* genotypes differed in Ca, Fe, Mg and Zn content from one season to another (Kopsell *et al.*, 2005). Similarly, Pandino *et al.* (2011) observed that season and location influenced levels of Ca, Fe, Cu and Zn in *Cynara cardunculus* var. *scolymus* genotypes. In a study to determine whether the amounts of leaf Ca, Fe and Mg were affected by dry or rainy seasons, Agbo *et al.* (2014) found that while Ca and Mg content increased in *Cleome gynandra* from the dry to the rainy season, the levels of Fe decreased.

While the physical properties, such as soil type and porosity, may have indirect effects on micronutrient content, the chemical properties of the soil determine the ability of the plant to absorb them (Oelberg, 1956). The addition of fertilisers also affects the nutritional quality of plants. Ayeni *et al.* (2010) established that supplying poultry manure or NPK fertiliser to *Lycopersicum esculentum* plants was effective in increasing leaf Ca and Mg content (Ewulo *et al.*, 2008). Safaa and Abd El Fattah (2007) found that nitrogen containing fertilisers and soil type both influenced and increased the levels of Fe, Mn and Zn in *Lactuca sativa* var. *capitata*

L. In a similar study, Musa *et al.* (2010) supplied *Corchorus olitorius* plants with nitrogen fertiliser and observed that Fe and Cu content decreased with fertiliser application, while Mg and Zn levels were not affected. In these studies, the method in which the plant material was analysed and the time of collection of plant material may have also influenced the micronutrient content of the plants.

2.5 Propagation of *Solanum nigrum*

2.5.1 Conventional propagation

Solanum nigrum plants have the ability to reproduce sexually via seed and vegetatively via cuttings (Chweya, 1997; Edmonds and Chweya, 1997), with the former being the most commonly-used method (Chweya, 1997; Taab and Anderson, 2009). In a study undertaken by Mwafusi (1992) to investigate the effect of propagation via seeds and cuttings on the growth and quality of *S. nigrum* plants, it was found that *S. nigrum* could be propagated via shoot cuttings but such plants appeared to grow less vigorously than those derived from seeds. A literature search found that no further studies have been undertaken on the use of cuttings of this species.

Solanum nigrum is an inbred species and its seeds are housed in prolific amounts in globose, dull purple-black berries (Basset and Munro, 1985; Stankiewics *et al.*, 2001). The seeds are equipped to survive in a dormant stage for prolonged periods of time, until provided with optimal conditions for germination and can, therefore, be stored in seed banks (Basset and Munro, 1985; Stankiewics *et al.*, 2001). Suthar *et al.* (2009) explained that in *S. nigrum* the mechanism of dormancy may be associated with the seeds' impermeable coat. The seeds can be germinated by removing them from the berries (fruit) and sowing them just below the surface of the soil (Taab and Anderson, 2008; Taab and Anderson, 2009). This is done by squeezing the seeds into a pulp and drying them at room temperature, away from the sun, as exposure may negatively affect germination (Keely and Thullen, 1983; Givelberg *et al.*, 1984; del Monte and Tarquis, 1997; Kremer and Lotz, 1998; Taab and Anderson, 2008). According to Edmonds and Chweya (1997), seeds can also be removed from the berries using a fermentation process or using hydrochloric acid.

The seeds of *S. nigrum* are commonly distributed by birds and cattle who consume and excrete them after they have been partially scarified by stomach acid (Basset and Munro, 1985; Barnea

et al., 1990; Stankiewics *et al.*, 2001). According to Ciarka and Gawronski (1996), this enhances seed germination. *Solanum nigrum* seedlings tend to self-pollinate and flower 5 to 7 weeks after germination, indicating the sexual maturity of the plant (Edmonds and Chweya, 1997; Kremer and Lotz, 1998). Within this period, the tender shoots and leaves are harvested and used as vegetables for the preparation of meals (Chweya, 1997; Jansen van Rensburg *et al.*, 2007).

2.5.2 *In vitro* propagation

Micropropagation is a method of mass-producing plants of a genotype with traits of interest (i.e. cloning), by manipulating the ability of a plant cell to regenerate whole plants *in vitro* through the use of exogenous plant growth regulators (PGRs) (Hartmann *et al.,* 1997; George *et al.*, 2008; Acquaah, 2012). It involves the growth of decontaminated plant material (explants), such as leaves, shoot tips, nodes and stems, in an aseptic environment which is later transferred to the external environment (George *et al.*, 2008). To ensure decontamination, *S. nigrum* explants are usually rinsed thoroughly under running tap water with a surfactant (Tween20® or Teepol®), prior to surface sterilisation which involves dipping the explant in 70 % alcohol and/or low levels of mercuric chloride ($HgCl_2$), followed by several rinses in sterile distilled water (Kolar *et al.,* 2008; Pandhure *et al.,* 2010; Sundari *et al.,* 2010; Verma *et al.,* 2010; Padmapriya *et al.,* 2011; Sridhar and Naidu, 2011a, b; Sarethy *et al.,* 2014; Geetha *et al.,* 2016). Nodes and leaf segments are the most commonly used explant types for establishing *in vitro* cultures of *S. nigrum* (Tables 5 and 6). Whole plants of this species have been regenerated *in vitro* mainly via direct (Table 5) or indirect (Table 6) organogenesis but can also be regenerated *in vitro* via the somatic embryogenesis pathway (Xu *et al.*, 2014).

Organogenesis

Solanum nigrum explants have been mainly cultured onto Murashige and Skoog (MS) (1962) media containing 30 g l^{-1} sucrose and various concentrations and combinations of PGRs, i.e. benzylaminopurine (BAP), kinetin (KIN), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (Bhat *et al.*, 2010; Sundari *et al.*, 2010; Verma *et al.*, 2010; Sridhar and Naidu, 2011a, b; Choudhary *et al.*, 2014; Geetha *et al.*, 2016). Most of the *in vitro* studies undertaken using *S. nigrum*, used BAP at the multiplication stage, alone or in combination with other PGRs (Table 5). Cytokinins, such as BAP, are recognised for their ability to enhance cell division, and to promote shoot multiplication and axillary bud

proliferation (George *et al.*, 2008). As part of the direct organogenesis pathway, the cytokinin KIN (0.25-5.4 mg l^{-1}) and other auxins were also used for shoot multiplication (Table 5) and

Table 5: List of reported micropropagation studies carried out on *Solanum nigrum* and combinations of plant growth regulators (PGRs) (mg l^{-1}) required by different explants for direct organogenesis protocols. BAP = benzylaminopurine, IAA = indole-3-acetic acid, IBA = indole-3-butyric acid, NAA = α-naphthalene acetic acid, 2, 4-D = 2, 4- dichlorophenoxyacetic acid, KIN = kinetin, TDZ = thidiazuron, NR = Not reported.

Explant type	Multiplication PGRs	Rooting PGRs	Reference
Leaves Internode Shoot tips	BAP [0.25-2.0] KIN [0.25-2.0]	IBA [0.5-5.0] IAA [0.5-5.0] NAA [0.5-5.0]	Hussanein and Soltan (2000)
Leaves	BAP [2.0]	IBA [1.0]	Kannan *et al.* (2006)
Leaves	BAP [0.25-2.0] KIN [0.25- 2.0]	IBA [0.25-2.0] IAA [0.25-2.0] NAA [0.25-2.0]	Bhat *et al.* (2010)
Leaves	BAP [3.0- 7.0] IBA [0.5]	IBA [0.5-2.5]	Pandhure *et al.* (2010)
Nodes	BAP [0.1-5.6]	IAA [0.2 and 1.0] IBA [1.0 and 4.0] NAA [0.2- 4.0]	Sundari *et al.* (2010)
Nodes	BAP [1.0]	NR	Verma *et al.* (2010)
Nodes	BAP [1.3-6.3] KIN [1.0-5.4]	IBA [1.0-5.0] NAA [0.9-4.7]	Padmapriya *et al.* (2011)
Leaves	BAP [0.5-3.0] KIN [0.5-3.0] alone or in combination with IAA [0.5] NAA [0.5] IBA [0.5]	IAA [0.25-1.0] NAA [0.25-1.0] IBA [0.25-1.0]	Sridhar and Naidu (2011b)

Table 5 continued

Explant type	Multiplication PGRs	Rooting PGRs	Reference
Shoot tips Nodes	BAP [1.0-5.0] KIN [1.0-5.0]	NAA [0.1-1.0]	Kavitha *et al.* (2012)
Nodes	BAP [0.25- 2.3]	IBA [0.6-4.0]	Rathore and Gupta (2013)
Nodes	IAA [0.0-5.0] NAA [1.0-5.0] BAP [0.0-1.0]	NR	Sarethy *et al.* (2014)
Axillary buds Leaves	BAP [1.0-2.0] KIN [1.0-2.0] in combination with IAA [0.5] NAA [0.5] and $AgNO_3$ [0.1-1.0]	IBA [1.0-2.0] NAA [1.0-2.0] IAA [1.0-2.0]	Geetha *et al.* (2016)
Leaves Nodes Petiole	TDZ [0.1-0.5]	NAA [0.09-0.3] IBA [0.1-0.4]	Afrasaib *et al.* (2017)

included NAA (0.5-5.0 mg l^{-1}), IBA (0.5 mg l^{-1}) TDZ (0.1-0.5 mg l^{-1}), and IAA (0.5-5.0 mg l^{-1}) (Hussanein and Soltan, 2000; Bhat *et al.*, 2010; Pandhure *et al.*, 2010; Padmapriya *et al.*, 2011; Sridhar and Naidu, 2011b; Kavitha *et al.*, 2012; Sarethy *et al.*, 2014; Afrasaib *et al.*, 2017).

The concentration of BAP used for each explant type differed among the various studies and ranged from 0.5 to 6.3 mg l^{-1} BAP (Table 5). Kannan *et al.* (2006), Bhat *et al.* (2010) and Sridhar and Naidu (2011b) found that 2.0 to 3.0 mg l^{-1} BAP was required to induce shoots in leaf explants. Sridhar and Naidu (2011b) reported 27 shoots/leaf explant when placed on media containing 3 mg l^{-1} BAP in combination with 0.5 mg l^{-1} IAA. Bhat *et al.* (2010) found that leaf explants produced 11 shoots/explant on media containing 2.0 mg l^{-1} BAP and 19 shoots/explant on leaf explants that were placed on 1.5 mg l^{-1} KIN (Bhat *et al.*, 2010). Pandhure *et al.* (2010) found that 6 mg l^{-1} BAP with 0.5 mg l^{-1} IAA resulted in 19 shoots/explant.

Sundari *et al.* (2010), Padmapriya *et al.* (2011) and Kavitha *et al.* (2012) found that 3 mg l^{-1} BAP was the most effective PGR concentration to induce shoots from nodal explants. They reported high percentages of nodal explants producing shoots on shoot multiplication media containing between 3.0 to 3.4 mg l^{-1} BAP. However, Kavitha *et al.* (2012) obtained 8 shoots/explant when nodal explants were placed on 3.0 mg l^{-1} BAP, whereas Sundari *et al.* (2010) only reported 2 shoots/nodal explant. Similar to the findings of Kavitha *et al.* (2012), Rathore and Gupta (2013) reported that nodal explants produced 8 to 10 shoots/explant, but on media containing 0.5 mg l^{-1} BAP. In a similar study, Sarethy *et al.* (2014) found that *S. nigrum* nodal explants produced 19 shoots/explant when placed on shoot multiplication medium containing 0.5 mg l^{-1} BAP in combination with 3 mg l^{-1} IAA.

The auxins most routinely used for rooting *in vitro* shoots of *S. nigrum* are IBA (0.25-5.0 mg l^{-1}), IAA (0.25-5.0 mg l^{-1}) and NAA (0.25-5.0 mg l^{-1}) (Table 5). High percentages (71-100 %) of shoots rooted on 0.5 mg l^{-1} IBA (Kolar *et al.,* 2008; Bhat *et al.,* 2010; Pandhure *et al.,* 2010; Swain *et al.,* 2010; Sridhar and Naidu, 2011a, b) and 1.0 mg l^{-1} IBA (Hussanein and Soltan; 2000; Kannan *et al,* 2006; Sundari *et al.*, 2010). Similar results (71-100 % rooting) were also reported when *S. nigrum* shoots were rooted on 0.5 mg l^{-1} BAP (Kolar *et al.,* 2008; Sridhar and Naidu, 2011a, b).

Indirect organogenesis occurs when an explant that is placed onto media containing high levels of auxins (and sometimes cytokinins) produces shoots from callus. Calli are made up of a mass of undifferentiated cells (Rao, 2004) which can be regenerated to produce shoots and then roots (Hartman *et al.,* 1997; George *et al.,* 2008). In *S. nigrum,* the cytokinin BAP (0.5 mg l^{-1}) is the most frequently used PGR for callus induction in leaves and nodal segments (Table 6) (Hussanein and Soltan, 2000; Kolar *et al.,* 2008; Swain *et al.,* 2010; Sridhar and Naidu, 2011a; Ewais *et al.,* 2015), in combination with other auxins such as 0.1 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D) (Hussanein and Soltan, 2000), 2 mg l^{-1} IAA (Kolar *et al.,* 2008) and 3 mg l^{-1} NAA (Sridhar and Naidu, 2011a). Leaf segments have been the preferred explants for inducing calli (Table 6). Ewais *et al.* (2015) found that 88 % of leaf explants produced calli. Sridhar and Naidu (2011a) also reported that although callus was produced from both nodal and leaf explants, leaf explants produced more calli than nodal explants, which were green and compact.

Table 6: List of reported micropropagation studies carried out on *Solanum nigrum* and combinations of plant growth regulators (PGRs) (mg l^{-1}) required by different explants for indirect organogenesis protocols. BAP = benzylaminopurine, IAA = indole-3-acetic acid, IBA = indole-3-butyric acid, NAA = α-naphthalene acetic acid, 2,4-D = 2,4-dichlorophenoxyacetic acid, KIN = kinetin, NR = Not reported.

Explant type	Callus induction PGRs	Multiplication PGRs	Rooting PGRs	Reference
Anthers	NAA [1.9] 2,4-D [2.2] KIN [2.2]	NAA [0.0-0.5] 2,4-D [0.0-0.5]	NR	Harn (1972)
Leaves Stems Shoot tips	BAP [0.6] NAA [1.0] 2,4-D [0.1]	BAP [0.25-2.0] KIN [0.25-2.0]	IBA [0.5-5.0] IAA [0.5-5.0] NAA [0.5-5.0]	Hussanein and Soltan (2000)
Leaves Nodes	IAA [2.0] BAP [0.5]	BAP [3.0-7.0] IAA [0.5] NAA [0.5]	IBA [0.5-2.5]	Kolar *et al.* (2008)
Leaves	BAP [3.0-7.0] IBA [0.5]	BAP [3.0-7.0] IBA [0.5]	IBA [0.5-2.5]	Pandhure *et al.* (2010)
Hypocotyl Segments	BAP [0.25] NAA [1.0]	BAP [2.0] NAA [0.5]	IBA [0.5]	Swain *et al.* (2010)
Leaves Nodes	2,4-D [0.5-2.0] IAA [0.5-3.0] NAA [0.5-3.0] In combination with BAP [0.5]	BAP [0.5-3.0] KIN [0.5] Alone or in combination with IAA [0.1 and 0.5] NAA [0.1 and 0.5]	IBA [0.25-1.0] NAA [0.25-1.0]	Sridhar and Naidu (2011a)
Leaves Shoot tips	2,4-D [0.2-0.4] KIN [0.5-1.0]	2,4-D [0.2-0.4] KIN [0.5-1.0]	NR	Choudhary *et al.* (2014)
Leaves	NAA [0.5-3.0] BAP [0.5]	NR	NR	Ewais *et al.* (2015)

Somatic embryogenesis

Somatic embryogenesis is the process by which somatic cells develop into embryos *in vitro*, and then germinate in a similar way to zygotic embryos (Kunitake and Mii, 1998; George *et al.*, 2008). As with organogenesis, the production of these somatic embryos may proceed directly from the explant or indirectly via a callus stage (Hartmann *et al.*, 1997; George *et al.*, 2008). Little information is available on the propagation of *S. nigrum* via somatic embryogenesis, other than a study by Xu *et al.* (2014), who obtained somatic embryo-like structures which they called frog egg-like bodies (FELBs). These were formed from the callus induced from leaf, stem and root explants, which were cultured onto media containing 2, 4-D and NAA. The best PGR concentration was 1 mg l^{-1} 2, 4-D that resulted in 100 % (leaves), 85 % (stems) and 93 % (roots) callus induction (Xu *et al.*, 2014). Xu *et al.* (2014) also noted that calli were only produced in the presence of 2, 4-D and that NAA failed to produce FELBs. All FELBs produced more than one plantlet when cultured onto shoot induction media containing 5 mg l^{-1} BAP in combination with 0.1 mg l^{-1} gibberellic acid (GA_3). While Xu *et al.* (2014) did report the transfer of *in vitro* shoots to a rooting medium, no further information on the composition of the media or findings on the effect of rooting medium on root production were reported.

2.6 *In vitro* conservation

In vitro micropropagation methods are predominantly used for mass production of selected plant genotypes and conservation of endangered ones (Engelmann, 1991; Bertrand-Desbrunais *et al.*, 1992; Engelmann and Engels, 2002). However, the use of such techniques may also be employed for storing plant material for subsequent propagation (Engelmann, 1991; Bertrand-Desbrunais *et al.*, 1992; Negash *et al.*, 2001; Rao, 2004; Fah, 2013). There are two approaches to *in vitro* storage: a) minimal growth storage and b) cryopreservation, and their use is dependent on the required duration of storage (Engelmann, 1991; Rao, 2004; Keller *et al.*, 2006; da Silva and Scherwinski-Pereira, 2011; Engelmann, 2011; Joshi and Jadhav, 2013). The former is best suited for short-to-medium term storage, whereas the latter facilitates long-term storage (Engelmann, 1991; Rao, 2004; Keller *et al.*, 2006; Ahmed and Anjum, 2010; Engelmann, 2011; Fah, 2013). These *in vitro* storage techniques have an advantage over *in situ* and *ex situ* conservation techniques, because they require minimal labour, reduced storage space, are free from contamination, have high multiplication rates and enable the exchange of plant material internationally (Engelmann, 1991; Bekheet, 2007; Engelmann, 2011; Pérez-

Molphe-Balch *et al.*, 2012). Although one of the objectives of the present study was to develop a protocol for *in vitro* minimal growth storage but not cryopreservation of selected *S. nigrum* genotypes, both are briefly discussed below.

a) Minimal growth storage

The main aim of minimal growth storage is to increase the interval between sub-cultures by limiting growth, thereby allowing aseptic plant material to be stored *in vitro* for approximately one to five years (Engelmann, 1991; Pérez-Tornero *et al.*, 1999; Engelmann and Engels, 2002; Rao, 2004; Bekheet, 2007; Cha-um and Kirdmanee, 2007; Ahmed and Anjum, 2010; Engelmann, 2011). However, for this technique to be successful, plant material must maintain its ability to regenerate and be rapidly micropropagated after the storage period (Witomska *et al.*, 2008; Pérez-Molphe-Balch *et al.*, 2012). Minimal plant growth is usually achieved by imposing one or more of the growth restrictions on the *in vitro* plantlets described below.

Culture medium

The culture medium can be modified by reducing the strength of MS basal nutrients and/or increasing or decreasing the concentration of sucrose in the medium (George *et al.*, 2008; Ahmed and Anjum, 2010; Engelmann, 2011). As there are no reports on minimal growth storage for *S. nigrum* germplasm, minimal growth storage of some other herbaceous plants are outlined here. Successful growth storage has been reported for many species, including *Mentha sp.* which was stored on hormone-free media containing ½ MS, 30 g l^{-1} sucrose and 15 g l^{-1} gelrite for a minimum of 24 months (Reed, 1999). Minimal-growth of *Elettaria cardomomum* (L.) Maton. was achieved for 18 months by storing explants on media containing ½ MS which included 1 mg l^{-1} BAP, 30 g l^{-1} sucrose and 7 g l^{-1} agar at 25º C (Tyagi *et al.*, 2009).

Growth retardants (e.g. abscisic acid) or osmotic substances (e.g. mannitol or sorbitol) may also be added in order to limit shoot growth (Engelmann, 1991; Cha-um and Kirdmanee, 2007; Akdemir *et al.*, 2010; da Silva and Scherwinski-Pereira, 2011; Engelmann; 2011; Pérez-Molphe-Balch *et al.*, 2012). Bekheet (2007) evaluated the effect of inducing osmotic stress on globe artichoke cultures by including either 40 g l^{-1} mannitol or 40 g l^{-1} sorbitol to the minimal growth storage media. The ability of these cultures to recover after minimal growth storage was screened over three, six, nine and twelve months. That study revealed that the percentage survival decreased from 100 % (with 4 shoots/explant) at three months to 20 % (with 1 shoot/explant) at twelve months (Bekheet, 2007).

Temperature

The storage temperature of cultures is dependent on the plant's sensitivity to low temperatures (Engelmann, 1991; Pérez-Molphe-Balch *et al.*, 2012). Cold-tolerant plants generally require low temperatures (0-5 ºC) in order to limit growth, whereas cold-sensitive plants require temperatures between 15 and 20 ºC (Cha-um and Kirdmanee, 2007; Witomska *et al.*, 2008; Engelmann, 2011; Fah, 2013). Reed (1999) reported successful storage of cold-tolerant *Mentha sp.* genotypes at 4 ºC for 30 months, while cold-sensitive genotypes survived only for 18 months and at 25 ºC. Cantos *et al.* (1998) reported successful growth reduction of *Atropa baetica* Willk. germplasm, a herbaceous plant belong to the family Solanaceae, by exposing cultures to 4 ºC or 25 ºC. Broccoli plantlets (*Brassica oleracea* L.) were successfully stored at 5 and 10 ºC for six weeks without any effect on their dry mass (Kubota and Kozai, 1994).

Other factors

The choice of culture vessel and the volume of culture medium play important roles in minimal growth storage (Engelmann, 1991) because they determine the availability of oxygen to the cultures (George *et al.*, 2008). Different explant types vary in their physiological condition and this influences the efficiency of storage under minimal growth conditions (Engelmann, 1991; Sharma and Nautiyal, 2009). Decreasing light intensity, in combination with temperature, is also sometimes effective in limiting *in vitro* plant growth (Engelmann, 1991; Pérez-Tornero *et al.*, 1999; Rao, 2004; Engelmann, 2011). Explants with differentiated cells, such as shoots, tend to be less susceptible to somaclonal variation in minimal growth storage than callus (Rao, 2004).

b) Cryopreservation

Cryopreservation is considered by some to be the only cost efficient and long-term conservation technique available (Engelmann, 2004; Cha-um and Kirdmanee, 2007; Engelmann, 2011). The technique is based on the reduction and eventual cessation of metabolic functions in plant material during storage at ultra-low temperatures in liquid nitrogen (-196°C), while retaining cellular viability (Engelmann, 1991; Bekheet, 2000 Engelmann and Engels, 2002; Rao, 2004; Engelmann, 2011; Fah, 2013). Theoretically, small pieces of plant material can be stored in this manner for an unlimited period of time (Engelmann, 1991; Engelmann and Engels, 2002; Fah, 2013). However, subsequent successful regeneration and propagation is a prerequisite (Rao, 2004). Cryopreserved cultures generally occupy a small volume of space, require minimal

maintenance and are protected from contamination (Engelmann and Engels, 2002; Engelmann, 2004; Rao, 2004; Engelmann, 2011).

There are two techniques of cryopreservation, i.e. freeze-induced dehydration and vitrification (Engelmann, 2004; Rao, 2004; Engelmann, 2011). The former requires plant material to be slowly cooled down to approximately - 40^{o} C prior to immersion in liquid nitrogen for rapid freezing (Engelmann, 2004; Rao, 2004; Keller *et al.*, 2006; Engelmann; 2011). Cryoprotectants, such as sucrose, mannitol, sorbitol or dimethylsulfoxide, are also used to prepare plant material for the freezing process so as to maintain the integrity of the plant tissues during cryopreservation (Engelmann, 1991; Rao, 2004). The latter technique is based on the ability to vitrify intracellular solutes in plant material by removing most, if not all, freezable water by means of air desiccation or osmotic dehydration of plant material prior to rapid freezing (Engelmann, 2004; Rao, 2004; Engelmann, 2011).

3. Materials and methods

3.1 Plant material

3.1.1 Whole plants

Whole plants of *S. nigrum*, 300-400 mm tall and containing ripe fruits, were collected from the Bisasar Road Landfill site (29° 49 '4" S, 30° 58' 43" E) (Durban, South Africa) and transported to the School of Life Sciences, University of KwaZulu-Natal (Durban, South Africa). They were planted in flower pots (260 mm diameter, 210 mm depth) containing soil and maintained in a shadehouse facility (Figure 1 A).

The plants were watered twice daily for 1 min by an automatic watering apparatus and treated on a weekly basis with the following systemic fungicides: 2 g l^{-1} Dithane® (mancozeb) (Kombat, South Africa) and 1 ml l^{-1} Supremo® (chlorothalonil) (Grovida, South Africa) as a foliar spray and 1 ml l^{-1} Chronos® (imidazole) (Makhro Agro, South Africa) and 1.25 ml l^{-1} Orius® (triazole) (Makhro Agro, South Africa) as a soil spray. They were also supplied with 1 ml l^{-1} Trelmix® (Hubers, South Africa) (22.6 g Fe kg^{-1}, 3.2 g Cu kg^{-1}, 2.9 Mn kg^{-1}, 2.4 g Zn kg^{-1}, 1.1 g B kg^{-1}, 0.3 g Mo kg^{-1}, 0.3 g Mg kg^{-1}) and 1 g l^{-1} Multifeed Orange® (193 g N kg^{-1}, 83 g P kg^{-1}, 158 g K kg^{-1}, 6.1 g S kg^{-1}, 4.6 g Mg kg^{-1}, 700 mg Zn kg^{-1}, 1054 mg B kg^{-1}, 63 mg Mo kg^{-1}, 751 mg Fe kg^{-1}, 273 mg Mn kg^{-1}, 75 mg Cu kg^{-1}) (Nulandis, South Africa) every alternate week, respectively.

As *S. nigrum* plants begin to flower, there is a reduction of vegetative growth and leaf yield (Chweya, 1997). Therefore, flower buds were regularly removed to encourage lateral growth in order to extend the harvesting period and prevent die-back. The shadehouse plants were used as a source of field-derived explants for subsequent *in vitro* investigations.

3.1.2 Seeds

Fruits (berries) were removed from the collected plants and the seeds extracted by squeezing out the fruit pulp onto dry paper towel and air-drying at room temperature for 24 h. The seeds were stored in an air-tight plastic container and placed in a cold room (5 ºC). Three weeks later, more seeds were extracted and air-dried as before. Thereafter, 40 chilled and 40 fresh seeds were chemically scarified with either 0.01 or 0.1 M HCl for 5 and 10 min before being sown into polystyrene seedling tray inserts (30 mm width, 100 mm depth) filled with soil. Seeds for

the control were sown directly into the soil without any scarification. The trays were housed in a mist tent inside a greenhouse (59.9-81.6 % relative humidity) and watered three times a day for 1 min by an automatic watering apparatus. Percentage germination was recorded after four weeks.

Two weeks later, the germinated seedlings were removed from the mist tent and transferred to larger flower pots (175 mm diameter, 135 mm depth). Solanaceous plants attract many harmful pests such as *Tetranychus evansi* (red spidermite) (Murungi *et al*., 2014) which was observed on some seedlings. Therefore, all seedlings were sprayed once with 2 ml l^{-1} Redspidercide® (tetradifon) (Efekto, South Africa) when spiders were observed. The seedlings were maintained in the greenhouse (Figure 1 B) and watered for 2 min every morning by an automatic watering system. They were also supplied with 5 g l^{-1} Multifeed Orange® on a weekly basis. Twelve to 14 weeks after germination, they were used as a source of seedling-derived explants for subsequent *in vitro* experiments.

Figure 1: (A) Field-derived plants of *Solanum nigrum* maintained in a shadehouse. (B) Seed-derived plants of *Solanum nigrum* maintained in a greenhouse.

3.2 Plant regeneration from nodal and leaf explants

3.2.1 Surface decontamination

Nodal and leaf explants from both seedling and field plants were used to determine the best source of explants for plant regeneration. Freshly harvested nodal and leaf explants from both seedling and field plants were separately washed under running tap water for 1 min, decontaminated with 1 % (v/v) sodium hypochlorite (NaOCl) containing a few drops of Tween 20® for 10 min, and washed three times (30 s each) with sterile distilled water. Nodal explants were also decontaminated with 1 % (v/v) NaOCl and Tween 20® and washed three times (30 s each) with sterile distilled water, prior to immersion in a filtered [using sterile 0.2 µm Acrodisc® filters (Pall Corporation Life Sciences, USA)] antibiotic wash containing 100 µg ml^{-1} Streptomycin and Penicillin for 30 or 60 min. Nodal explants were then aseptically trimmed to a length of 15 mm and leaf explants were trimmed to produce 15 mm x 15 mm segments.

3.2.2 Media preparation

Explants were cultured onto full strength Murashige and Skoog (MS) (1962) (Highveld Biological, South Africa) basal salt medium with vitamins, 30 g l^{-1} sucrose and 10 g l^{-1} agar-agar (Sigma, South Africa). The pH was adjusted to 5.8 ± 0.2 before autoclaving at 121 °C and 1.2 kg cm^{-2} for 20 min. All culture vessels were sealed with parafilm and maintained in a growth room at 16 h light (200 µmol m^{-2} s^{-1})/ 8 h dark photoperiod, at 25 and 23 °C, respectively.

3.2.3 Multiplication

Individual decontaminated explants were placed in culture tubes (25 mm diameter, 90 mm depth) containing 5 ml of medium. Nodal explants from field plants were cultured onto shoot multiplication media containing 1 or 3 mg l^{-1} benzylaminopurine (BAP) (Kavitha *et al.*, 2012). Media, that included 3 mg l^{-1} BAP, for nodal explants from seedling and field plants were prepared with or without both 1 ml l^{-1} Methylene blue® and 1 ml l^{-1} Previcur® (propamocarb hydrochloride) (R.T Chemicals, South Africa). The cultures with 1 or 3 mg l^{-1} BAP without Methylene blue® and Previcur® were kept in a growth room for two weeks, whereas those with 3 mg l^{-1} BAP, Methylene blue® and Previcur® were maintained in the growth room for two weeks with the explants being sub-cultured onto fresh media after one week. Results recorded included percentage contamination, percentage necrosis, time taken to produce visible shoots,

percentage explants with shoots and number of shoots/explant. As bacterial contamination was too extensive, nodal explants were not elongated and rooted.

Decontaminated leaf explants from seedling and field plants were cultured onto medium containing 3 mg l^{-1} BAP, without the addition of Methylene blue® (Sridhar and Naidu, 2011b). The cultures were maintained in the growth room for four weeks (seed-derived explants) and seven weeks (field-derived explants). The recorded results included percentage contamination, percentage necrosis, time taken to produce visible shoots, percentage explants with shoots and number of shoots/explant but no statistical comparisons were done due to the difference in culture period.

3.2.4 Elongation and rooting

Subsequent to multiplication, shoot clumps of ± 10 shoots arising directly from leaf explants were placed onto 20 ml medium (as described in section 3.2.2) in culture bottles (50 mm diameter, 90 mm depth) containing 0.1 mg l^{-1} BAP and 1 ml l^{-1} Methylene blue®. These cultures were maintained in a growth room for one week (seed-derived explants) or two weeks (field-derived explants) to elongate shoots to 10-20 mm tall. After elongation, individual shoots (10 to 20 mm tall) were rooted on 5 ml of three different rooting media in culture tubes: A) 4.4 g l^{-1} MS salts and vitamins, 30 g l^{-1} sucrose, 10 g l^{-1} agar, 1 ml l^{-1} Methylene blue®; B) as for A but including 0.5 mg l^{-1} IBA (indole-3-butyric acid) (Sridhar and Naidu, 2011b); or C) as for A but including 1 mg l^{-1} IAA (indole-3-acetic acid) (Bhat *et al.*, 2010). These cultures were maintained in the growth room for three weeks. Results recorded included % rooted shoots and shoot length.

3.2.5 Acclimatisation

Plants (30 to 120 mm tall) were removed from the culture tubes and the roots were rinsed in distilled water to remove excess agar. The plants were placed individually in flower pots (100 mm diameter, 80 mm depth) containing soil. Each pot was watered with ¼ strength MS basal salt medium with vitamins, individually covered with clear plastic bags and maintained in a growth room at 16 h light (200 µmol m^{-2} s^{-1})/ 8 h dark photoperiod, at 25 and 23 °C, respectively (Swain *et al.*, 2010). After one week, the bags were perforated and one week later, removed completely. The potted plants were then transferred to a mist tent in a greenhouse for one week and watered by an automatic watering apparatus for 1 min twice a day. The plants

were then removed, transferred to larger flowers pots (175 mm diameter, 135 mm depth) containing soil and watered by an automatic watering apparatus for 2 min every morning for two weeks in the greenhouse. Results recorded included percentage survival, shoot length, and fresh and dry masses of the shoots and roots.

3.3 Determination of calcium and iron content in leaves

Leaves were harvested from plants, dried in an oven at 85 ºC for 48 h and ground to a fine powder using a mortar and pestle. Thereafter, 0.15 g dry mass of plant was digested in 5 ml nitric acid for 10 min on a hotplate under a fume hood (Nayar *et al.*, 1975). Each sample was then diluted to 25 ml with Millipore® water, filtered into a polypropylene vial using a 0.2 µm sterile Acrodisc® filter (Pall Corporation Life Sciences, USA) and analysed for calcium (Ca) and iron (Fe) content using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Perkin Elmer 5300, Germany). Total Ca content was expressed as mg of Ca per 100 g of dry leaf weight through a calibration curve with Ca standards. The calibration curve range was 10-150 mg l^{-1}. Total Fe content was expressed as mg of Fe per 100 g of dry leaf weight through a calibration curve with Fe standards. The calibration curve range was 0.05-10 mg l^{-1}. All analyses were performed in triplicate.

3.4 Screening and micropropagation of selected genotypes

3.4.1 Screening

Fifty, 6-week old seed-germinated plants, with 8-10 leaves and 250 mm tall, were screened for their Ca and Fe levels. The fourth, fifth and sixth leaves (± 2.2 g fresh mass) were harvested from each plant and assayed for Ca and Fe, as presented in section 3.3.

3.4.2 Selection and micropropagation

Two genotypes for each of the following categories: a) high Ca (G5 and G20); b) high Fe (G6 and G15); c) low Ca (G43 and G45) and; d) low Fe (G35 and G50) were selected for micropropagation using the protocol for leaf explants (as discussed in section 3.2). Hormone-free MS medium (rooting medium A, as described in section 3.2.4) was used for rooting *in vitro* shoots of the selected genotypes. After three weeks, the acclimatisation substrate included 1 soil : 2 vermiculite (w/w) (1S : 2V). After two weeks of acclimatisation in plastic bags in a growth room (as presented in section 3.2.5), half of the selected cloned genotypes were

transferred to larger flower pots (175 mm diameter, 135 mm depth) containing 1S : 2V while the other half were transferred into flower pots of the same size containing soil. All pots were maintained in the greenhouse, as described in section 3.2.5. Results were recorded as explained in section 3.2. The number of branches, number of leaves, shoot and root lengths, fresh and dry shoot and root masses and root:shoot dry masses were recorded at the start of acclimatisation and after 6 weeks *ex vitro* (which included 2 weeks acclimatisation).

3.4.3 The effect of physiological age and substrate on growth and calcium and iron content

One genotype of each of the following categories: a) high Ca (G20); b) high Fe (G6); c) low Ca (G43); and d) low Fe (G35) was selected to evaluate the effect of physiological age (i.e. 4, 6 and 8 weeks *ex vitro* growth) and substrate type (i.e. soil and 1S : 2V) on growth and Ca and Fe content of clones of the selected genotypes of *S. nigrum* (Figure 2). Results for micropropagation were recorded as described in section 3.4.2. After 4, 6 and 8 weeks of *ex vitro* growth (which included two weeks acclimatisation), a portion of plants were harvested for each genotype. Results recorded for *ex vitro* growth were for the number of leaves, shoot and root fresh and dry masses and root:shoot dry mass. The levels of Ca and Fe were determined as outlined in section 3.3.

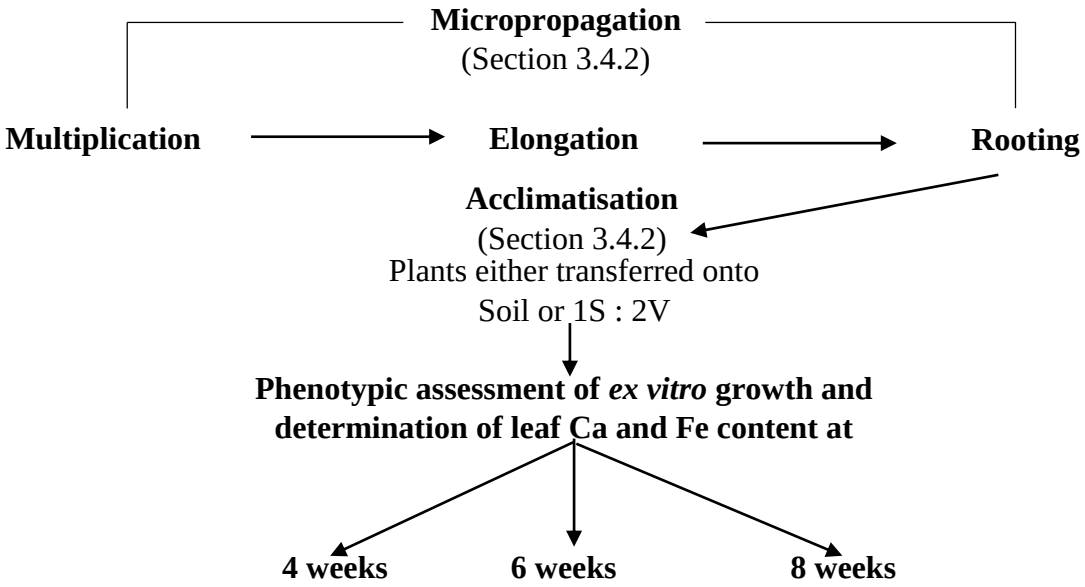


Figure 2: Experimental design for the study on the effect of physiological age and substrate type on the *ex vitro* growth and Ca and Fe content of the clones of the selected genotypes of *Solanum nigrum.*

3.5 Minimal growth storage

Four-week old *in vitro* shoots of the selected genotypes (G5, G6, G15, G20, G35, G43, G45 and G50) were transferred from shoot multiplication medium onto two types of minimal growth media in culture bottles (50 mm diameter, 90 mm depth): A) ⅓ MS basal salt medium with vitamins, supplemented with 10 g l^{-1} sucrose, 1 ml l^{-1} Methylene blue® and 10 g l^{-1} agar and B) as for A but with ¼ MS basal salt medium with vitamins and 5 g l^{-1} sucrose. The cultures were maintained in a growth room for eight weeks at 16 h light (200 µmol m^{-2} s^{-1})/ 8 h dark photoperiod, at 25 and 23 °C, respectively.

After eight weeks, individual shoots that appeared viable were retrieved from storage and transferred onto 3 mg l^{-1} BAP shoot multiplication medium (as described in section 3.2.3) for four weeks and placed under standard growth room conditions (as presented in section 3.2.2). Results were recorded for the number of shoots/explant. Thereafter, shoots (± 10 mm) were transferred to elongation medium, followed by rooting on hormone-free MS medium (rooting medium A; as discussed in section 3.2.4) and acclimatisation (as described in section 3.2.5).

3.6 Statistical analyses

Data were analysed using the statistical package SPSS (Version 22 and 23). The data were tested for normal distribution and significant differences between two treatments using an unpaired t-test and amongst three or more treatments using an analysis of variance (ANOVA) followed by a Tukey's post-hoc test. In some cases, data were $\log_{10}$ transformed prior to carrying out appropriate statistical tests. A probability of $p \leq 0.05$ was considered significant.

4. Results

4.1 Seed germination

Freshly harvested and chilled seeds were sourced from *S. nigrum* field plants, germinated in potting soil and housed in a greenhouse. After 4 weeks, the germination percentages of the controls of the fresh (28 ± 5 %) and chilled (5 ºC; 3 weeks) (60 ± 7 %) seeds were considered too low for the purpose of this study (Figure 3). Therefore, to enhance germination, seeds were chemically scarified by immersion in two concentrations of HCl (0.01 and 0.1 M) for 5 and 10 min each. Scarification increased the percentage germination of the fresh and the chilled seeds in comparison to the control (Figure 3). However, the percentage germination was not significantly different between the 5 and 10 min treatments in HCl (0.01 or 0.1 M) (Figure 3). The overall percentage germination was significantly higher in chilled (60-95 %) than in fresh (27-60 %) seeds. Seed germination was optimal (almost 100 %) when the seeds were pre-chilled and then chemically scarified with 0.01 M HCl for 10 min. The seedlings from this treatment were then used as a source of explants for subsequent *in vitro* experiments.

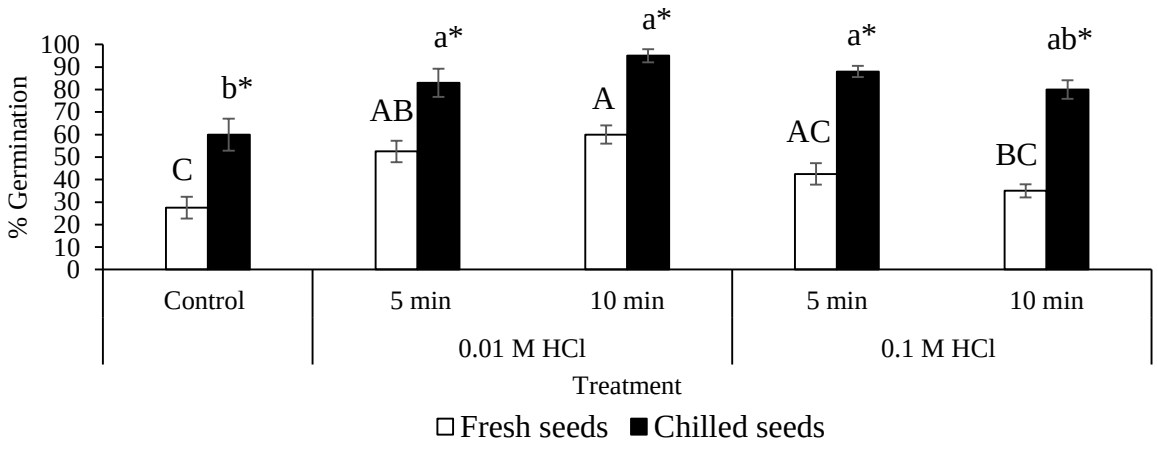


Figure 3: The effect of immersion time and concentration of HCl on the % germination of fresh and chilled *Solanum nigrum* seeds 4 weeks after sowing. Data were analysed between seed types for each treatment using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=40), across treatments for freshly harvested seeds and across treatments for chilled seeds using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=40). Different uppercase letters denote a statistical significance across treatments for fresh seeds and different lowercase letters denote a statistical significance across treatments for chilled seeds. * denotes a statistical significance between fresh seeds and chilled seeds for each treatment.

4.2 Strategies for the establishment of a decontamination protocol

4.2.1 Preliminary studies on the decontamination of nodal explants

Solanum nigrum nodes, excised from field plants kept in a shadehouse, were decontaminated with 1 % (v/v) NaOCl and Tween 20® for 10 min (as described in section 3.2.1) and placed onto shoot multiplication media (as presented in sections 3.2.2 and 3.2.3) containing either 1 or 3 mg l^{-1} benzylaminopurine (BAP). Shoots developed within 5 days of culture on media containing both concentrations of BAP. However, some explants began to exhibit fungal and bacterial contamination within 3 days of culture (Table 7).

For all tested parameters, no significant differences were noted between explants placed on 1 and 3 mg l^{-1} BAP (Table 7). However, there were high levels of fungal and bacterial contamination on explants cultured on both BAP-containing media (Table 7). A high percentage of explants produced shoots directly from their nodal region (88 ± 7.2 % and 100 ± 0.0 % on 1 and 3 mg l^{-1} BAP, respectively) but they eventually became contaminated or necrotic. Results for the percentage explants with shoots were recorded when the shoots were first observed (3-5 days) at the nodal region prior to the onset of contamination or necrosis. The nodal explants produced only one shoot/explant even though 88 ± 7.2 and 100 ± 0.0 % of explants produced shoots on 1 and 3 mg l^{-1} BAP, respectively. High percentages (75 ± 0.0 and 81 ± 11.9 % on 1 and 3 mg l^{-1} BAP, respectively) of nodal explants also produced calli at the base of the shoot (Table 7). As there were no significant differences in the effect of BAP concentration on shoot production, in subsequent studies, all explants were cultured on 3 mg l^{-1} BAP.

Table 7: The effect of the decontamination protocol and media containing 1 and 3 mg l^{-1} BAP on % fungal and bacterial contamination, % necrosis, % explants forming callus, % explants with shoots and the number of shoots/explant of nodes of *Solanum nigrum* derived from field plants. Data were recorded after 2 weeks and were analysed using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=16). For all parameters, there were no significant differences between explants placed on 1 and 3 mg l^{-1} BAP.

BAP (mg l^{-1})	% Contamination		% Necrosis	% Explants with shoots	# Shoots/ explant	% Explants forming callus
	Fungal	Bacterial				
1	50±10.2	13±7.2	63±7.2	88±7.2	1±0.0	75±0.0
3	25±10.2	31±15.7	38±12.5	100±0.0	1±0.2	81±11.9

In conclusion, the nodal explants had high percentages of contamination (fungal and bacterial) and necrosis. Therefore, decontamination of the nodes had to be further investigated (section 4.2.2).

4.2.2 Decontamination of explants from seedling and field plants

Nodal explants

In an attempt to obtain aseptic explant material, nodal segments from seedling and field plants were subjected to three decontamination protocols (as described in section 3.2.1) (Table 8) and thereafter placed on shoot multiplication medium containing 3 mg l^{-1} BAP (as presented in section 3.2.2). The explants were sub-cultured onto fresh shoot multiplication medium after 1 week and the incidence of contamination was monitored over 2 weeks. New shoots (Figure 4) began to develop on the explants of both seedling and field plants within 5 days of being placed onto shoot multiplication medium.

Table 8: The effect of applying decontamination protocols A, B and C on % fungal and bacterial contamination, % necrosis, % explants with shoots and number of shoots/explant of *Solanum nigrum* seed- and field-derived nodal explants after 2 weeks of culture on shoot multiplication medium containing 3 mg l^{-1} BAP. Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=16). Different lowercase letters denote a statistical significance amongst protocols A, B and C for each explant source. Different uppercase letters denote a statistical significance for each parameter (i.e. within a column). A = control; B = as for protocol A, followed by the addition of 1 ml l^{-1} Methylene blue® and 1 ml l^{-1} of the fungicide Previcur® in the shoot multiplication medium and a 30 min antibiotic treatment in 100 µg ml^{-1} Streptomycin and Penicillin; C = as for protocol B but for 60 min in the antibiotic treatment.

Explant source	Protocol	% Contamination		% Necrosis	% Explants with shoots	# Shoots/ explant
		Fungal	Bacterial			
Seed-derived	A	31 ± 6.5^{Aa}	94 ± 6.2^{Aa}	0 ± 0.0^{Bb}	100 ± 0.0^{Aa}	1 ± 0.0^{Aa}
	B	0 ± 0.0^{Bb}	69 ± 6.2^{ABb}	13 ± 7.2^{ABb}	56 ± 6.2^{BCb}	1 ± 0.0^{Aa}
	C	0 ± 0.0^{Bb}	44 ± 6.2^{Bc}	56 ± 6.2^{Aa}	69 ± 6.2^{BCb}	1 ± 0.0^{Aa}
Field-derived	A	56 ± 18.7^{Aa}	63 ± 7.2^{ABa}	0 ± 0.0^{Bb}	44 ± 11.9^{Cb}	2 ± 0.0^{Aa}
	B	38 ± 12.5^{Aa}	50 ± 10.2^{ABa}	25 ± 10.2^{Aa}	88 ± 7.2^{ABa}	1 ± 0.5^{Aa}
	C	0 ± 0.0^{Bb}	50 ± 20.4^{ABa}	50 ± 20.4^{Aa}	44 ± 6.2^{Cb}	1 ± 0.2^{Aa}

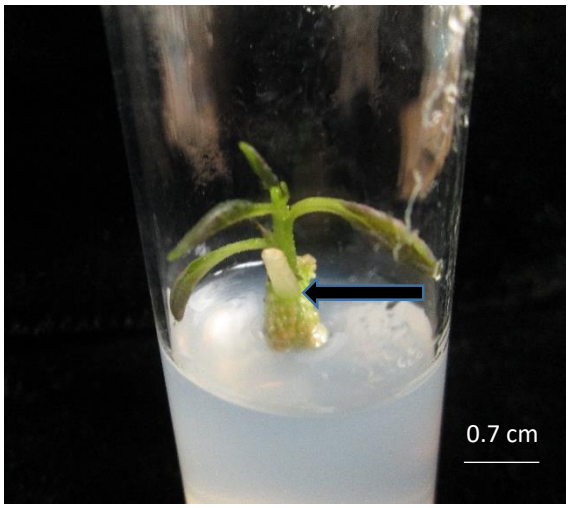


Figure 4: New shoot forming (as indicated by the arrow) from a seed-derived nodal explant, on shoot multiplication medium containing 3 mg l^{-1} BAP, prior to the onset of contamination or necrosis.

A preliminary study (protocol A) was done using the initial decontamination protocol for nodal explants. The percentages of fungal and bacterial contamination of the two tested explant sources, which resulted after this protocol, were high but not significantly different to each other (Table 8). No necrosis was observed for either explant source. There was, however, a significant difference in the percentage explants forming shoots, with the explants from seedling plants producing a significantly higher percentage of new shoots (100 ± 0.0 %) than those from the field plants (44 ± 6.2 %).

Since no aseptic material was produced using protocol A (Table 8), two more rigorous ones were tested on the nodal explants. Explants were immersed in a filtered antibiotic solution containing 100 µg ml^{-1} Streptomycin and Penicillin for 30 (protocol B) or 60 (protocol C) min subsequent to decontamination of explants (as described in section 3.2.1). For both protocols B and C, 1 ml l^{-1} Previcur® and 1 ml l^{-1} Methylene blue® were incorporated into the shoot multiplication medium. The inclusion of Previcur® in protocols B and C successfully eliminated fungal contamination in seed-derived explants. However, fungal contamination persisted in the field-derived explants exposed to protocol B (38 ± 12.5 %) but was overcome with protocol C (Table 8).

Bacterial contamination still occurred in the explants from the seedling and field plants (Figures 5 A and B) (Table 8), despite the addition of 1 ml l^{-1} Methylene blue® to the shoot multiplication

medium and their treatment with 100 µg ml^{-1} Streptomycin and Penicillin for 30 (protocol B) or 60 (protocol C) min. However, a significant decrease in bacterial contamination was recorded for seed-derived explants with increased immersion time in the antibiotic treatment, with that of explants treated with decontamination protocol C being significantly the lowest (44 ± 6.2 %).

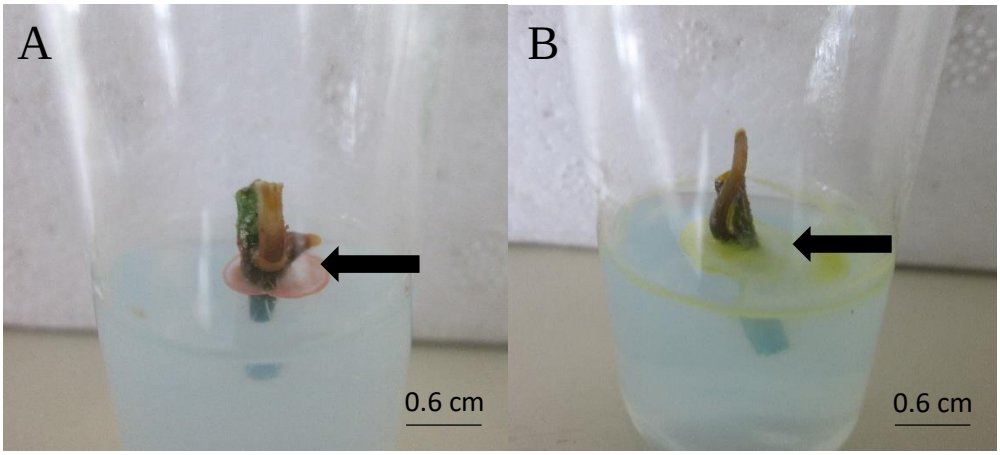


Figure 5: Bacterial contamination exhibited by nodal explants of *Solanum nigrum* (as indicated by the arrows) from seedling (A) and field (B) plants.

On the other hand, similar results were obtained, in terms of bacterial contamination, amongst protocols A (63 ± 7.2 %), B (50 ± 10.2 %) and C (50 ± 20.4 %) (Table 8) for field-derived nodal explants. For both explant sources, the bacterial contamination observed on them after treatment with each protocol, was considered too high for the purposes of this study. Additionally, increasing explant immersion time in the antibiotic solution (30 min for protocol B and 60 min for protocol C) increased necrosis from 13 ± 7.2 to 56 ± 6.2 % in seedling-derived explants and from 25 ± 10.2 to 50 ± 20.4 % in field-derived explants.

The percentage of seed-derived explants which produced shoots using protocols B (56 ± 6.2 %) and C (69 ± 6.2 %) was significantly lower than that of protocol A (100 ± 0.0 %) and the percentage of field-derived explants which formed shoots using protocol B (88 ± 7.2 %) was significantly higher than those from protocols A (44 ± 11.9 %) and C (44 ± 6.2 %). The newly formed shoots elongated on the shoot multiplication medium. However, they became

contaminated or necrotic prior to transfer onto rooting medium. All explants produced only one shoot regardless of the decontamination protocol or whether they were sourced from seedling or field plants. The exception was that field-derived explants subjected to protocol A produced two shoots/explant (Table 8).

The results of this study showed that some of the decontamination efforts described above, were not successful in producing aseptic nodal explant material because of the high recorded percentages of contamination and necrosis and the low numbers of shoots/explant. Consequently, leaf segments were investigated as an alternate source of explants.

Leaf explants

Leaf explants, as used by Sridhar and Naidu (2011b), were decontaminated with 1 % (v/v) NaOCl and Tween 20® for 10 min (as described in section 3.2.1). The percentage contamination, percentage necrosis and number of shoots/explant were monitored over 4 weeks for leaf explants from seedling plants and over 7 weeks for those from field plants. No fungal contamination was observed in either of these cultures. Due to the difference in the culture periods of the two explant sources, no statistical comparisons between seed- and field-derived leaf explants were done. The percentage bacterial contamination obtained using leaf segments (10 ± 4.4 % for seed- and 13 ± 9.9 % for field-derived explants) (Table 8) was deemed acceptable for the purpose of this study.

4.3 Establishing a direct organogenesis protocol using leaf explants

4.3.1 Shoot multiplication using leaf explants

Solanum nigrum leaf explants, excised from a variety of seedling and field plants, were decontaminated and placed onto shoot multiplication medium containing 3 mg l^{-1} BAP (as discussed in sections 3.2.2 and 3.2.3). They began curling after 3 days of culture (Figures 6 A and 7 A), and visible shoots were observed developing directly on them after 2 weeks (seed-derived) and 4 weeks (field-derived) on shoot multiplication medium, respectively (Figures 6 B and 7 B). After shoot initiation, growth of the leaf explants was monitored over 4 and 7 weeks for seed- and field-derived leaf explants, respectively (Table 9). The number of shoots/explant obtained from field-derived explants (20 ± 1.6 shoots/explant) were much lower than those obtained from seed-derived explants (50 ± 1.1 shoots/explant) (Table 9).

Table 9: A summary of the effect of micropropagation on the shoot multiplication, rooting and acclimatisation responses and the post-acclimatisation yield of *Solanum nigrum* leaf explants from seedling and field plants. Results for % bacterial contamination, % necrosis, and the number of shoots/explant were recorded after 4 weeks for seed-derived and 7 weeks for field-derived explants, % rooting after 3 weeks and % survival after 2 weeks of acclimatisation were recorded for both explant sources. The yield of plants/explant was calculated after 2 weeks acclimatisation. Data for % rooted shoots, % survival and post-acclimatisation yield of plants/explant were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$). Different lowercase letters denote a statistical significance for each explant source. n =20-30 (seed-derived); n =14-30 (field-derived). PGR = Plant growth regulator, IAA = indole-3-acetic acid, IBA = indole-3-butyric acid

Source	Shoot multiplication			Rooting		Acclimatisation	
	% Bacterial contamination	% Necrosis	# Shoots explant	PGR* (mg l^{-1})	% Rooted shoots	% Survival	Yield of plants/explant
Seed-derived	10±4.4	10±4.4	50±1.1	0	80±0.0[a]	100±0.0[a]	25±0.5[b]
				1 (IAA)	95±5.0[a]	100±0.0[a]	30±0.6[a]
				0.5 (IBA)	95±5.0[a]	93±6.6[a]	28±0.6[a]
Field-derived	13±9.9	40±5.1	20±1.6	0	75±10.0[a]	19±8.1[b]	1±0.3[a]
				1 (IAA)	80±0.0[a]	46±8.1[ab]	2±0.3[a]
				0.5 (IBA)	75±19.1[a]	59±12.4[a]	2±0.3[a]

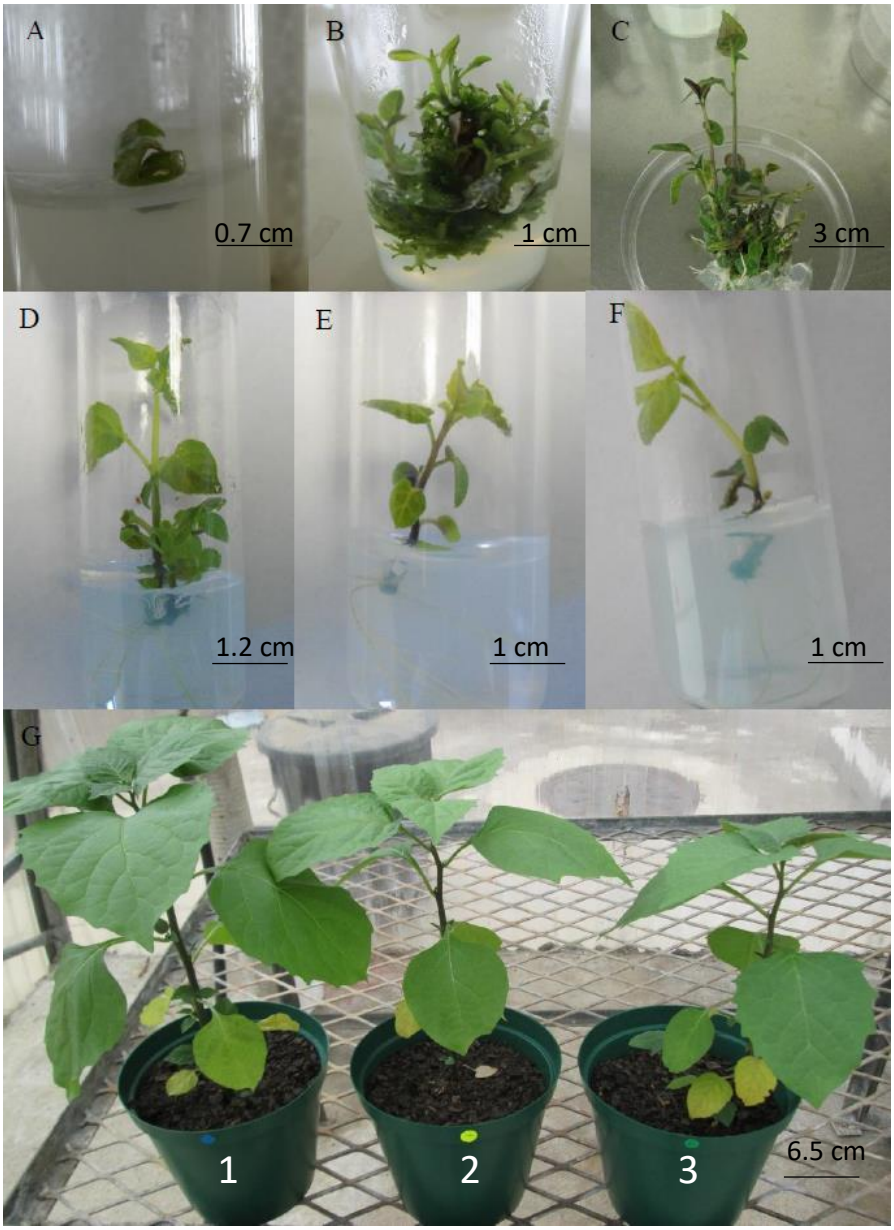


Figure 6: Illustration of *in vitro* culture and stages of plantlet regeneration via direct organogenesis using leaf explants of *Solanum nigrum* from seedling plants. (A) Decontaminated explant exhibiting curling on shoot multiplication medium containing 3 mg l^{-1} BAP after 3 days. (B) Shoots produced on shoot multiplication medium after 4 weeks. (C) Clumps of shoots on elongation medium containing 0.1 mg l^{-1} BAP. Rooting on (D) hormone-free MS medium, (E) 1 mg l^{-1} IAA and (F) 0.5 mg l^{-1} IBA after 3 weeks. (G) Acclimatised plants from the different rooting media (1. hormone-free MS medium, 2. 1 mg l^{-1} IAA and 3. 0.5 mg l^{-1} IBA).

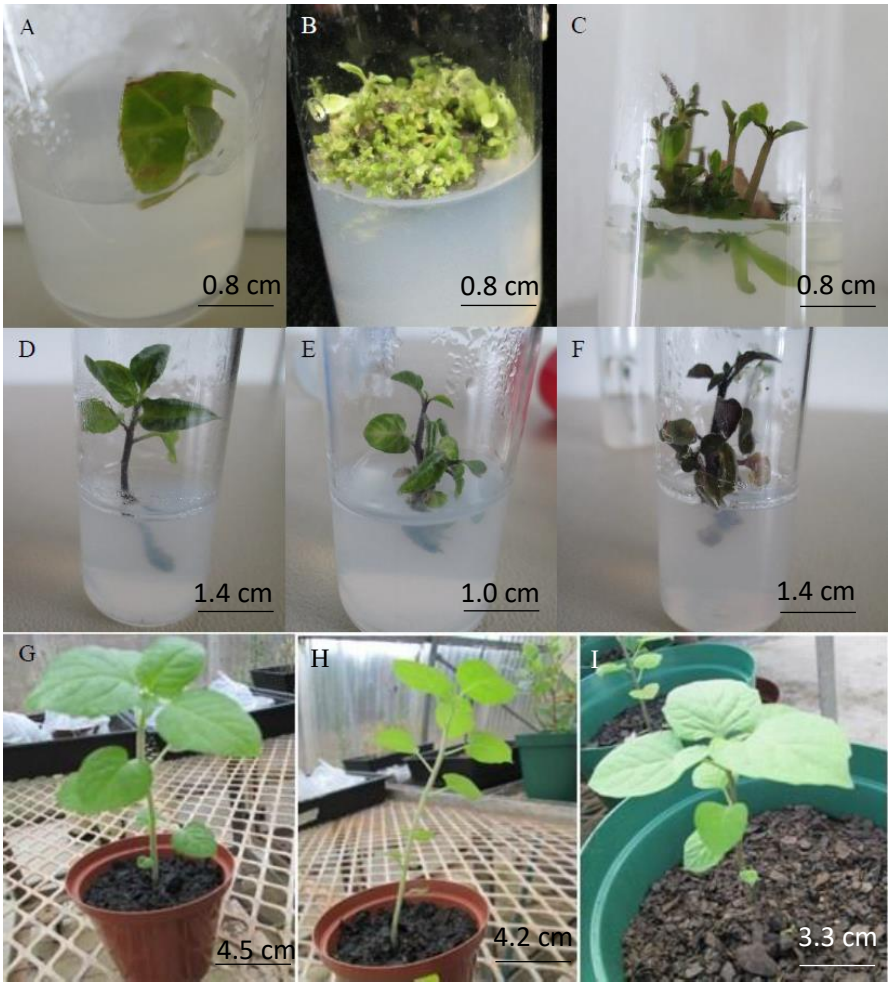


Figure 7: Illustration of *in vitro* culture and stages of plantlet regeneration via direct organogenesis using leaf explants of *Solanum nigrum* from field plants. (A) Decontaminated explant exhibiting curling on shoot multiplication medium containing 3 mg l^{-1} BAP after 3 days. (B) Shoots produced on multiplication medium after 7 weeks. (C) Clumps of shoots on elongation medium containing 0.1 mg l^{-1} BAP. Rooting on (D) hormone-free MS medium, (E) 1 mg l^{-1} IAA and (F) 0.5 mg l^{-1} IBA after 3 weeks. Acclimatised plants from (G) hormone-free MS medium, (H) 1 mg l^{-1} IAA and (I) 0.5 mg l^{-1} IBA.

4.3.2 Elongation and rooting of *in vitro* shoots

The shoot clumps formed during shoot multiplication were transferred to elongation medium containing 0.1 mg l^{-1} BAP (as described in sections 3.2.2 and 3.2.4). All the shoots of the seed- and field-derived explants elongated to 10-20 mm tall (Figures 6 C and 7 C). Those from seed-derived explants were the quickest to respond (1 week) than those from field-derived explants (2 weeks). As spontaneous rooting was exhibited by some shoots during elongation, it was postulated that they would root efficiently on media containing no or low concentrations of plant growth regulators (PGRs). Rooting was, therefore, investigated on hormone-free MS medium and on media containing low concentrations of PGRs (1 mg l^{-1} indole-3-acetic acid (IAA) or 0.5 mg l^{-1} indole-3-butyric acid (IBA)) to determine the best media composition for promoting root morphogenesis of *in vitro* shoots.

Visible roots were observed on most shoots of the seed- and field-derived explants (Figures 6 D-F and 7 D-F) during the second week of rooting. *In vitro* shoots from seed-derived explants were green during rooting on all media (Figure 6 D-F). However, *in vitro* shoots from field-derived explants were yellow on 1 mg l^{-1} IAA (Figure 7 E) and purple on 0.5 mg l^{-1} IBA (Figure 7 F), but they became green during acclimatisation (Figure 7 G). The *in vitro* field-derived shoots rooted on hormone-free MS medium were green (Figure 7 D). Regardless, after 3 weeks on the three tested rooting media, no significant differences were observed in the percentage of rooted shoots (Table 9).

4.3.3 Acclimatisation and post-acclimatisation yield of the cloned plants

The rooted plantlets were individually acclimatised (as described in section 3.2.5) for 2 weeks in pots (100 mm diameter, 80 mm depth) containing soil. The rooting media history had no significant effect on percentage survival of the clones produced from seed-derived explants (Table 9) (Figure 6 G). However, it had a significant effect on the percentage survival of the clones produced from field-derived explants, as plants from hormone-free MS medium resulted in a significantly lower percentage survival (19 ± 8.1 %) than those rooted on 0.5 mg l^{-1} IBA (59 ± 12.4 %) (Table 9).

Significantly higher post-acclimatisation yields, in terms of plants/explant, were recorded for the seed-derived clones previously rooted on 1 mg l^{-1} IAA (30 ± 0.6) and 0.5 mg l^{-1} IBA (28 ± 0.6) than those previously rooted on hormone-free MS medium (25 ± 0.5) (Table 9). There

were no significant differences in the post-acclimatisation yield of the field-derived clones previously rooted on hormone-free MS medium, 1 mg l^{-1} IAA and 0.5 mg l^{-1} IBA (Table 9). While no statistical comparisons could be done due to the differences in the culture period used for the two explant sources, it was apparent that the leaf explants from seedling plants produced a higher post-acclimatisation yield (25-30 plants/explant) over a shorter period of time than those from field plants (1-2 plants/explant) (Table 9).

It is clear, therefore, that seed-derived leaf explants responded better to micropropagation and acclimatisation than the field-derived leaf explants. Hence, due to their high post-acclimatisation yields, leaf explants from seedling plants were used in subsequent studies.

4.3.4 Phenotypic assessment of the *ex vitro* growth of the cloned plants

Some *ex vitro* growth parameters (shoot length and shoot and root fresh and dry masses) were recorded for the cloned plants of the seed- and field-derived explants. The shoot lengths of the cloned plants were measured at the start (w_0) and after 2 weeks (w_2) of acclimatisation for seed- and field-derived cloned plants (Table 10). Shoot and root fresh and dry masses of the cloned plants were recorded at 4 weeks *ex vitro* when they began to produce flowers (Table 10), but the flowers were removed before masses were determined.

There were no significant differences in the shoot lengths of the seed-derived cloned plants that were rooted on the different media types at w_0. However, significant differences were observed in the shoot lengths of the field-derived cloned plants- the shoots of plants rooted on 0.5 mg l^{-1} IBA (73 ± 7.2 mm) were significantly shorter than those of plants rooted on hormone-free MS medium (105 ± 5.3 mm) and 1 mg l^{-1} IAA (96 ± 6.2 mm) (Table 10). After 2 weeks of acclimatisation, the shoots of the seed-derived cloned plants previously rooted on hormone-free MS medium (117 ± 11.4 mm) were significantly longer than those of plants rooted on 0.5 mg l^{-1} IBA (74 ± 8.7 mm), while the shoot lengths of the field-derived cloned plants were not significantly different to each other (Table 10).

The shoot and root fresh and dry masses of the seed-derived clones, previously rooted on hormone-free MS medium, were significantly greater (9.7 ± 1.5 and 10.4 ±1.5 g for shoot and root fresh masses, respectively, and 1.4 ± 0.2 and 0.8 ± 0.1 g for shoot and root dry mass, respectively) than those of the clones previously rooted on 0.5 mg l^{-1} IBA (4.5 ± 1.1 and 3.9 ±

Table 10: The effect of rooting media history on the shoot lengths and, shoot and root fresh and dry masses of clones produced from seed- and field-derived leaf explants of *Solanum nigrum.* The shoot lengths were recorded at the start (w_0) and after 2 weeks (w_2) of acclimatisation and the fresh and dry masses of shoots and roots of clones of the *Solanum nigrum* plants recorded after 4 weeks *ex vitro* (including 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=15). Different lowercase letters denote a statistical significance for each parameter for each explant source. PGR* = Plant growth regulator, w_0** = End of rooting and the start of acclimatisation, w_2*** = After 2 weeks acclimatisation

Source	Rooting	Shoot length (mm)		Shoot (g)		Root (g)		Root: Shoot
	PGR* (mg l^{-1})	w_0**	w_2***	Fresh Mass	Dry Mass	Fresh Mass	Dry Mass	
Seed- derived	0	56±5.4^{a}	117±11.4^{a}	9.7±1.5^{a}	1.4±0.2^{a}	10.4±1.5^{a}	0.8±0.1^{a}	1 ±0.1^{a}
	1 (IAA)	57±3.9^{a}	93±9.7^{ab}	8.7±1.4^{ab}	1.3±0.3^{ab}	8.1±0.9^{ab}	0.7±0.1^{ab}	1±0.1^{a}
	0.5 (IBA)	60±5.3^{a}	74±8.7^{b}	4.5±1.1^{b}	0.5±0.2^{b}	3.9±1.0^{b}	0.3±0.1^{b}	1±0.2^{a}
Field- derived	0	105±5.3^{a}	112±11.0^{a}	0.8±0.4^{a}	0.1±0.0^{a}	1.1±0.4^{a}	0.1±0.0^{a}	1±0.0^{a}
	1 (IAA)	96±6.2^{a}	98±15.7^{a}	1.0±0.3^{a}	0.1±0.0^{a}	1.8±0.4^{a}	0.5±0.1^{a}	3±0.9^{a}
	0.5 (IBA)	73±7.2^{b}	105±11.4^{a}	1.8±0.4^{a}	0.2±0.0^{a}	3.0±0.7^{a}	0.6±0.1^{a}	2±0.7^{a}

1.0 g for shoot and root fresh masses, respectively, and 0.5 ± 0.2 and 0.3 ± 0.1 g shoot and root dry masses, respectively) (Table 10). Rooting media history had no significant effects on the shoot and root fresh and dry masses of the field-derived cloned plants (Table 10). This was also true for the root:shoot dry masses of both the seed- and field-derived clones (Table 10).

In conclusion, the clones of seed-derived leaf explants, previously rooted on hormone free MS medium and 1 mg l^{-1} IAA, had the greatest shoot and root fresh and dry masses after 4 weeks of *ex vitro* growth (which included 2 weeks acclimatisation). In subsequent studies, seed-derived leaf explants were used for shoot induction and multiplication, and hormone-free MS medium was used for rooting the *in vitro* shoots.

4.4 Screening, selection and micropropagation of the calcium and iron genotypes

4.4.1 Screening and selection of the genotypes

A population of fifty 6-week old seedlings was chosen to screen for leaf calcium (Ca) and iron (Fe). The Ca content ranged from 331.05 to 916.30 mg 100 g^{-1} dry mass (DM) (Figure 8) and the levels of Fe ranged from 0.64 to 14.95 mg 100 g^{-1} DM (Figure 9), with significant differences detected amongst many different genotypes. Based on these results, eight genotypes were selected for micropropagation: a) high Ca (G5 and G20); b) low Ca (G43 and G45); c) high Fe (G6 and G15); and d) low Fe (G35 and G50) hereafter refered to as selected genotypes.

4.4.2 Direct organogenesis of the selected genotypes

In vitro propagation of the selected genotypes

Leaf segments of the selected genotypes were used as a source of explants (section 3.4.2) for their micropropagation. As previously observed, the explants began to produce visible shoots after 2 weeks on the shoot multiplication medium. After a further 2 weeks, significantly high percentages of explants (75-92 %) from most of the genotypes produced shoots (Table 11). The exception was the low percentage of shoots from the G50 explants (25 ± 8.2 %). Significant differences were observed in the number of shoots/explant amongst the selected genotypes (Table 11), with G5 (25 ± 4.1), G6 (26 ± 3.0), G20 (24 ± 3.1) and G35 (22 ± 3.3) producing the least number of shoots/explant and G43 (51 ± 5.8) the highest. All the shoots elongated to 10-20 mm tall, regardless of genotype. They were then transferred onto hormone-free MS medium for rooting, as the plants that were previously rooted on this medium survived acclimatisation (Table 9). There were no significant differences in the percentages of rooted

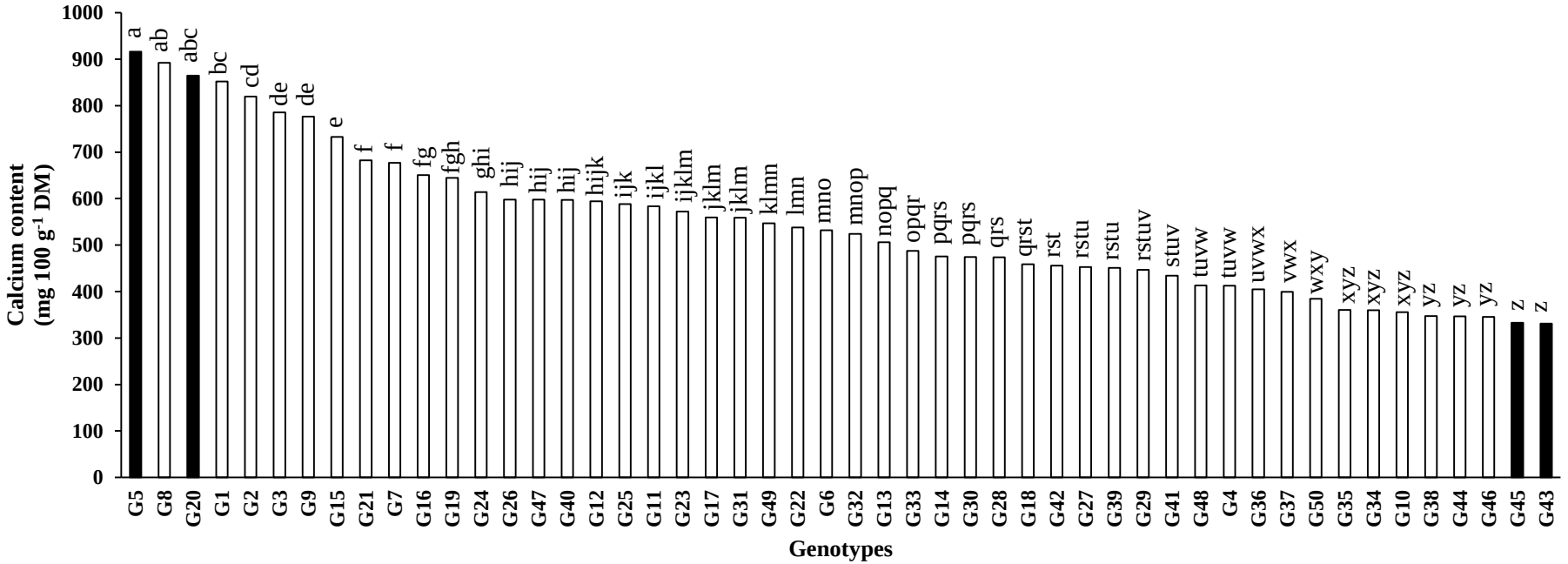


Figure 8: Leaf calcium content (mg 100 g^{-1} DM) of different 6-week old *Solanum nigrum* genotypes (G). Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=50). Mean values with different letters denote a statistical significance amongst the genotypes. Shaded bars represent the calcium content of the genotypes selected for further study.

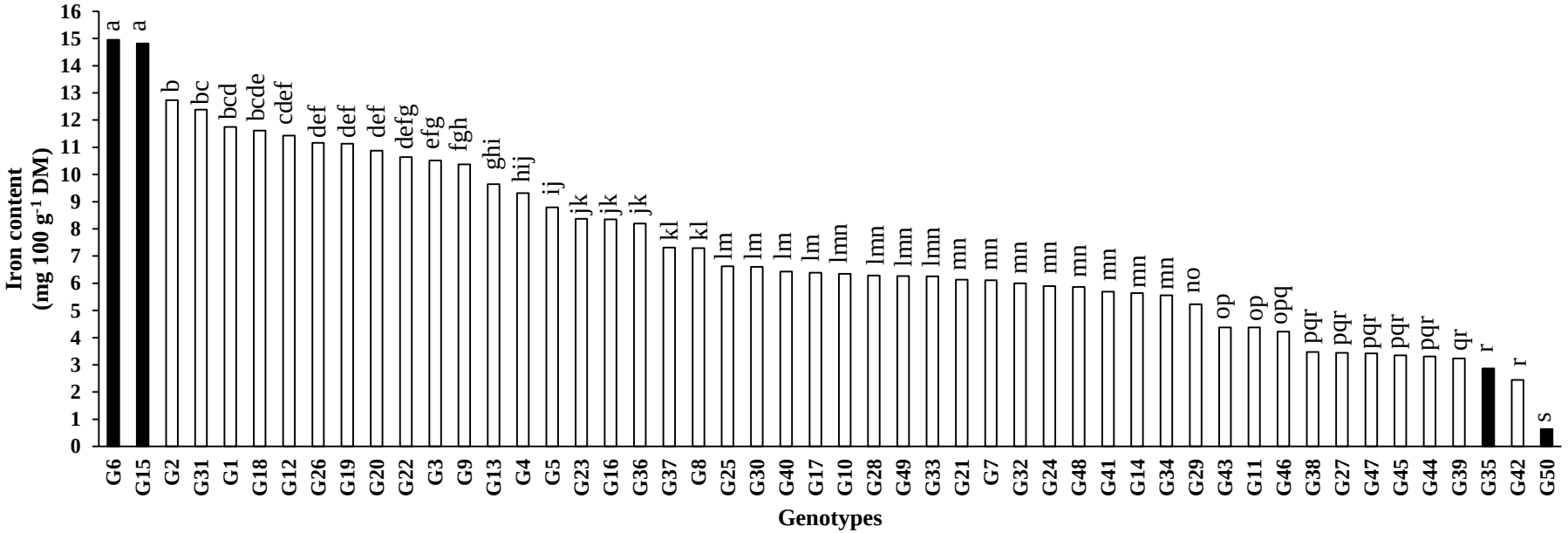


Figure 9: Leaf iron content (mg 100 g^{-1} DM of different 6-week old *Solanum nigrum* genotypes (G). Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=50). Mean values with different letters denote a statistical significance amongst the genotypes. Shaded bars represent the iron content of the genotypes selected for further study.

shoots amongst the tested genotypes (Table 11).

Acclimatisation and post-acclimatisation yield of the clones of the selected genotypes

The major goal of the research programme, of which the present study was only the initial component, is to provide community gardens (which usually have low fertile soil) with a natural breeding population of *S. nigrum* plants with high Ca and Fe contents. Therefore, a mix of 1 soil : 2 vermiculite (w/w) (1S : 2V) was used to represent a substrate with medium to low levels of nutrients. All plants were individually acclimatised in flower pots (100 mm diameter, 80 mm depth) containing 1S : 2V for 2 weeks. After this period, 92-100 % of the clones of all the tested genotypes survived acclimatisation (Table 11). After acclimatisation, the post-acclimatisation yield of G43 (32 ± 3.6 plants/explant) was significantly higher than those of G5 (13 ± 2.1 plants/explant), G6 (16 ± 1.8 plants/explant), G20 (16 ± 2.0 plants/explant), G35 (11 ± 1.6 plants/explant) and G50 (14 ± 1.6 plants/explant).

In conclusion, there were no differences amongst the genotypes with regards to the ability of the shoots to root and in the plantlet survival after acclimatisation but there were some phenotypic differences amongst the tested genotypes with regards to the percentage of explants with shoots, number of shoots/explant and subsequent post-acclimatisation yield of plants/explant.

4.4.3 Phenotypic assessment of the *ex vitro* growth of the clones of the selected genotypes

Some *ex vitro* growth parameters, i.e. the number of branches, number of leaves, shoot length, root length and the fresh and dry masses of the shoots and roots were recorded for the clones of the tested genotypes (Tables 12-15). This was done at the start of acclimatisation (w_0) and after 6 weeks *ex vitro* (w_6) growth (which included 2 weeks acclimatisation) in soil or in 1S : 2V. These parameters were not recorded for the parent plants as it required harvesting and destroying them. Therefore, comparisons of these tested parameters between each parent plant and its clones were not performed.

Number of branches and leaves

The number of branches (1-3) was not significantly different amongst the cloned genotypes both at the start of acclimatisation (w_0) and after 6 weeks of *ex vitro* growth (w_6) in soil (Table 12). However, there were significant differences amongst them when grown in 1S : 2V (Table

Table 11: A summary of the effect of the established micropropagation protocol on the shoot multiplication, rooting and acclimatisation responses and the subsequent post-acclimatisation yield of the selected *Solanum nigrum* genotypes. Results for % explants with shoots and number of shoots/explant were recorded after 4 weeks, % rooting was recorded after 3 weeks, % survival and yield of plants/explant were recorded and calculated after 2 weeks of acclimatisation. Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=8-30). Different lowercase letters denote a statistical significance amongst genotypes for each parameter (within a column).

Genotype	Shoot Multiplication		Rooting	Acclimatisation	Yield of plants/ explant
	% Explants with shoots	# Shoots/explant	% Rooted shoots	% Survival	
G5	75±16.2^{a}	25±4.1^{b}	100±0.0^{a}	92±4.8^{a}	13±2.1^{b}
G6	92±8.2^{a}	26±3.0^{b}	90±4.4^{a}	92±4.8^{a}	16±1.8^{b}
G15	83±16.7^{a}	28±3.8^{ab}	97±3.3^{a}	96±4.0^{a}	19±2.5^{ab}
G20	83±9.5^{a}	24±3.1^{b}	97±3.3^{a}	96±4.0^{a}	16±2.0^{b}
G35	75±16.0^{a}	22±3.3^{b}	90±6.8^{a}	100±0.0^{a}	11±1.6^{b}
G43	83±9.5^{a}	51±5.8^{a}	100±0.0^{a}	100±0.0^{a}	32±3.6^{a}
G45	75±16.0^{a}	43±9.5^{ab}	100±0.0^{a}	100±0.0^{a}	24±5.3^{ab}
G50	25±8.2^{b}	45±5.0^{ab}	93±6.6^{a}	100±0.0^{a}	14±1.6^{b}

12), with the G15 (4 ± 1.1) clones developing a significantly higher number of branches than the G35 (1 ± 0.0), G43 (1 ± 0.3), G45 (1 ± 0.1) and G50 (1 ± 0.0) clones. For each genotype, there was no significant difference in the number of branches between w_0 and w_6 in both the tested substrates. The exception was the G5 clones, which had significantly higher numbers of branches at w_6 in soil (3 ± 0.6) and in 1S : 2V (3 ± 0.8) than at w_0 (1 ± 0.2). For each tested genotype, there were similar numbers of branches between the clones kept in soil and 1S:2V.

At the start of acclimatisation (w_0), the clones of G15 (20 ± 1.9) had a significantly higher number of leaves than the clones of G5 (12 ± 1.5), G20 (12 ± 1.6), G35 (10 ± 1.4), G43 (13 ± 1.6), G45 (8 ± 0.6) and G50 (8 ± 1.7). After growing for 6 weeks (w_6) in soil, the number of leaves of the G15 (25 ± 4.1) clones was significantly higher than those of the G20 (12 ± 1.0), G35 (11 ± 2.1), G45 (9 ± 1.3) and G50 (10 ± 2.4) clones. In 1S : 2V, the G5 (21 ± 3.7), G15 (22 ± 5.6) and G20 (22 ± 2.1) clone's leaf numbers were significantly higher than those of the G35 (10 ± 0.4), G45 (11 ± 1.3) and G50 (9 ± 0.5) clones. In soil, the number of leaves of each genotype did not change significantly between w_0 and w_6. In 1S : 2V, the G5 and G20 clones (21 ± 3.7 and 22 ± 2.1, respectively) had significantly higher numbers of leaves at w_6 than at w_0 (12 ± 1.5 and 12 ± 1.6, respectively). At w_6, the number of leaves of the G20 clones was significantly higher in 1S : 2V (22 ± 2.1) than in soil (12 ± 1.0).

Shoot and root lengths

Prior to acclimatisation (w_0), no significant differences were found amongst the clones of the tested genotypes regarding shoot length (Table 13). At w_6 in soil, the shoots of the G5 (90 ± 10.4 mm) clones were significantly the shortest. In 1S : 2V, the G5 (143 ± 8.5 mm), G15 (147 ± 11.6 mm), G20 (175± 15.0 mm), G35 (152 ± 9.2 mm), G43 (145 ± 9.4 mm) and G50 (144± 9.5 mm) clones' shoots were significantly longer than those of the G6 (103 ± 9.1 mm) and G45 (116± 8.9 mm) clones. When grown in soil, the G15, G20, G35, G43, G45 and G50 clones had significantly longer shoots at w_6 than at w_0 (Table 13). In 1S : 2V, except for the G6 clones, the shoots of all the tested genotypes were significantly longer at w_6 than at w_0. After 6 weeks of growth, the G5 clones had significantly longer shoots in 1S : 2V (143 ± 8.5 mm) than in soil (90 ± 10.4 mm).

In both soil and 1S : 2V, the roots of all of the cloned genotypes were significantly longer w_6 than at w_0 (Table 13). At w_0, the G6 (53 ± 3.8 mm) and G20 (51 ± 6.7 mm) clones had significantly longer roots than the G43 (30 ± 1.2 mm) clones. After 6 weeks of *ex vitro* growth

Table 12: The number of branches and leaves of clones of the selected *Solanum nigrum* genotypes at the start of acclimatisation (w_0) and after 6 weeks *ex vitro* growth (which included 2 weeks acclimatisation) (w_6) in either soil or 1 soil : 2 vermiculite (1S : 2V). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=8). Different uppercase letters denote a statistical significance for acclimatisation time and substrate type at w_0, w_6 (soil) and w_6 (1S : 2V) for each genotype for each parameter and different lowercase letters denote a statistical significance amongst genotypes for each parameter at each acclimatisation time (i.e. within columns). w_0 = end of rooting and start of acclimatisation, $w_{6\ soil}$ = maintained *ex vitro* for 6 weeks in soil (which included 2 weeks acclimatisation), $w_{6\ 1S:2V}$ = maintained *ex vitro* for 6 weeks in 1 soil : 2 vermiculite (which included 2 weeks acclimatisation)

Genotype	Number of branches			Number of leaves		
	W0	**W6 soil**	**W6 1S : 2V**	**W0**	**W6 soil**	**W6 1S : 2V**
G5	1 ± 0.2^{Ba}	3 ± 0.6^{Aa}	3 ± 0.8^{Aab}	12 ± 1.5^{Bb}	15 ± 2.9^{ABab}	21 ± 3.7^{Aa}
G6	2 ± 0.4^{Aa}	3 ± 1.0^{Aa}	3 ± 0.5^{Aab}	15 ± 2.5^{Aab}	17 ± 2.6^{Aab}	13 ± 1.9^{Aab}
G15	3 ± 0.3^{Aa}	3 ± 0.7^{Aa}	4 ± 1.1^{Aa}	20 ± 1.9^{Aa}	25 ± 4.1^{Aa}	22 ± 5.6^{Aa}
G20	2 ± 0.3^{Aa}	2 ± 0.3^{Aa}	3 ± 0.4^{Aab}	12 ± 1.6^{Bb}	12 ± 1.0^{Bb}	22 ± 2.1^{Aa}
G35	2 ± 0.3^{Aa}	2 ± 0.4^{Aa}	1 ± 0.0^{Ab}	10 ± 1.4^{Ab}	11 ± 2.1^{Ab}	10 ± 0.4^{Ab}
G43	2 ± 0.3^{Aa}	2 ± 0.5^{Aa}	1 ± 0.3^{Ab}	13 ± 1.6^{Ab}	16 ± 3.1^{Aab}	13 ± 1.6^{Aab}
G45	1 ± 0.2^{Aa}	1 ± 0.1^{Aa}	1 ± 0.1^{Ab}	8 ± 0.6^{Ab}	9 ± 1.3^{Ab}	11 ± 1.3^{Ab}
G50	1 ± 0.3^{Aa}	2 ± 0.3^{Aa}	1 ± 0.0^{Ab}	8 ± 1.7^{Ab}	10 ± 2.4^{Ab}	9 ± 0.5^{Ab}

Table 13: The shoot and root lengths of clones of the selected *Solanum nigrum* genotypes at the start of acclimatisation (w_0) and after 6 weeks *ex vitro* growth (which included 2 weeks acclimatisation) (w_6) in either soil or 1 soil : 2 vermiculite (1S : 2V). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=8). Different uppercase letters denote a statistical significance for acclimatisation time and substrate type at w_0, w_6 (soil) and w_6 (1S : 2V) for each genotype for each parameter and different lowercase letters denote a statistical significance amongst genotypes for each parameter at each acclimatisation time (i.e. within columns). w_0 = end of rooting and start of acclimatisation, $w_{6\ soil}$ = maintained *ex vitro* for 6 weeks in soil (which included 2 weeks acclimatisation), $w_{6\ 1S:2V}$ = maintained *ex vitro* for 6 weeks in 1 soil : 2 vermiculite (which included 2 weeks acclimatisation)

Genotype	Shoot length (mm)			Root length (mm)		
	W0	W6 soil	W6 1S : 2V	W0	W6 soil	W6 1S : 2V
G5	79±7.8^{Ba}	90±10.4^{Bb}	143±8.5^{Aa}	43±5.2^{Cab}	204±30.7^{Ba}	391±46.1^{Aa}
G6	84±8.9^{Aa}	112±6.1^{Aab}	103±9.1^{Ab}	53±3.8^{Ba}	214±26.6^{Aa}	261±28.1^{Aab}
G15	88±7.9^{Ba}	143±10.1^{Aa}	147±11.6^{Aa}	41±3.6^{Bab}	195±34.7^{Aa}	240±19.8^{Ab}
G20	85±10.6^{Ba}	153±18.5^{Aa}	175±15.0^{Aa}	51±6.7^{Ca}	221±30.5^{Ba}	359±52.5^{Aab}
G35	66±10.6^{Ba}	142±13.4^{Aa}	152±9.2^{Aa}	36±4.2^{Cab}	192±21.8^{Ba}	322±45.3^{Aab}
G43	80±8.8^{Ba}	140±27.4^{Aa}	145±9.4^{Aa}	30±1.2^{Cb}	190±33.0^{Ba}	290±23.0^{Aab}
G45	69±11.9^{Ba}	140±13.1^{Aa}	116±8.9^{Ab}	34±5.2^{Cab}	208±13.6^{Ba}	273±18.7^{Aab}
G50	63±7.8^{Ba}	113±12.1^{Aab}	144±9.5^{Aa}	33±5.2^{Cab}	213±32.9^{Ba}	294±14.2^{Aab}

(w_6) in soil, there were no significant differences amongst the tested genotypes regarding root length (Table 13). However, in 1S : 2V, the clones of G5 (391 ± 46.1 mm) had significantly longer roots than the clones of G15 (240 ± 19.8 mm). When grown for 6 weeks *ex vitro* (w_6), the roots of the G5 (391 ± 46.1 mm), G20 (359 ± 52.5 mm), G35 (322 ± 45.3 mm), G43 (290 ± 23.0 mm), G45 (273 ± 18.7 mm) and G50 (294 ± 14.2 mm) clones were significantly longer in 1S : 2V than in soil (204 ± 30.7, 221 ± 30.5, 192 ± 21.8, 190 ± 33.0, 208 ± 13.6 and 213 ± 32.9 mm, respectively).

Shoot and root fresh masses

At w_0, the shoot fresh mass of the G15 (0.7 ± 0.0 g) clones was significantly greater than that of the G5 (0.4 ± 0.1 g), G20 (0.4 ± 0.1 g), G35 (0.2 ± 0.1 g), G43 (0.2 ± 0.1 g) and G45 (0.4 ± 0.1 g) clones (Table 14). After 6 weeks (w_6) growth in soil, there were no significant differences amongst the cloned genotypes in terms of their shoot fresh mass (Table 14). However, under 1S : 2V conditions, the shoot fresh mass of the G20 (2.7 ± 0.2 g) clones was significantly higher than those of the other tested genotypes (1.1-1.8 g). In soil, the shoot fresh masses of the G35, G43, G45 and G50 clones were significantly greater at w_6 (1.5 ± 0.4, 1.8 ± 0.6, 1.5 ± 0.3, 1.3 ± 0.3 g, respectively) than at w_0 (0.2 ± 0.1, 0.2 ± 0.1, 0.4 ± 0.1, 0.5 ± 0.1 g, respectively) (Table 14). In 1S : 2V, most of the tested genotypes had significantly greater shoot fresh masses at w_6 than at w_0. The exceptions were the shoot fresh masses of the G6 and G15 clones, which were the same between w_0 and w_6. At w_6, the clones of G5 and G20 had significantly greater shoot fresh masses in 1S : 2V (1.7 ± 0.1 and 2.7 ± 0.2 g, respectively) than in soil (0.8 ± 0.2 and 1.2 ± 0.3 g, respectively).

At w_0, the root fresh mass of the G15 (0.6 ± 0.2 g) clones was significantly greater than that of the G43 (0.1 ± 0.0 g) clones. When grown in soil for 6 weeks (w_6), the G6 (3.9 ± 1.3 g), G15 (2.3 ± 0.5 g), G20 (2.2 ± 0.6 g), G43 (2.7 ± 0.4 g) and G45 (2.7 ± 0.6 g) clones' root fresh masses were significantly greater than that of the G5 (0.6 ± 0.2 g) clones. Under 1S : 2V conditions, the clones of G20 (6.3 ± 0.8 g) had significantly the highest root fresh mass. In both substrates, the root fresh masses of each of the tested genotypes were significantly greater at w_6 than at w_0. The only exception was G5 clones in soil, which had the same root fresh mass between w_0 and w_6 (Table 14). At w_6, the G5 and G20 clones' root fresh masses were significantly greater in 1S : 2V (4.4 ± 0.8 and 6.3 ± 0.8 g, respectively) than in soil (0.6 ± 0.2 and 2.2 ± 0.6 g, respectively).

Table 14: The shoot and root fresh masses of clones of the selected *Solanum nigrum* genotypes at the start of acclimatisation (w_0) and after 6 weeks *ex vitro* growth (which included 2 weeks acclimatisation) (w_6) in either soil or 1 soil : 2 vermiculite (1S : 2V). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=8). Different uppercase letters denote a statistical significance for acclimatisation time and substrate type at w_0, w_6 (soil) and w_6 (1S : 2V) for each genotype for each parameter and different lowercase letters denote a statistical significance amongst genotypes for each parameter at each acclimatisation time (i.e. within columns). w_0 = end of rooting and start of acclimatisation, $w_{6\ soil}$ = maintained *ex vitro* for 6 weeks in soil (which included 2 weeks acclimatisation), $w_{6\ 1S:2V}$ = maintained *ex vitro* for 6 weeks in 1 soil : 2 vermiculite (which included 2 weeks acclimatisation)

Genotype	Fresh mass (g)					
	Shoots			Roots		
	W0	W6 soil	W6 1S : 2V	W0	W6 soil	W6 1S : 2V
G5	0.4 ± 0.1^{Bb}	0.8 ± 0.2^{Ba}	1.7 ± 0.1^{Ab}	0.5 ± 0.1^{Bab}	0.6 ± 0.2^{Bb}	4.4 ± 0.8^{Aab}
G6	0.6 ± 0.1^{Aab}	1.4 ± 0.5^{Aa}	1.1 ± 0.1^{Ab}	0.3 ± 0.1^{Bab}	3.9 ± 1.3^{Aa}	2.3 ± 0.3^{Abc}
G15	0.7 ± 0.0^{Aa}	1.1 ± 0.2^{Aa}	1.1 ± 0.1^{Ab}	0.6 ± 0.2^{Ba}	2.3 ± 0.5^{Aa}	3.8 ± 0.8^{Aac}
G20	0.4 ± 0.1^{Bb}	1.2 ± 0.3^{Ba}	2.7 ± 0.2^{Aa}	0.3 ± 0.1^{Cab}	2.2 ± 0.6^{Ba}	6.3 ± 0.8^{Aa}
G35	0.2 ± 0.1^{Bb}	1.5 ± 0.4^{Aa}	1.5 ± 0.1^{Ab}	0.2 ± 0.1^{Bab}	2.0 ± 0.7^{Aab}	3.0 ± 0.5^{Abc}
G43	0.2 ± 0.1^{Bb}	1.8 ± 0.6^{Aa}	1.6 ± 0.1^{Ab}	0.1 ± 0.0^{Bb}	2.7 ± 0.4^{Aa}	2.8 ± 0.5^{Abc}
G45	0.4 ± 0.1^{Bb}	1.5 ± 0.3^{Aa}	1.2 ± 0.1^{Ab}	0.3 ± 0.1^{Bab}	2.7 ± 0.6^{Aa}	1.6 ± 0.3^{Ac}
G50	0.5 ± 0.1^{Bab}	1.3 ± 0.3^{Aa}	1.8 ± 0.2^{Ab}	0.4 ± 0.1^{Bab}	1.8 ± 0.5^{Aab}	2.8 ± 0.5^{Abc}

Shoot and root dry masses

In both substrate types, the clones of each tested genotype grew well over the 6 week *ex vitro* (w_6) growth period, as the dry masses of their shoots were significantly higher at this point in time than at w_0 (Table 15). At w_0, the shoot dry masses of the cloned genotypes were not significantly different amongst each other (Table 15). However, at w_6 in soil, that of the G50 (0.4 ± 0.1 g) was significantly greater than that of the G5 (0.1 ± 0.0 g) clones. In 1S : 2V, the shoot dry mass of the G20 (0.5 ± 0.0 g) clones was significantly greater than those of the clones of the all other tested genotypes (0.2-0.3 g). At w_6, the G20 clones had a significantly greater shoot dry mass in 1S : 2V (0.5 ± 0.0 g) than in soil (2.0 ± 0.0 g).

As expected, for each tested genotype, their root dry masses were significantly greater at w_6 (irrespective of substrate type) than at w_0. At w_0 and at w_6 in soil, there were no significant differences in the root dry masses amongst the tested genotypes (Table 15). At w_6, under 1S : 2V conditions, the clones of G20 (0.7 ± 0.1 g) had a significantly greater root dry mass than the clones of G6 (0.1 ± 0.1 g), G43 (0.1 ± 0.1 g), G45 (0.1 ± 0.1 g) and G50 (0.2 ± 0.1 g). At w_6, the clones of some of the tested genotypes (i.e. G6, G43 and G45) had significantly greater root dry masses when kept in soil (0.8 ± 0.3, 0.6 ± 0.2 and 0.6 ± 0.2 g, respectively) than when grown in 1S : 2V (0.1 ± 0.1, 0.1 ± 0.1 and 0.1 ± 0.1 g, respectively). However, the G20 clone's root dry mass was significantly greater in 1S : 2V (0.7 ± 0.1 g) than in soil (0.4 ± 0.1 g).

Root:shoot dry masses

At w_0 and at w_6 in soil, the root:shoot dry masses of the tested genotypes were not significantly different amongst each other (Table 16). At w_6 in 1S : 2V, the clones of G15 (3.0 ± 0.5) had a significantly greater root:shoot dry mass than the clones of G6 (0.7 ± 0.2), G43 (0.9 ± 0.4), G45 (0.9 ±0.4) and G50 (0.9 ± 0.3). In soil, the G6 and G15 clones had significantly greater root:shoot dry masses at w_6 (3.2 ± 0.7 and 3.3 ± 1.0, respectively) than at w_0 (0.9 ± 0.2 and 0.8 ± 0.2, respectively). Under 1S : 2V conditions, except for the clones of G15, there were no significant differences in the root:shoot dry masses of each of the other genotypes between w_0 and w_6 (Table 16). At w_6, the root:shoot dry masses of the G6 clones was significantly greater in soil (3.2 ± 0.7) than in 1S : 2V (0.7 ± 0.2).

Table 15: The shoot and root dry masses of clones of the selected *Solanum nigrum* genotypes at the start of acclimatisation and after 6 weeks *ex vitro* growth (which included 2 weeks acclimatisation) (w_6) in either soil or 1 soil : 2 vermiculite (1S : 2V). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=8). Different uppercase letters denote a statistical significance for acclimatisation time and substrate type at w_0, w_6 (soil) and w_6 (1S : 2V) for each genotype for each parameter and different lowercase letters denote a statistical significance amongst genotypes for each parameter at each acclimatisation time (i.e. within columns). w_0 = end of rooting and start of acclimatisation, $w_{6\ soil}$ = maintained *ex vitro* for 6 weeks in soil (which included 2 weeks acclimatisation), $w_{6\ 1S:2V}$ = maintained *ex vitro* for 6 weeks in 1 soil : 2 vermiculite (which included 2 weeks acclimatisation)

Genotype	Dry mass (g)					
	Shoots			Roots		
	W0	W6 soil	W6 1S : 2V	W0	W6 soil	W6 1S : 2V
G5	0.04 ± 0.01^{Ba}	0.1 ± 0.0^{Ab}	0.3 ± 0.0^{Ab}	0.06 ± 0.01^{Ba}	0.4 ± 0.2^{Aa}	0.5 ± 0.1^{Aab}
G6	0.06 ± 0.01^{Ba}	0.2 ± 0.0^{Aab}	0.2 ± 0.0^{Ab}	0.04 ± 0.01^{Ca}	0.8 ± 0.3^{Aa}	0.1 ± 0.1^{Bc}
G15	0.07 ± 0.00^{Ba}	0.2 ± 0.0^{Aab}	0.2 ± 0.0^{Ab}	0.06 ± 0.01^{Ba}	0.7 ± 0.2^{Aa}	0.5 ± 0.2^{Aac}
G20	0.04 ± 0.01^{Ca}	0.2 ± 0.0^{Bab}	0.5 ± 0.0^{Aa}	0.05 ± 0.01^{Ca}	0.4 ± 0.1^{Ba}	0.7 ± 0.1^{Aa}
G35	0.07 ± 0.04^{Ba}	0.2 ± 0.1^{Aab}	0.3 ± 0.0^{Ab}	0.04 ± 0.01^{Ba}	0.4 ± 0.1^{Aa}	0.4 ± 0.1^{Aac}
G43	0.03 ± 0.00^{Ba}	0.3 ± 0.1^{Aab}	0.2 ± 0.0^{Ab}	0.03 ± 0.01^{Ca}	0.6 ± 0.2^{Aa}	0.1 ± 0.1^{Bc}
G45	0.03 ± 0.00^{Ba}	0.3 ± 0.1^{Aab}	0.2 ± 0.0^{Ab}	0.03 ± 0.01^{Ca}	0.6 ± 0.2^{Aa}	0.1 ± 0.1^{Bc}
G50	0.06 ± 0.01^{Ba}	0.4 ± 0.1^{Aa}	0.3 ± 0.0^{Ab}	0.06 ± 0.01^{Ba}	0.5 ± 0.2^{Aa}	0.2 ± 0.1^{Abc}

Table 16: The root:shoot dry masses of clones of the selected *Solanum nigrum* genotypes at the start of acclimatisation (w_0) and after 6 weeks *ex vitro* growth (which included 2 weeks acclimatisation) (w_6) in either soil or 1 soil : 2 vermiculite (1S : 2V). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=8). Different uppercase letters indicate a statistical significance for acclimatisation time and substrate type at w_0, w_6 (soil) and w_6 (1S : 2V) for each genotype and different lowercase letters denote a statistical significance amongst genotypes for each parameter at each acclimatisation time (i.e. within columns). w_0 = end of rooting and start of acclimatisation, $w_{6\ soil}$ = maintained *ex vitro* for 6 weeks in soil (which included 2 weeks acclimatisation), $w_{6\ 1S:2V}$ = maintained *ex vitro* for 6 weeks in 1 soil : 2 vermiculite (which included 2 weeks acclimatisation)

Genotype	Root:Shoot		
	W0	**W6 soil**	**W6 1S : 2V**
G5	1.8 ± 0.5^{Aa}	2.0 ± 0.6^{Aa}	1.7 ± 0.3^{Aab}
G6	0.9 ± 0.2^{Ba}	3.2 ± 0.7^{Aa}	0.7 ± 0.2^{Bb}
G15	0.8 ± 0.2^{Ba}	3.3 ± 1.0^{Aa}	3.0 ± 0.5^{Aa}
G20	1.3 ± 0.3^{Aa}	2.1 ± 0.3^{Aa}	1.6 ± 0.2^{Aab}
G35	1.4 ± 0.5^{Aa}	1.9 ± 0.3^{Aa}	1.6 ± 0.5^{Aab}
G43	1.5 ± 0.5^{Aa}	2.2 ± 0.6^{Aa}	0.9 ± 0.4^{Ab}
G45	1.5 ± 0.5^{Aa}	2.2 ± 0.6^{Aa}	0.9 ± 0.4^{Ab}
G50	1.4 ± 0.4^{Aa}	1.6 ± 0.6^{Aa}	0.9 ± 0.3^{Ab}

In conclusion, at the start of acclimatisation (w_0) and after 6 weeks of *ex vitro* growth (w_6) in soil and in 1S : 2V, there was some variation amongst the tested genotypes with regards to the tested growth parameters (*viz.* number of branches and leaves, shoot and root lengths and fresh and dry masses and root:shoot dry masses). More genotypes were significantly affected by substrate with regards to their root parameters than their shoot parameters. The genotypes that had the most plasticity in terms of their shoot and root growth in the two tested substrates were G5 and G20.

4.4.4 Leaf calcium and iron content of the clones of the selected genotypes and their parents

The main objective of the present study was to compare the leaf Ca and Fe levels of the cloned genotypes, in soil and in 1 soil : 2 vermiculite (1S : 2V), to each other and to that of the parent genotype. The genotypes used in this experiment contained either high Ca (G5 and G20), high Fe (G6 and G15), low Ca (G43 and G45) or low Fe (G35 and G50). The cloned genotypes were kept in soil or 1S : 2V for 6 weeks (which included 2 weeks acclimatisation) and the parent genotype was grown in soil for 6 weeks. The leaves of *S. nigrum* are usually harvested and eaten when the plants reach maturity, i.e. when the plants begin to flower (Jimoh *et al.*, 2010). Six weeks of growth were chosen in the present study because the parent genotypes and their clones began to flower (reached maturity) then.

When grown in soil, the levels of Ca were not significantly different amongst the clones of the tested genotypes (Figure 10 A). However, in 1S : 2V, the clones of G20 (875 ± 52 mg 100 g^{-1} dry mass [DM]) had a significantly higher level of Ca than the G45 (619 ± 67 mg 100 g^{-1} DM) clones. In terms of Fe content, when grown in soil, the G50 clones had significantly higher Fe (10 ± 1.5 mg 100 g^{-1} DM) than the G35 (5 ± 1.1 mg 100 g^{-1} DM) clones (Figure 10 B). Under 1S : 2V conditions, the levels of Fe were not significantly different amongst the tested genotypes (Figure 10 B).

The type of tested substrate did not affect the Ca levels of the G43 and G45 clones and the Fe contents of the G6, G15 and G35 clones. However, it did affect the Ca contents of the G5 and the G20 clones, which were significantly higher in 1S : 2V (811 ± 66 and 875 ± 52 mg 100 g^{-1} DM, respectively) than in soil (437 ± 113 and 332 ± 62 mg 100 g^{-1} DM, respectively). The clones of G50 were also affected by substrate and had a significantly higher concentration of Fe in soil (10 ± 1.5 mg 100 g^{-1} DM) than in 1S : 2V (5 ± 1.0 mg 100 g^{-1} DM).

When grown in soil, only the clones of G43 had the same level of Ca as its parent. The G5 (437 ± 113 mg 100 g^{-1} DM) and the G20 (332 ± 62 mg 100 g^{-1} DM) clones had significantly lower Ca contents and the G45 clones significantly higher levels (533 ± 36 mg 100 g^{-1} DM) than their parent genotypes (916 ± 6 mg 100 g^{-1} DM for G5, 865 ± 5 mg 100 g^{-1} DM for G20 and 333 ± 4 mg 100 g^{-1} DM for G43). In soil, the G35 parent and clones had the same amount of Fe. The

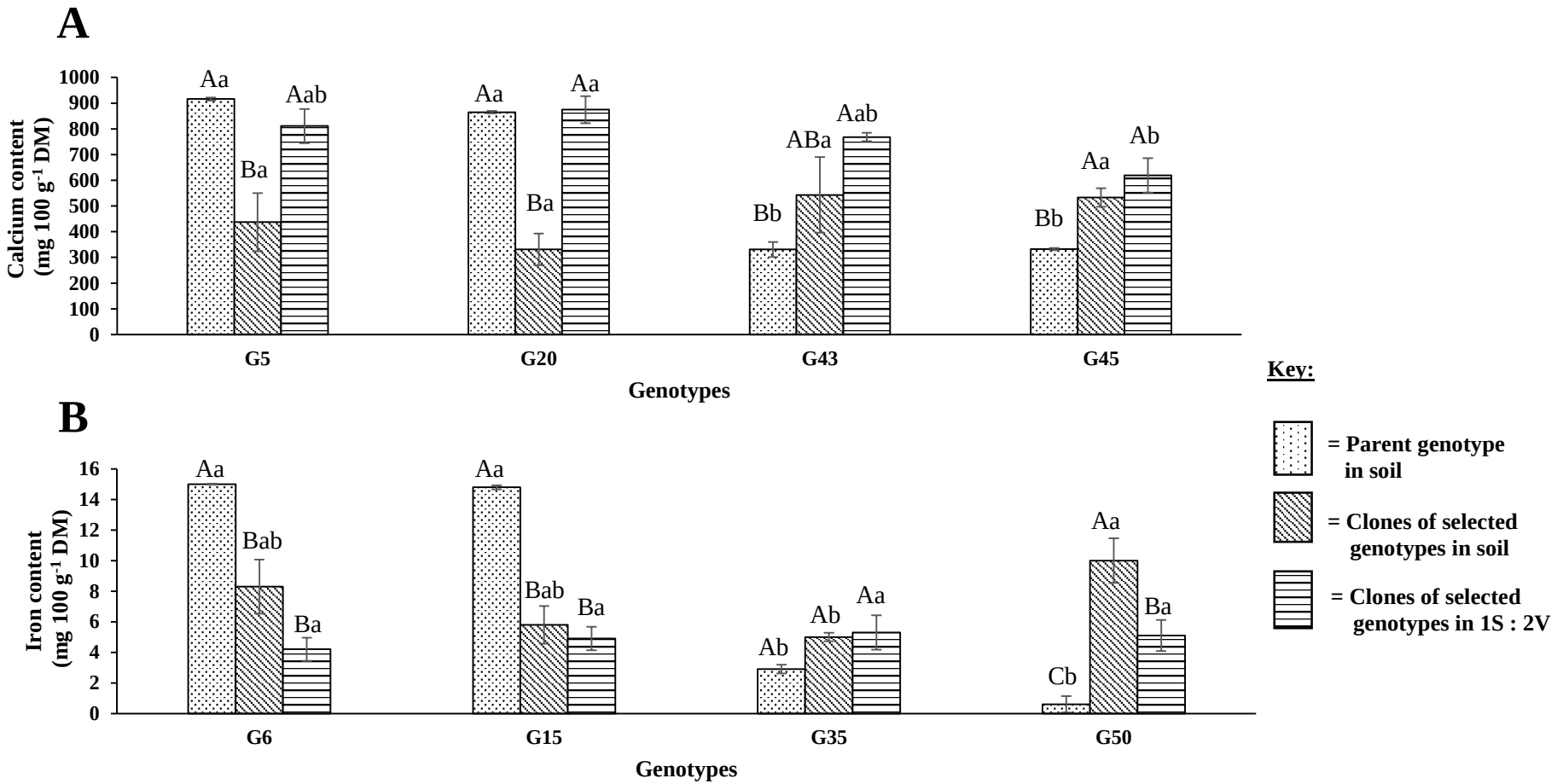


Figure 10: Comparison of Ca (A) and Fe (B) contents (mg 100 g^{-1} DM) amongst the clones of the selected genotypes (G) of *Solanum nigrum*, grown in soil and 1 soil : 2 vermiculite (1S : 2V), and the parent genotype. Leaf Ca and Fe contents of the parent genotypes were determined 6 weeks after germination and those of their clones were determined 6 weeks *ex vitro* (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n=3). Different uppercase letters indicate a statistical significance within a genotype. Different lowercase letters indicate a statistical significance across all the parent genotypes and across all the clones of the selected genotypes grown in each substrate type.

Fe levels of the G6 (8 ± 1.8 mg 100 g^{-1} DM) and G15 (6 ± 1.2 mg 100 g^{-1} DM) clones were significantly lower and that of the G50 (10 ± 1.5 mg 100 g^{-1} DM) clones was ten times higher than their parents (15 ± 0.1 mg 100 g^{-1} DM for G6, 15 ± 0.1 mg 100 g^{-1} DM for G15 and 1 ± 0.3 mg 100 g^{-1} DM for G50).

Under 1S : 2V conditions, the Ca levels of the G5 and G20 clones and the Fe content of the G35 clones were the same as their respective parents. In terms of Ca contents, those of the clones of G43 and G45 were significantly higher (768 ± 16 and 619 ± 67 mg 100 g^{-1} DM, respectively) than their parents (331 ± 29 mg 100 g^{-1} DM for G43 and 333 ± 4 mg 100 g^{-1} DM for G45). With regards to the Fe contents, those of the G6 (4 ± 0.8 mg 100 g^{-1} DM) and G15 (5 ± 0.8 mg 100 g^{-1} DM) clones were significantly lower than their parents (15 ± 0.1 mg 100 g^{-1} DM for G6 and 15 ± 0.1 mg 100 g^{-1} DM for G15). The level of Fe of G50 was five times higher in the clones (5 ± 1.0 mg 100 g^{-1} DM) than in the parent (1 ± 0.3 mg 100 g^{-1} DM).

The significant differences in the levels of Ca and/or Fe amongst the parent genotypes and their clones (as seen in Figure 10) were used to deduce a 'ranking' system (Table 17). The established 'ranking' of Ca of the parent genotypes was G5=G20>G43=G45. When maintained in 1S : 2V, the clones of G20 had a significantly higher level of Ca than the G45 clones. This trend was also recorded for the respective parent genotypes. The 'ranking' of the parent genotypes' leaf Fe was G6=G15>G35=G50. In 1S : 2V, the G50 clones had a significantly higher Fe content than the G35 clones. This was different to the 'ranking' that was established for the respective parent genotypes.

Table 17: Illustration of the significant differences in the leaf Ca and Fe contents of the parent genotypes and their clones that were grown in soil or 1 soil : 2 vermiculite (1S : 2V). Data are as from Figure 10 and were analysed using a one-way ANOVA ($p \leq 0.05$) followed by a post-hoc Tukey test.

Parent genotype and clones	Significant difference in Ca content	Significant difference in Fe content
Parent genotypes	G5=G20>G43=G45	G6=G15>G35=G50
Clones of genotypes (Soil)	None	G50>G35
Clones of genotypes (1S : 2V)	G20>G45	None

As mentioned above, the leaf Ca and Fe contents of the 6-week old seedling parents (*viz.* G5, G6, G15, G20, G35, G43, G45 and G50) were compared with those of their 6-week old *ex vitro* clones that were kept in soil or in 1S : 2V. The results showed that in soil, the Ca content of the G43 clones and the Fe level of the G35 clones were the same as their respective parents. Similarly, in 1S : 2V, this was recorded for the Ca contents of the G5, G20 and the Fe concentration of the G35 parents and their clones. Substrate type influenced the amount of Ca in the leaves of the G5 and G20 clones and the Fe content of the G50 clones.

4.5 The effect of physiological age on *ex vitro* growth and, calcium and iron content of the clones of the selected genotypes

As presented and discussed above (Figure 10 and Table 17), there were some significant differences in the Ca and Fe contents of the cloned genotypes when grown in the two substrates (i.e. soil and 1 soil : 2 vermiculite [1S : 2V]) and when compared with their respective parents. Therefore, a subsequent study was undertaken to evaluate the effects of physiological age and substrate type on the *ex vitro* growth and leaf Ca and Fe levels of four of the eight selected genotypes of *S. nigrum, viz*. G15 (high Fe), G20 (high Ca), G35 (low Fe) and G43 (low Ca). The *in vitro* shoots of these genotypes were micropropagated as per the established protocol (section 3.4.2). As mentioned previously (sections 4.3.1 and 4.4.2), the explants began to produce visible shoots after 2 weeks on shoot multiplication medium. Two weeks later, the greatest percentage explants producing shoots were from G15 (93 ± 6.7 %) and G43 (93 ± 6.7 %) (Table 18). The shoots of all selected genotypes were elongated to 10-20 mm tall and transferred onto hormone-free MS medium for rooting (Table 18), where all (100 %) of them rooted. Thereafter, they all survived acclimatisation (Table 18). The highest post-acclimatisation yield/explant was obtained in G15 (28 ± 1.3), followed by G43 (23 ± 1.0), G20 (19 ± 1.2), and G35 (18 ± 1.2). Except for the G20 clones, the plant yields of these genotypes differed when compared with those obtained previously (section 4.4.2).

Table 18: A summary of the effect of the established micropropagation protocol on the shoot multiplication, rooting and acclimatisation responses and, the subsequent post-acclimatisation yield of four of the selected *Solanum nigrum* genotypes. Results for % explants with shoots and number of shoots/explant were recorded after 4 weeks, % rooting was recorded after 3 weeks, % survival was recorded and yield of plants/explant was calculated after 2 weeks acclimatisation. Data were analysed using a one-way ANOVA and a post hoc Tukey test (mean ± SE, $p \leq 0.05$, n =10-50). Different lowercase letters denote a statistical significance within a column for each parameter.

Genotype	Shoot Multiplication		Rooting	Acclimatisation	Yield of plants/ explant
	% Explants with shoots	# Shoots /explant	% Rooted shoots	% Survival	
G15	93±6.7^{a}	35±1.6^{a}	100±0.0^{a}	100±0.0^{a}	28±1.3^{a}
G20	67±6.7^{b}	32±2.0^{ab}	100±0.0^{a}	100±0.0^{a}	19±1.2^{c}
G35	67±6.7^{b}	31±2.0^{ab}	100±0.0^{a}	100±0.0^{a}	18±1.2^{c}
G43	93±6.7^{a}	29±1.2^{b}	100±0.0^{a}	100±0.0^{a}	23±1.0^{b}

4.5.1 The effect of physiological age on the *ex vitro* growth of the selected cloned genotypes

The number of leaves and, shoot and root fresh and dry masses of the clones of the selected genotypes that were grown in soil or in 1 soil : 2 vermiculite (1S : 2V) were recorded at 4, 6 and 8 weeks *ex vitro* (which included 2 weeks acclimatisation). As previously stated, comparisons of these tested parameters between each parent plant and its clones were not performed as recording some of the parameters required harvesting and destroying the parent plants.

Number of leaves

The physiological age (4, 6 and 8 weeks *ex vitro*) of the G15 clones grown in both substrates and the G20 clones in soil had no significant effect on their numbers of leaves (Table 19). However, in 1S : 2V, the number of leaves of the G20 clones was significantly higher at 8 (25 ± 1.2) than at 4 (17 ± 1.9) and 6 (18 ± 0.6) weeks. The clones of G35 that were grown in both soil and 1S : 2V, had significantly higher leaf numbers at 8 (22 ± 2.6 and 22 ± 2.6, respectively) than at 4 (12 ± 1.5 and 12 ± 0.7, respectively) and 6 (15 ± 1.5 and 14 ± 0.7, respectively) weeks. In soil and 1S : 2V, the leaf numbers of the G43 clones were significantly higher at 6 (16 ± 0.5

Table 19: The number of leaves of the clones of the selected *Solanum nigrum* genotypes over 8 weeks *ex vitro* growth (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=3) and analysed between soil and 1 soil : 2 vermiculite (1S : 2V) for each genotype at each week using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=3). The interactions amongst genotype, physiological age and substrate type were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). Different uppercase letters denote a statistical significance for individual genotypes amongst sampling times for each substrate type; different lowercase letters denote a statistical significance amongst genotypes for each substrate type at each week (within a column); z denotes a statistical significance between soil and 1S : 2V for each genotype at each week. * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Genotype	**Number of leaves**					
	4 weeks		**6 weeks**		**8 weeks**	
	Soil	**1S : 2V**	**Soil**	**1S : 2V**	**Soil**	**1S : 2V**
G15	13 ± 0.3^{Ab}	17 ± 2.0^{Aa}	13 ± 1.9^{Ab}	17 ± 2.6^{Aa}	20 ± 3.2^{Aa}	20 ± 0.3^{Aab}
G20	23 ± 1.5^{Aa}	17 ± 1.9^{Ba}	23 ± 2.6^{Aa}	18 ± 0.6^{Ba}	23 ± 3.0^{Aa}	25 ± 1.2^{Aa}
G35	12 ± 1.5^{Bb}	12 ± 0.7^{Ba}	15 ± 1.5^{Bb}	14 ± 0.7^{Ba}	22 ± 2.6^{Aa}	22 ± 2.6^{Aa}
G43	9 ± 0.3^{Bb}	12 ± 0.7^{Baz}	16 ± 0.5^{Ab}	15 ± 0.9^{Aa}	16 ± 0.0^{Aa}	16 ± 0.7^{Ab}
Genotype x Physiological age			3.766^{*}			
Genotype x Substrate type			3.992^{*}			
Physiological age x Substrate type			0.548^{NS}			
Genotype x Physiological age x Substrate type			1.785^{NS}			

and 15 ± 0.9, respectively) and 8 (16 ± 0.0 and 16 ± 0.7, respectively) than at 4 (9 ± 0.3 and 12 ± 0.7, respectively) weeks.

Amongst the clones of the selected genotypes, there were some significant differences in the numbers of leaves at each tested physiological age (i.e. at 4, at 6 and at 8 weeks *ex vitro*) in both soil and 1S : 2V. After 4 and 6 weeks of *ex vitro* growth in soil, the G20 clones had significantly higher numbers of leaves (23 ± 1.5 at 4 and 23 ± 2.6 at 6 weeks) than the clones of the other tested genotypes (9-13 at 4 and 13-16 at 6 weeks) (Table 19). However, after growing under the same conditions (i.e. soil) for 8 weeks, there were no significant differences in the number of leaves amongst the clones of the selected genotypes. In 1S : 2V, there were similar numbers of leaves amongst the clones at 4 and at 6 weeks *ex vitro* (Table 19). At 8 weeks in 1S : 2V, the clones of G20 and G35 had significantly higher numbers of leaves (25 ± 1.2 and 22 ± 2.6, respectively) than the clones of G43 (16 ± 0.7). Except for the G43 clones at 4 weeks (9 ± 0.3 in soil and 12 ± 0.7 in 1S : 2V), the numbers of leaves of the clones of the tested genotypes were not influenced by substrate type (Table 19).

Further statistical analyses of the data (Table 19) showed that the numbers of leaves amongst the clones of the selected genotypes were significantly affected by the genotype x physiological age ($p \leq 0.05$) and genotype x substrate type ($p \leq 0.05$) interactions. This indicates that the number of leaves of clones of each genotype was affected by the tested physiological ages and the two substrate types.

Fresh and dry masses of shoots

The physiological age of the G15 clones had no effect on their shoot fresh masses over the 8 week growth period, irrespective of the substrate in which they were grown (Table 20). In soil, the shoot fresh mass of the G20 clones was significantly greater at 8 (3.0 ± 0.3 g) than at 4 (1.5 ± 0.1 g) weeks; however, when kept in 1S : 2V, there were no changes in their fresh masses during the 8 weeks of *ex vitro* growth. When grown in soil, the shoot fresh masses of the G35 clones did not change significantly over time but those of the clones in 1S : 2V were significantly different to each other at 4 (1.1 ± 0.1 g), 6 (1.8 ± 0.1 g) and 8 (2.4 ± 0.2 g) weeks. After growing in soil and in 1S : 2V, the shoot fresh masses of the G43 clones were significantly greater at 8 (1.8 ± 0.1 and 2.9 ± 0.2 g, respectively) than at 4 (1.0 ± 0.2 and 1.8 ± 0.1 g, respectively) and 6 (1.0 ± 0.0 and 2.1 ± 0.1 g, respectively) weeks.

Table 20: The shoot fresh mass (g) of the clones of the selected *Solanum nigrum* genotypes over 8 weeks *ex vitro* growth (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=3) and analysed between soil and 1 soil : 2 vermiculite (1S : 2V) for each genotype at each week using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=3). The interactions amongst genotype, physiological age and substrate type were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). Different uppercase letters denote a statistical significance for individual genotypes amongst sampling times for each substrate type; different lowercase letters denote a statistical significance amongst genotypes for each substrate type at each week (within a column); z denotes a statistical significance between soil and 1S : 2V for each genotype at each week. * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Genotype	**Shoot Fresh Mass (g)**					
	4 weeks		**6 weeks**		**8 weeks**	
	Soil	**1S : 2V**	**Soil**	**1S : 2V**	**Soil**	**1S : 2V**
G15	1.8 ± 0.2^{Aa}	1.6 ± 0.2^{Aa}	2.2 ± 0.1^{Aa}	2.2 ± 0.2^{Aa}	2.1 ± 0.1^{Ab}	2.0 ± 0.4^{Aa}
G20	1.5 ± 0.1^{Ba}	1.7 ± 0.0^{Aa}	2.1 ± 0.2^{ABa}	1.9 ± 0.2^{Aa}	3.0 ± 0.3^{Aa}	2.2 ± 0.1^{Aa}
G35	1.6 ± 0.2^{Aa}	1.1 ± 0.1^{Cb}	1.7 ± 0.2^{Aa}	1.8 ± 0.1^{Ba}	1.5 ± 0.2^{Ab}	2.4 ± 0.2^{Aaz}
G43	1.0 ± 0.2^{Ba}	1.8 ± 0.1^{Baz}	1.0 ± 0.0^{Bb}	2.1 ± 0.1^{Baz}	1.8 ± 0.1^{Ab}	2.9 ± 0.2^{Aaz}
Genotype x Physiological age			3.406*			
Genotype x Substrate type			15.080*			
Physiological age x Substrate type			1.078^{NS}			
Genotype x Physiological age x Substrate type			3.671*			

The differences in shoot fresh masses amongst the genotypes were assessed at each tested physiological age. At 4 weeks in soil, there were no significant differences in the shoot fresh masses of the selected cloned genotypes (Table 20). However, after 4 weeks in 1S : 2V, the shoot fresh masses of the G15 (1.6 ± 0.2 g), G20 (1.7 ± 0.0 g) and G43 (1.8 ± 0.1 g) clones were significantly greater than that of the G35 (1.1 ± 0.1 g) clones. When grown under soil conditions for 6 weeks, the G15 (2.2 ± 0.1 g), G20 (2.1 ± 0.2 g) and G35 (1.7 ± 0.2 g) clones had significantly greater shoot fresh masses than the G43 (1.0 ± 0.0 g) clones. By 8 weeks, that of the clones of G20 (3.0 ± 0.3 g) was significantly higher than those of the clones of G15 (2.1 ± 0.1 g), G35 (1.5 ± 0.2 g) and G43 (1.8 ± 0.1 g). However, at 6 and 8 weeks in 1S : 2V, there were no significant differences in the shoot fresh masses amongst the clones of the selected genotypes.

Regarding the effect of substrate type on shoot fresh mass, that of the G43 clones was significantly higher in 1S : 2V (1.8 ± 0.1 at 4, 2.1 ± 0.1 at 6 and 2.9 ± 0.2 g at 8 weeks) than in soil (1.0 ± 0.2 at 4, 1.0 ± 0.0 at 6 and 1.8 ± 0.1 g at 8 weeks). At 8 weeks, the shoot fresh mass of the G35 clones was significantly greater in 1S : 2V (2.4 ± 0.2 g) than in soil (1.5 ± 0.2 g).

The shoot fresh masses of the clones of the selected genotypes were significantly affected by the genotype x physiological age ($p \leq 0.05$), genotype x substrate type ($p \leq 0.05$) and genotype x physiological age x substrate type ($p \leq 0.05$) interactions. Therefore, in each of the two substrates, the clones of each genotype displayed different shoot fresh masses at the various tested physiological ages.

In terms of shoot dry mass, those of the G15 clones, grown in both soil and 1S : 2V, were significantly greater at 8 (0.43 ± 0.03 and 0.43 ± 0.06 g, respectively) than at 4 (0.14 ± 0.01 and 0.14 ± 0.0 g, respectively) and 6 (0.23 ± 0.02 and 0.28 ± 0.03 g, respectively) weeks (Table 21). When kept in soil, the shoot dry mass of the G20 clones was significantly greater at 6 (0.36 ± 0.07 g) and 8 (0.47 ± 0.04 g) than at 4 (0.13 ± 0.0 g) weeks. Under the same conditions (i.e. soil), the clones of G35 and G43 had significantly greater shoot dry masses at 8 (0.33 ± 0.04 and 0.33 ± 0.03 g, respectively) than at 4 (0.13 ± 0.01 and 0.13 ± 0.01 g, respectively) and 6 (0.19 ± 0.02 and 0.16 ± 0.0 g, respectively) weeks. When grown in 1S : 2V, the G20 and G35 clone's shoot dry masses did not significantly change, regardless of their physiological age (Table 21). The G43 clones that were kept in 1S : 2V had significantly greater shoot dry masses at 8 (0.52 ± 0.02 g) and 6 (0.45 ± 0.04 g) than at 4 (0.24 ± 0.02 g) weeks *ex vitro*.

Table 21: The shoot dry mass (g) of the clones of the selected *Solanum nigrum* genotypes over 8 weeks *ex vitro* growth (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=3) and analysed between soil and 1 soil : 2 vermiculite (1S : 2V) for each genotype at each week using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=3). The interactions amongst genotype, physiological age and substrate type were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). Different uppercase letters denote a statistical significance for individual genotypes amongst sampling times for each substrate type; different lowercase letters denote a statistical significance amongst genotypes for each substrate type at each week (within a column); z denotes a statistical significance between soil and 1S : 2V for each genotype at each week. * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Genotype	**Shoot Dry Mass (g)**					
	4 weeks		**6 weeks**		**8 weeks**	
	Soil	**1S : 2V**	**Soil**	**1S : 2V**	**Soil**	**1S : 2V**
G15	0.14 ± 0.01^{Ca}	0.14 ± 0.00^{Ca}	0.23 ± 0.02^{Bab}	0.28 ± 0.03^{Bb}	0.43 ± 0.03^{Aa}	0.43 ± 0.06^{Aab}
G20	0.13 ± 0.0^{Ba}	0.25 ± 0.08^{Aa}	0.36 ± 0.07^{Aa}	0.30 ± 0.04^{Aab}	0.47 ± 0.04^{Aaz}	0.35 ± 0.02^{Abc}
G35	0.13 ± 0.01^{Ba}	0.17 ± 0.02^{Aa}	0.19 ± 0.02^{Bb}	0.26 ± 0.04^{Ab}	0.33 ± 0.04^{Aaz}	0.24 ± 0.02^{Ac}
G43	0.13 ± 0.01^{Ba}	0.24 ± 0.02^{Baz}	0.16 ± 0.0^{Bb}	0.45 ± 0.04^{Aaz}	0.33 ± 0.03^{Aa}	0.52 ± 0.02^{Aaz}
Genotype x Physiological age			1.626^{NS}			
Genotype x Substrate type			11.782*			
Physiological age x Substrate type			8.414*			
Genotype x Physiological age x Substrate type			3.014*			

When grown for 4 weeks in both substrates, there were no significant differences in the shoot dry masses amongst the clones of the selected genotypes (G15, G20, G45 and G35) (Table 21). After 6 weeks in soil, the G20 (0.36 ± 0.07) clones had a significantly higher shoot dry mass than the G35 (0.19 ± 0.02 g) and G43 (0.16 ± 0.0 g) clones. In 1S : 2V, that of the G43 (0.45 ± 0.04 g) clones was significantly higher than those of the G15 (0.28 ± 0.03 g) and G35 (0.26 ± 0.04 g) clones. At 8 weeks *ex vitro* in soil, there were no significant differences in the shoot dry masses amongst the clones. When kept in 1S : 2V for 8 weeks, the clones of G43 (0.52 ± 0.02 g) had a significantly greater shoot dry mass than the clones of G20 (0.35 ± 0.02 g) and G35 (0.24 ± 0.02 g).

The effect of substrate type was observed for the shoot dry mass of the G43 clones, which was significantly higher when the clones were kept in 1S : 2V (0.24 ± 0.02 g at 4, 0.45 ± 0.04 g at 6 and 0.52 ± 0.02 g at 8 weeks) than in soil (0.13 ± 0.01 g at 4, 0.16 ± 0.0 g at 6 and 0.33 ± 0.03 g at 8 weeks) (Table 21). At 8 weeks, the G20 and G35 clones had significantly greater shoot dry masses in soil (0.47 ± 0.04 and 0.33 ± 0.04 g, respectively) than in 1S : 2V (0.35 ± 0.02 and 0.24 ± 0.02 g, respectively).

The shoot dry masses of the selected cloned genotypes were not affected by the genotype x physiological age ($p \geq 0.05$) interaction (Table 21) but they were significantly affected by the genotype x substrate type ($p \leq 0.05$), physiological age x substrate type ($p \leq 0.05$) and genotype x physiological age x substrate type ($p \leq 0.05$) interactions. Hence, the shoot dry masses of the tested genotypes varied at each tested physiological age in both soil and 1S : 2V.

Fresh and dry masses of roots

When grown both in soil and in 1S : 2V, the effect of physiological age was observed in the root fresh masses of the G15 clones - they were significantly greater at 8 (2.7 ± 0.2 and 3.5 ± 0.3 g, respectively) than at 4 (1.3 ± 0.1 and 1.3 ± 0.0 g, respectively) and 6 (1.7 ± 0.2 and 2.1 ± 0.3 g, respectively) weeks (Table 22). This was also seen in the root fresh masses of the G20 clones after growing in both substrate types. When maintained in soil, the G35 clones had significantly greater root fresh masses at 6 (2.1 ± 0.1 g) and 8 (2.3 ± 0.2 g) than at 4 (1.4 ± 0.2 g) weeks. In 1S : 2V, the clones of G35 had a significantly greater root fresh mass at 8 (1.9 ± 0.0 g) than at 4 (1.3 ± 0.1 g) weeks. The root fresh mass of the G43 clones was not affected by physiological age, irrespective of substrate type (Table 22).

Table 22: The root fresh mass (g) of the clones of the selected *Solanum nigrum* genotypes over 8 weeks *ex vitro* growth (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=3) and analysed between soil and 1 soil : 2 vermiculite (1S : 2V) for each genotype at each week using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=3). The interactions amongst genotype, physiological age and substrate type were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). Different uppercase letters denote a statistical significance for individual genotypes amongst sampling times for each substrate type; different lowercase letters denote a statistical significance amongst genotypes for each substrate type at each week (within a column); z denotes a statistical significance between soil and 1S : 2V for each genotype at each week. * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Genotype	**Root Fresh Mass (g)**					
	4 weeks		**6 weeks**		**8 weeks**	
	Soil	**1S : 2V**	**Soil**	**1S : 2V**	**Soil**	**1S : 2V**
G15	1.3 ± 0.1^{Bb}	1.3 ± 0.0^{Bb}	1.7 ± 0.2^{Bb}	2.1 ± 0.3^{Ba}	2.7 ± 0.2^{Aab}	3.5 ± 0.3^{Aa}
G20	2.0 ± 0.1^{Baz}	1.5 ± 0.1^{Bb}	2.4 ± 0.2^{Baz}	1.7 ± 0.2^{Ba}	3.8 ± 0.5^{Aaz}	2.4 ± 0.1^{Ab}
G35	1.4 ± 0.2^{Bb}	1.3 ± 0.1^{Bb}	2.1 ± 0.1^{Aab}	1.7 ± 0.2^{ABa}	2.3 ± 0.2^{Abcz}	1.9 ± 0.0^{Ab}
G43	0.9 ± 0.1^{Ab}	2.5 ± 0.1^{Aaz}	1.0 ± 0.1^{Ac}	2.6 ± 0.4^{Aaz}	1.4 ± 0.2^{Ac}	2.8 ± 0.3^{Aabz}
Genotype x Physiological age			5.030*			
Genotype x Substrate type			34.269*			
Physiological age x Substrate type			0.223^{NS}			
Genotype x Physiological age x Substrate type			1.425*			

After growing for 4 weeks in soil, the root fresh mass of the G20 (2.0 ± 0.1 g) clones was significantly greater than those of the G15 (1.3 ± 0.1 g), G35 (1.4 ± 0.2 g) and G43 (0.9 ± 0.1 g) clones. Under 1S : 2V conditions at 4 weeks, the clones of G43 (2.5 ± 0.1 g) had a significantly higher root fresh mass than the clones of G15 (1.3 ± 0.0 g), G20 (1.5 ± 0.1 g) and G35 (1.3 ± 0.1 g). When maintained for 6 weeks in soil, the G20 (2.4 ± 0.2 g), G43 (1.0 ± 0.1 g) and G15 (1.7± 0.2 g) clone's root fresh masses were significantly different to each other but in 1S : 2V, they were not. When kept in soil for 8 weeks, the G20 (3.8 ± 0.5 g) clones had a significantly greater root fresh mass than the G35 (2.3 ± 0.2 g) and G43 (1.4 ± 0.2 g) clones. However, after growing for 8 weeks in 1S : 2V, the clones of G15 (3.5 ± 0.3) had a significantly greater root fresh mass than the clones of G20 (2.4 ± 0.1 g) and G35 (1.9 ± 0.0 g).

The type of substrate affected the root fresh masses of some of the clones of the selected genotypes. The clones of G20 had a significantly higher root fresh mass in soil (2.0 ± 0.1 g at 4, 2.4 ± 0.2 g at 6 and 3.8 ± 0.5 g at 8 weeks) than in 1S : 2V (1.5 ± 0.1 g at 4, 1.7 ± 0.2 g at 6 and 2.4 ± 0.1 g at 8 weeks) (Table 22). This was also observed in the G35 clones at 8 weeks *ex vitro*. In contrast, the G43 clone's root fresh mass was significantly higher in 1S : 2V (2.5 ± 0.1 g at 4, 2.6 ± 0.4 g at 6 and 2.8 ± 0.3 g at 8 weeks) than in soil (0.9 ± 0.1 g at 4, 1.0 ± 0.1 g at 6 and 1.4 ± 0.2 g at 8 weeks).

The root fresh masses of the clones of the selected genotypes were significantly affected by the genotype x physiological age ($p \leq 0.05$), genotype x substrate type ($p \leq 0.05$) and genotype x physiological age x substrate type ($p \leq 0.05$) interactions. Thus, in soil and in 1S : 2V, the root fresh masses of the clones of each genotype differed at the different tested physiological ages.

The root dry mass of the G15 clones increased significantly with time (i.e. physiological age) in both soil (0.15 ± 0.01 g at 4, 0.16 ± 0.02 g at 6 and 0.28 ± 0.02 g at 8 weeks) and 1S : 2V (0.12 ± 0.01 g at 4, 0.20 ± 0.01 g at 6 and 0.37 ± 0.07 g at 8 weeks). This was also recorded for the root dry mass of the G20 clones, irrespective of substrate type (Table 23). When grown in soil, the root dry mass of the G35 clones also increased with physiological age (0.16 ± 0.03 g at 4, 0.18 ± 0.01 g at 6 and 0.31 ± 0.02 g at 8 weeks). However, over the 8 weeks of *ex vitro* growth in 1S:2V, there was no change in the root dry mass of the G35 clones. Similarly, in soil, the root dry mass of the G43 clones was not significantly affected by physiological age. However, when grown in 1S : 2V, their root dry masses were significantly greater at 6 (0.33 ± 0.03 g) and 8 (0.37 ± 0.03 g) than at 4 (0.19 ± 0.02 g) weeks.

Table 23: The root dry mass (g) of the clones of the selected *Solanum nigrum* genotypes over 8 weeks *ex vitro* growth (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=3) and between soil and 1 soil : 2 vermiculite (1S : 2V) for each genotype at each week using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=3). The interactions amongst genotype, physiological age and substrate type were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). Different uppercase letters denote a statistical significance for individual genotypes amongst sampling times for each substrate type; different lowercase letters denote a statistical significance amongst genotypes for each substrate type at each week (within a column); z denotes a statistical significance between soil and 1S : 2V for each genotype at each week. * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Genotype	**Root Dry Mass (g)**					
	4 weeks		**6 weeks**		**8 weeks**	
	Soil	**1S : 2V**	**Soil**	**1S : 2V**	**Soil**	**1S : 2V**
G15	0.15 ± 0.01^{Bb}	0.12 ± 0.01^{Ca}	0.16 ± 0.02^{Bb}	0.20 ± 0.01^{Bb}	0.28 ± 0.02^{Aab}	0.37 ± 0.07^{Aa}
G20	0.20 ± 0.00^{Ba}	0.16 ± 0.02^{Ba}	0.24 ± 0.01^{Ba}	0.19 ± 0.02^{Bb}	0.43 ± 0.10^{Aa}	0.32 ± 0.01^{Aa}
G35	0.16 ± 0.03^{Bab}	0.14 ± 0.01^{Aa}	0.18 ± 0.01^{Bab}	0.20 ± 0.02^{Ab}	0.31 ± 0.02^{Aabz}	0.19 ± 0.04^{Ab}
G43	0.14 ± 0.01^{Ab}	0.19 ± 0.02^{Ba}	0.14 ± 0.01^{Ab}	0.33 ± 0.03^{Aaz}	0.16 ± 0.04^{Ab}	0.37 ± 0.03^{Aaz}
Genotype x Physiological age			1.915^{NS}			
Genotype x Substrate type			19.101*			
Physiological age x Substrate type			1.866^{NS}			
Genotype x Physiological age x Substrate type			1.555^{NS}			

At both 4 and 6 weeks in soil, the clones of G20 (0.20 ± 0.0 and 0.24 ± 0.01 g, respectively) had a significantly greater root dry mass than the G15 (0.15 ± 0.01 and 0.16 ± 0.02 g, respectively) and G43 (0.14 ± 0.01 and 0.14 ± 0.01 g, respectively) clones. After 8 weeks in soil, the root dry mass of G20 was significantly greater (0.43 ± 0.10 g) than that of G43 (0.16 ± 0.04 g) (Table 23). Under 1S : 2V conditions, the root dry masses of the 4-week old cloned genotypes were similar but at 6 weeks that of the clones of G43 was significantly higher than those of the G15 (0.20 ± 0.01 g), G35 (0.20 ± 0.02 g) and G20 (0.19 ± 0.02 g) clones; by 8 weeks the G15 (0.37 ± 0.07 g), G20 (0.32 ± 0.01 g) and G43 (0.37 ± 0.03 g) clones had significantly greater root dry masses than the G35 (0.19 ± 0.04 g) clones.

The root dry masses of the clones of the selected genotypes were not influenced by substrate type (Table 23). The exceptions were the G43 clones at 6 (0.14 ± 0.01 g in soil and 0.33 ± 0.03 g in 1S : 2V) and 8 (0.16 ± 0.04 g in soil and 0.37 ± 0.03 g in 1S : 2V) weeks and the G35 clones at 8 weeks (0.31 ± 0.02 g in soil and 0.19 ± 0.04 g in 1S : 2V).

Further statistical analyses indicated that the clones of the selected genotypes were significantly affected by the genotype x substrate type ($p \leq 0.05$) interaction, implying that the root dry masses of the clones of each genotype differed in the two substrates.

Root:shoot dry masses

The root:shoot dry masses of the G15 and G20 clones were positively affected by their physiological age in soil but not in 1S : 2V (Table 24). In both the substrates, the root:shoot dry mass of the G35 and G43 clones were similar, irrespective of their physiological age.

At 4 weeks in soil, this ratio was significantly higher in the G20 clones (1.5 ± 0.0) than in the G43 clones (0.9 ± 0.1). After 4 weeks in 1S : 2V and 6 and 8 weeks in both substrates, no significant differences were found amongst the clones of the selected genotypes with regards to root:shoot dry masses (Table 24).

With the exception of the G15 clones at 8 weeks (0.6 ± 0.0 and 0.9 ± 0.1 in soil and 1S : 2V, respectively) and the G20 clones at 4 weeks (1.5 ± 0.0 and 0.8 ± 0.2 in soil and 1S : 2V, respectively), the root:shoot dry masses of the clones of the selected genotypes were not significantly affected by substrate type (Table 24).

Table 24: The root:shoot dry masses of the clones of the selected *Solanum nigrum* genotypes over 8 weeks *ex vitro* growth (which included 2 weeks acclimatisation). Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$; n=3) and analysed between soil and 1 soil : 2 vermiculite (1S : 2V) for each genotype at each week using an unpaired t-test (mean ± SE, $p \leq 0.05$, n=3). The interactions amongst genotype, physiological age and substrate type were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). Different uppercase letters denote a statistical significance for individual genotypes amongst sampling times for each substrate type; different lowercase letters denote a statistical significance amongst genotypes for each substrate type at each week (within a column); z denotes a statistical significance between soil and 1S : 2V for each genotype at each week. * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Genotype	**Root:Shoot**					
	4 weeks		**6 weeks**		**8 weeks**	
	Soil	**1S : 2V**	**Soil**	**1S : 2V**	**Soil**	**1S : 2V**
G15	1.1 ± 0.1^{Aab}	0.8 ± 0.1^{Aa}	0.7 ± 0.1^{Ba}	0.7 ± 0.1^{Aa}	0.6 ± 0.0^{Ba}	0.9 ± 0.1^{Aaz}
G20	1.5 ± 0.0^{Aaz}	0.8 ± 0.2^{Aa}	0.7 ± 0.1^{Ba}	0.6 ± 0.1^{Aa}	0.9 ± 0.2^{Ba}	0.9 ± 0.1^{Aa}
G35	1.3 ± 0.2^{Aab}	0.9 ± 0.2^{Aa}	1.0 ± 0.2^{Aa}	0.8 ± 0.0^{Aa}	1.0 ± 0.2^{Aa}	0.8 ± 0.1^{Aa}
G43	0.9 ± 0.1^{Ab}	0.8 ± 0.1^{Aa}	0.9 ± 0.1^{Aa}	0.7 ± 0.0^{Aa}	0.5 ± 0.1^{Aa}	0.7 ± 0.0^{Aa}
Genotype x Physiological age	1.600^{NS}					
Genotype x Substrate type	2.695^{NS}					
Physiological age x Substrate type	6.223*					
Genotype x Physiological age x Substrate type	0.918^{NS}					

The physiological age x substrate type ($p \leq 0.05$) interaction significantly affected the root:shoot dry masses of the clones of the selected genotypes. This suggests that the root:shoot dry masses of clones differed at the different tested physiological ages when grown in both soil and 1S : 2V.

In conclusion, the shoot growth (*viz.* number of leaves and fresh and dry masses) of the clones of the selected genotypes was less affected by physiological age than the root growth (*viz.* fresh and dry masses). Substrate type affected the root fresh mass of the G20 clones and the shoot and root fresh and dry masses of the G43 clones throughout the tested growth period. The shoot and root growth of the clones of the other genotypes were mainly affected by substrate type toward the end of the tested growth period (i.e. 8 weeks).

4.5.2 The effect of physiological age on the levels of leaf calcium and iron of the clones of the selected genotypes

The leaf Ca contents of the G20 and G43 clones did not change with advancing physiological age, regardless of the substrate in which they were grown (Table 25). When maintained in soil for 4 weeks, the Ca level of the G20 clones (843 ± 68 mg 100 g^{-1} dry mass [DM]) was significantly higher than that of the G43 clones (591 ± 74 mg 100 g^{-1} DM; Table 25) but it became significantly lower at 6 weeks (675 ± 57 mg 100 g^{-1} DM in G20 and 862 ± 14 mg 100 g^{-1} DM in G43). By 8 weeks, the amount of Ca between the G20 and G43 clones was not significantly different. In 1S : 2V, the clones of G20 and G43 had similar levels of Ca up to week 6 but at week 8 the clones of G20 (825 ± 52 mg 100 g^{-1} DM) had a significantly higher level of leaf Ca than the clones of G43 (581 ± 32 mg 100 g^{-1} DM).

The clones of G20 had similar levels of Ca to its parent at all tested physiological ages, regardless of substrate type (Table 25). After growing in soil for 4 weeks, the G43 clones (591 ± 74 mg 100 g^{-1} DM) had a similar level of Ca to its parent (331 ± 29 mg 100 g^{-1} DM). However, at 6 and 8 weeks (862 ± 14 and 676 ± 117 mg 100 g^{-1} DM, respectively) the Ca levels were two times or more higher in the clones than the parent genotype (331 ± 29 mg 100 g^{-1} DM). At 6 weeks under 1S : 2V conditions, the Ca content of the G43 clones (785 ± 103 mg 100 g^{-1} DM) was more than double that of its parent (331 ± 29 mg 100 g^{-1} DM) and similar at 4 (597 ± 87 mg 100 g^{-1} DM) and 8 (581 ± 32 mg 100 g^{-1} DM) weeks *ex vitro*.

Table 25: Comparison of leaf Ca and Fe contents (mg $100g^{-1}$ DM) amongst the clones of the selected *Solanum nigrum* genotypes (G), kept on two substrates, over 8 weeks *ex vitro* growth with those of their respective parent genotype. Data were analysed between substrate types for each genotype; between G20 and G43 or G15 and G35 for each substrate type at 4, at 6 and at 8 weeks *ex vitro* using unpaired t-tests (mean ± SE, $p \leq 0.05$, n=3) and amongst the clones of the selected genotypes at 4, 6 and 8 weeks *ex vitro* and the parent genotype for each genotype and substrate type using a one-way ANOVA and post-hoc Tukey test (mean ± SE, $p \leq 0.05$, n=3). The data for parent genotypes were as from Figures 8 and 9. Different uppercase letters indicate a significant difference amongst the parent genotype and its clones (at 4, 6 and 8 weeks) for each individual genotype when kept in soil. Different lowercase letters indicate a statistical significance amongst the parent genotype and its clones (at 4, 6 and 8 weeks) for each individual genotype when kept in 1 soil : 2 vermiculite (1S : 2V). An * indicates a statistical significance between G20 and G43 or G15 and G35 for each substrate type at 4, at 6 and at 8 weeks *ex vitro*. A ^{Z} indicates a statistical significance between substrate types for each genotype at 4, at 6 and at 8 weeks *ex vitro*.

Genotype		**Parent 'Ranking'**	**Substrate**	**Weeks**			
				6	**4**	**6**	**8**
				Parent genotype	**Clones of the selected genotypes**		
Ca	**G20**	**High**	**Soil**	864 ± 5^{Aa}	$843\pm68^{A*}$	675 ± 57^{A}	674 ± 74^{A}
			1S : 2V	-	752 ± 29^{a}	775 ± 34^{a}	$825\pm52a^{*}$
	G43	**Low**	**Soil**	331 ± 29^{Bb}	591 ± 74^{AB}	$862\pm14^{A*}$	676 ± 117^{A}
			1S : 2V	-	597 ± 87^{ab}	785 ± 103^{a}	581 ± 32^{ab}
Fe	**G15**	**High**	**Soil**	15.0 ± 0.1^{Aa}	7.9 ± 0.6^{B}	7.6 ± 1.1^{B}	4.1 ± 0.2^{C}
			1S : 2V	-	8.8 ± 0.4^{b}	6.1 ± 0.3^{c}	5.2 ± 0.3^{cZ}
	G35	**Low**	**Soil**	3.0 ± 0.3^{Bb}	7.5 ± 0.1^{A}	6.1 ± 0.4^{A}	$6.6\pm0.6^{A*}$
			1S : 2V	-	10.0 ± 1.7^{a}	5.8 ± 0.4^{b}	6.0 ± 0.4^{b}

The effects of physiological age and substrate type on Fe content were also assessed for the clones of G15 and G35. When grown in soil, the G15 clone's Fe content decreased with increasing physiological age and was significantly higher at 4 (7.9 ± 0.6 mg 100 g^{-1} DM) and 6 (7.5 ± 1.1 mg 100 g^{-1} DM) than at 8 weeks (4.1 ± 0.2 mg 100 g^{-1} DM). Under 1S : 2V conditions, the Fe content of the clones of G15 decreased at an earlier physiological age than that of the clones kept in soil- the clones had a significantly higher level of Fe at 4 (8.8 ± 0.3 mg 100 g^{-1} DM) than at 6 (6.1 ± 0.3 mg 100 g^{-1} DM) and 8 (5.3 ± 0.3 mg 100 g^{-1} DM) weeks. When grown in soil, no significant differences were detected in the Fe contents of the G35 clones at all tested physiological ages (Table 25). However, in 1S : 2V, the Fe levels of the G35 clones were significantly higher at 4 (10 ± 1.7 mg 100 g^{-1} DM) than at 6 (6 ± 0.4 mg 100 g^{-1} DM) and 8 (6 ± 0.4 mg 100 g^{-1} DM) weeks.

At 4 and 6 weeks in soil, there were no significant differences in the Fe contents between the clones of G15 and G35 (Table 25). However, after growing for 8 weeks in soil, the clones of G15 (4.1 ± 0.2 mg 100 g^{-1} DM) had a significantly lower Fe level than the G35 clones (6.6 ± 0.6 mg 100 g^{-1} DM). Under 1S : 2V conditions, the levels of Fe between the G15 and G35 clones were the same at all physiological ages (Table 25).

At each physiological age and in both the substrates, the clones of G15 had significantly lower levels of Fe than the parent genotype (Table 25). In contrast, when grown in soil, the Fe content of the G35 clones was more than double that of its parent genotype (3.0 ± 0.3 mg 100 g^{-1} DM) at all the tested physiological ages (7.5 ± 0.1 at 4, 6.1 ± 0.4 at 6 and 6.6 ± 0.6 mg 100 g^{-1} DM at 8 weeks) (Table 25). Under 1S : 2V conditions at 4 weeks, the level of Fe in the clones of G35 (10.0 ± 1.7 mg 100 g^{-1} DM) was three times or more higher than that of its parent (3.0 ± 0.3 mg 100 g^{-1} DM) but by 6 (5.8 ± 0.4 mg 100 g^{-1} DM) and 8 (6.0 ± 0.4 mg 100 g^{-1} DM) weeks, the Fe levels decreased and were similar to that of its parent.

Further statistical analyses of the data, using a three-way ANOVA, showed that the Ca and Fe levels of the clones of the selected genotypes were significantly affected by the genotype x physiological age interaction (Table 26). This suggests that the Ca and Fe contents of the clones of each genotype differed at the different tested physiological ages. Only Fe was affected by the physiological age x substrate type interaction ($p \leq 0.05$), implying that the Fe content of the clones differed (decreased) at different physiological ages when grown in both tested substrates.

Table 26: The interaction amongst genotype, physiological age and substrate type on the leaf Ca and Fe contents of the clones of the selected *Solanum nigrum* genotypes. Data were analysed using a three-way ANOVA (F value, $p \leq 0.05$, n=3). * denotes a statistical significance, NS denotes no statistical significance for each interaction.

Interaction	Ca	Fe
Genotype x Physiological age	5.169*	7.068*
Genotype x Substrate type	1.866^{NS}	0.023^{NS}
Physiological age x Substrate type	0.286^{NS}	3.616*
Genotype x Physiological age x Substrate type	1.746^{NS}	2.992^{NS}

This study was done to investigate the effect of physiological age and substrate type on the levels of Ca and Fe in the leaves of the clones of four of the selected *S. nigrum* genotypes, *viz*. G15 (high Fe), G20 (high Ca), G35 (low Fe) and G43 (low Ca). Using the significant differences in the Ca (G20 and G43) and/or Fe (G15 and G35) contents amongst the clones of the tested genotypes at 4, 6 and 8 weeks *ex vitro* in both soil and 1S : 2V (Table 25), 'rankings' were established and compared with those of the parent genotypes (Table 27). The leaf Ca contents of the clones of G20 and G43 differed from their parent genotypes at 6 and 8 weeks in soil and at 4 and 6 weeks in 1S : 2V (Table 27). However, at 4 weeks in soil and at 8 weeks in 1S : 2V, the G20 and G43 clones maintained similar levels of Ca to those of their parent genotypes (G20>G43) (Table 27).

Table 27: Significant differences in the levels of Ca and Fe of the parent genotypes at weeks and their clones at 4, 6 and 8 weeks *ex vitro*. The clones were grown in soil or 1 soil : 2 vermiculite (1S : 2V). Data for the parent genotypes are as from Figure 10 and for the clones of the selected genotypes are as from Table 25.

Parent genotype and clones	Week	Ca content	Fe content
Parent genotype	**6**	G20>G43	G15>G35
Clones of the selected genotypes (soil)	**4**	G20>G43	None
	6	G43>G20	None
	8	None	G35>G15
Clones of the selected genotypes (1S : 2V)	**4**	None	None
	6	None	None
	8	G20>G43	None

In conclusion, physiological age had no effect on the Ca contents of the G20 and G43 clones in both substrates and the Fe level of the G35 clones in soil. However, it negatively affected the Fe contents of the G15 clones in the two substrates and the G35 clones in 1S : 2V. When compared with its parent genotype in soil, the G20 clones had the same Ca level at all tested physiological ages. At 4 weeks in soil, this was observed for the Ca content of G43. Subsequent to growing for 4 weeks in soil and 8 weeks in 1S : 2V, the Ca content of the G20 clones was greater than that of the G43 clones, i.e. G20 > G43. This trend was also recorded for their parent plants. In terms of Fe, the 'ranking' of the clones of G15 and G35 in both the substrates and at all the tested ages differed from that of their parents due to them having similar amounts of Fe.

4.6 Preliminary studies on the minimal growth storage of *Solanum nigrum*

As *S. nigrum* is a short-lived seasonal plant, a constant supply of explant material from the parent genotype for additional studies is not always available. Hence, one of the objectives of the present study was to undertake some investigations towards a short-term storage protocol for *in vitro S. nigrum* shoots for future studies. Toward this end, the effect of two media compositions on minimal growth storage and the ability of the *in vitro* plants to regenerate and form whole plants after an 8-week storage period were tested.

In vitro shoots, from the eight genotypes (i.e. G5, G6, G15, G20, G35, G43, G45 and G50), stored on ⅓ MS + 10 g l^{-1} sucrose, multiplied, elongated and produced roots within the first week of storage. After the first week, the *in vitro* plants began to lose colour (from green to pale whitish green) and developed white callus on the stems (Figure 11 A). *In vitro* shoots of the eight tested genotypes, which were stored on ¼ MS + 5 g l^{-1} sucrose also multiplied, elongated and produced roots within the first week of storage. After their first week on ¼ MS + 5 g l^{-1} sucrose, *in vitro* plants became hyperhydric (Figure 11 B; Saher *et al.*, 2003; Kevers *et al.*, 2004) and remained in this state for the duration of the minimal growth storage period.

After being stored for 8 weeks under minimal growth conditions, individual shoots from both storage media were retrieved and transferred onto shoot multiplication medium for 4 weeks to determine recovery, which was assessed as number of shoots/explant. The shoots retrieve from both storage media began to multiply within 2 weeks of being on the shoot multiplication medium.

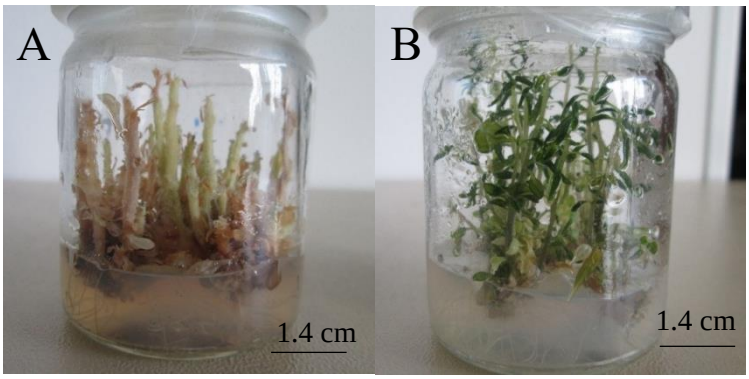


Figure 11: *In vitro* shoots of *Solanum nigrum* on ⅓ MS + 10 g l^{-1} sucrose (A) and ¼ MS + 5 g l^{-1} sucrose (B) displaying hyperhydricity after 8 weeks of storage in a growth room at 16 h light (200 µmol m^{-2} s^{-1})/ 8 h dark photoperiod, at 25 and 23 °C, respectively.

After 4 weeks on shoot multiplication medium, the selected genotypes that had been previously stored on ⅓ MS + 10 g l^{-1} sucrose showed significant differences in the number of shoots/explant, in that G20 (10 ± 1.5) and G15 (13 ± 1.9) were significantly higher than those of G5 (5 ± 0.8), G6 (4 ± 0.6), G43 (5 ± 0.5) and G50 (5 ± 0.3) (Figure 12). In contrast, there were no significant differences in the number of shoots/explant amongst the selected genotypes that had been stored on ¼ MS + 5 g l^{-1} sucrose (Figure 12).

The G6 (9 ± 2.4 shoots), G43 (12 ± 1.4 shoots), G45 (13 ± 0.8 shoots) and G50 (9 ± 0.9 shoots) explants that were on ¼ MS + 5 g l^{-1} sucrose (Figure 13 A) produced significantly higher numbers of shoots/explant than those on ⅓ MS + 10 g l^{-1} sucrose (4 ± 0.6; 5 ± 0.5; 7 ± 1.1 and 5 ±0.3 shoots, respectively) (Figure 14). The genotypes that were stored on ¼ MS + 5 g l^{-1} sucrose were, therefore, chosen and successfully elongated (Figure 13 B), rooted (Figure 13 C) and acclimatised (Figure 13 D). All shoots rooted and survived acclimatisation. The results from the present study showed that ¼ MS containing 5 g l^{-1} sucrose was an appropriate medium to store *S. nigrum in vitro* cultures for a minimum of 8 weeks without sub-culturing, while retaining the ability of these shoots to produce whole plants.

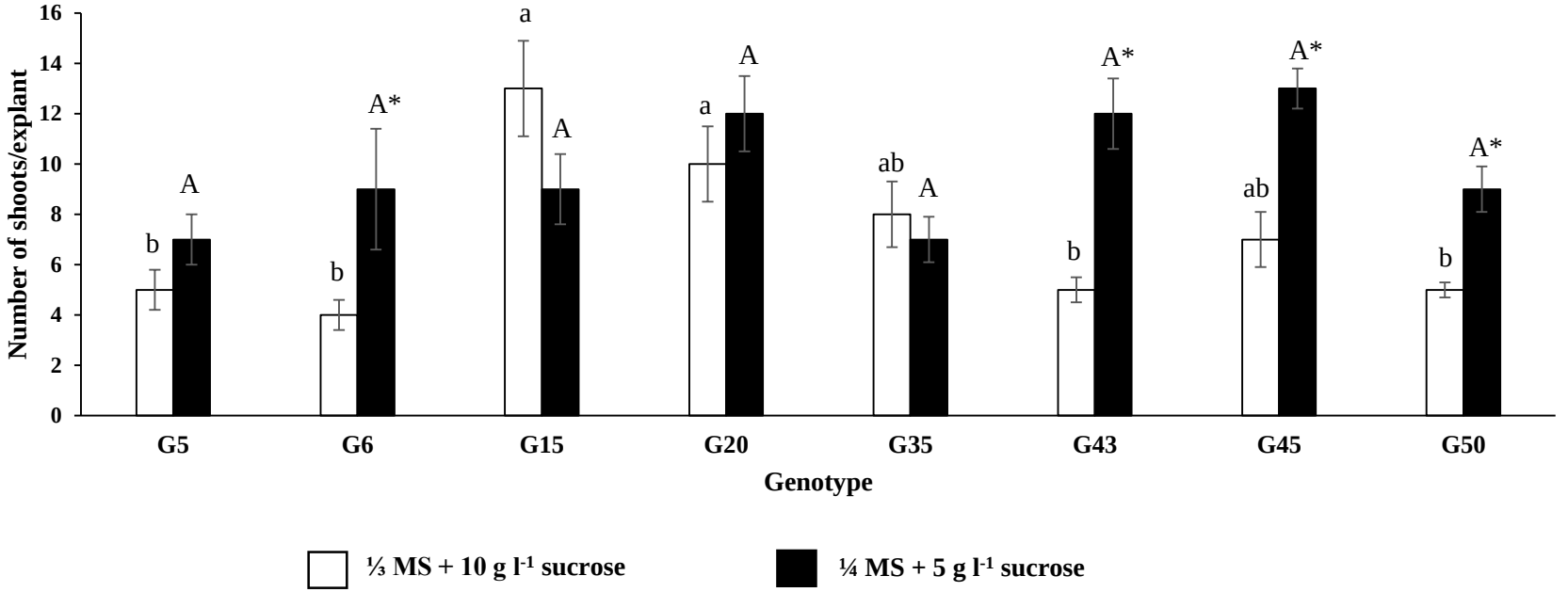


Figure 12: The effect of shoot multiplication medium containing 3 mg l^{-1} BAP on the number of shoots/explant of the selected genotypes of *Solanum nigrum in vitro* shoots after 8 weeks of minimal growth storage in either ⅓ MS + 10 g l^{-1} sucrose or ¼ MS + 10 g l^{-1} sucrose. Data were analysed using a one-way ANOVA and a post-hoc Tukey test (mean ± SE, $p \leq 0.05$, n=8). Lowercase letters denote a statistical significance across genotypes recovered from ⅓ MS + 10 g l^{-1} sucrose and uppercase letters denote a statistical significance across genotypes recovered from ¼ MS + 5 g l^{-1} sucrose; * indicates a statistical significance between the two media types for each genotype.

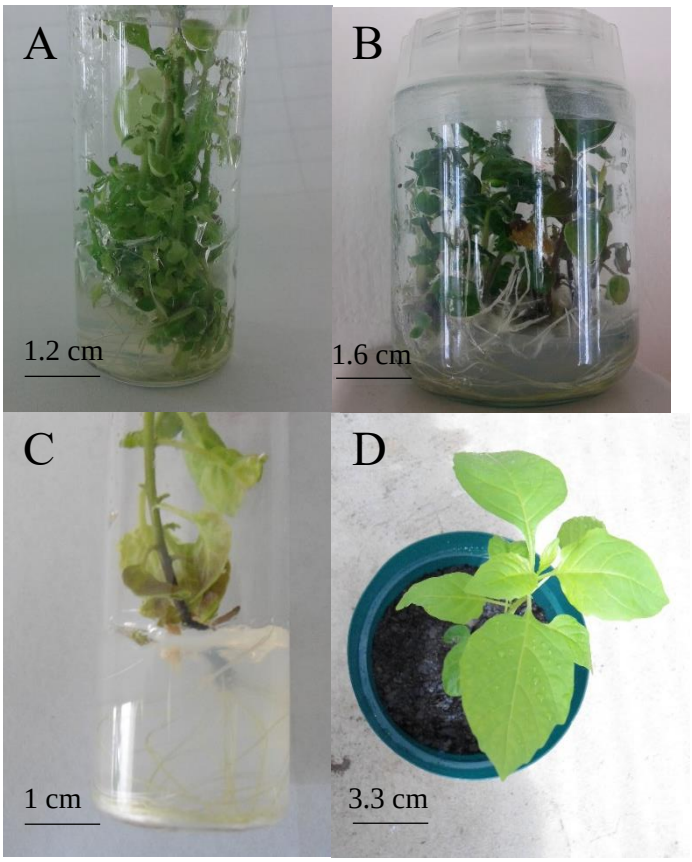


Figure 13: Direct organogenesis of *Solanum nigrum* shoots, derived from minimal growth media containing ¼ MS + 5 g l^{-1} sucrose after 8 weeks. (A) *In vitro* shoots after 4 weeks on shoot multiplication medium containing 3 mg l^{-1} BAP. (B) *In vitro* shoots after 1 week on elongation medium containing 0.1 mg l^{-1} BAP. (C) Rooted *in vitro* shoots after 3 weeks on hormone-free MS medium. (D) A plant after 4 weeks acclimatisation.

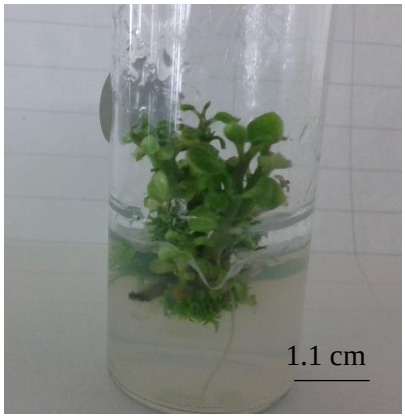


Figure 14: *In vitro Solanum nigrum* shoots, derived from minimal growth media containing ⅓ MS + 10 g l^{-1} sucrose after 4 weeks on shoot multiplication medium containing 3 mg l^{-1} BAP.

5. Discussion and conclusions

In South Africa many poor and rural communities are deficient in important micronutrients such as calcium (Ca) and iron (Fe) due to their diet being mainly cereal-based (Faber and Wenhold, 2007; Labadarios *et al.,* 2008; Faber *et al*., 2010; Berti *et al.,* 2014; Njume *et al.,* 2014; Mchiza *et al.*, 2015; van der Hoeven *et al.*, 2015; Chakona and Shackleton, 2017; Ngoroyemoto *et al.,* 2017). Some communities supplement their diet by consuming the leaves of African leafy vegetables (ALVs) (Steyn *et al.*, 2001; Aphane *et al*., 2003; Oniang'o *et al.,* 2004; Faber and Wenhold, 2007; Uusiku *et al.,* 2010; Ebert, 2014; Njume *et al.,* 2014; Seeiso and Materechera, 2014; Bvenura and Afolayan, 2015; Byrnes *et al.*, 2017; Ngoroyemoto *et al.,* 2017) because they are easily obtained (collected from the wild or cultivated in gardens) and are rich sources of micronutrients (Shukla *et al.,* 2006; Vorster *et al*., 2007). However, in a plant population, different genotypes differ in their levels of micronutrients (Beebe *et al.*, 2000 [*Phaseolus vulgaris* L.]; Kopsell *et al.,* 2005 [*Brassica oleracea*]; Shukla *et al.,* 2006 [*Amaranthus sp.*]; Modi, 2007 [*Amaranthus sp.*]; Pandino *et al.,* 2011 [*Cynara cardunculus* var. *scolymus*]; Lewu *et al.,* 2012 [*Brassica oleracea*]; Baloyi *et al.,* 2013 [*Lablab purpureus*]; Kachiguma *et al.,* 2015 [*Amaranthus sp.*]; Byrnes *et al.,* 2017 [*Amaranthus sp.*]). Micropropagation techniques can be used as tools to clone ALV genotypes with high levels of important micronutrients (Dubois, 2009; Tadele and Assefa, 2012).

In this study, *Solanum nigrum* was of particular interest as it is one of many ALVs that are consumed by communities in SA (Steyn *et al*., 2001; Jansen van Rensburg *et al.*, 2007; Bvenura and Afolayan, 2014a, b; Ngoroyemoto *et al.,* 2017). This plant has been previously micropropagated and protocols from the previous studies were adapted in the present study to establish a micropropagation protocol for *S. nigrum* using nodal and leaf explants from greenhouse-germinated seedlings and locally grown field plants. In addition, a population of fifty 6-week old seedling plants was screened using inductively coupled plasma-optical emission spectrometry (ICP-OES) for the determination of leaf Ca and Fe levels (Figures 8 and 9). Amongst the fifty screened genotypes, there was variation in the amounts of Ca (331.05-916.30 mg 100 g^{-1} dry mass [DM]) and Fe (0.64-14.95 mg 100 g^{-1} DM). Based on these results, two genotypes each were selected for high Ca (G5 and G20) and Fe (G6 and G15) or low Ca (G43 and G45) and Fe (G35 and G50) (hereafter referred to as selected genotypes). The established micropropagation protocol was applied to leaf explants of these selected genotypes

to determine its effect on their *in vitro* and *ex vitro* growth responses. In addition, the effects of substrate type and physiological age on the Ca and Fe levels of the clones were investigated.

5.1 Establishment of a direct organogenesis protocol for *Solanum nigrum* and its application to the selected genotypes

There are numerous reports for the *in vitro* micropropagation of *S. nigrum* using direct (Hassanein and Soltan, 2000; Kannan *et al.,* 2006; Bhat *et al.*, 2010; Pandhure *et al.*, 2010; Sundari *et al.,* 2010; Verma *et al.,* 2010; Sridhar and Naidu, 2011b; Kavitha *et al.*, 2012; Padmapriya *et al.,* 2011; Rathore and Gupta, 2013; Sarethy *et al.*, 2014) and indirect (Harn, 1972; Hassanein and Soltan, 2000; Kolar *et al.*, 2008; Pandhure *et al.*, 2010; Swain *et al.,* 2010) organogenesis. Based on direct organogenesis protocols reported by Sridhar and Naidu (2011b) and Kavitha *et al.* (2012), the present study set out to establish a micropropagation protocol for populations of *S. nigrum* grown around Durban, South Africa, using nodal and leaf explants from seedling and field-derived plants. Direct organogenesis was used to propagate the explants because true-to-type clones were the desired result. In this pathway of morphogenesis, shoot production proceeds without a callus phase, plantlet production occurs over a shorter period of time, albeit with a lower yield, than that of indirect organogenesis and chances of there being somaclonal variation in the clones are less likely (Karp, 1994; Hartmann *et al.*, 1997; George *et al.,* 2008; Bairu *et al.,* 2011; Acquaah, 2012).

Obtaining aseptic explants is an important prerequisite to the success of any micropropagation protocol (George *et al.,* 2008). In studies on *S. nigrum*, many workers have decontaminated nodal and leaf explants using mercuric chloride (Kolar *et al.,* 2008; Pandhure *et al.,* 2010; Sundari *et al.,* 2010; Verma *et al.*, 2010; Padmapriya *et al.,* 2011; Sridhar and Naidu, 2011a, b; Kavitha *et al.*, 2012; Rathore and Gupta, 2013) but this chemical was not tested in the current study as it is hazardous to humans (carcinogenic) and toxic to explants (Hammond *et al.,* 2014; Wee *et al.,* 2015; Mahmoud and Al-Ani, 2016). Therefore, in the present study, the nodal and leaf explants from seedling and field-derived plants were decontaminated with 1 % (v/v) NaOCl for 10 min. While 80 and 47 % of leaf explants from seedling and field parents, respectively, were successfully decontaminated, nodal explants had to be further treated with Methylene blue®, fungicides and antibiotics (Table 8). However, these treatments were not effective in reducing the fungal and bacterial contamination on the nodes and no further attempts of decontaminating the nodes were pursued. These findings were consistent with those

of a study by Wee *et al.* (2015), who found that the nodal explants of *Sauropus androgynous* (an underutilised leafy vegetable) were more contaminated than leaf explants after being treated with 70 % (v/v) ethanol for 1 min and 20 % NaOCl for 20 min.

Shoots were induced from both the nodal and leaf explants when cultured onto shoot multiplication medium containing 3 mg l^{-1} benzylaminopurine (BAP). However, at the end of this stage, leaf explants from seedling and field plants produced more shoots/explant (20 and 50 shoots, respectively) than the nodes (1 and 1-2 shoots, respectively) (Tables 8 and 9). Other workers, who also used 3 mg l^{-1} BAP to multiply *S. nigrum*, found that leaf segments produced 18 shoots/explant (Sridhar and Naidu, 2011b) whereas nodes produced 2 (Sundari *et al*., 2010) and 8 (Kavitha *et al.,* 2012) shoots/explant. The result reported by Kavitha *et al.* (2012) was higher than that of the present study and the differences in the results might be due to differences in genotype (Gubis *et al.*, 2003; George *et al.,* 2008; Latif *et al.*, 2014; Kareem *et al.,* 2016) and geographical location (Hartmann *et al.*, 1997; Zhao *et al.,* 2013; Gashi *et al.*, 2015; Kareem *et al.*, 2016).

Subsequent to these comparisons, the *in vitro* shoots obtained from leaf explants of seedlings and field plants were selected to investigate rooting on media containing low (*viz.* 1 mg l^{-1} indole-3-acetic acid [IAA] and 0.5 mg l^{-1} indole-3-butyric acid [IBA]) or no plant growth regulators (PGRs). After 3 weeks on the three tested rooting media, no significant differences were observed in the rooting percentage of shoots obtained from both the seed- and field-derived explants (Table 9). However, high percentages of the shoots, produced from seedling and field-derived leaf explants, rooted- 80 ± 0.0 and 75 ± 10.0 % on hormone-free MS medium, 95 ± 5.0 and 80 ± 0.0 % on 1 mg l^{-1} IAA and, 95 ± 5.0 and 75 ± 19.1 % on 0.5 mg l^{-1} IBA, respectively). In other studies on *S. nigrum*, researchers found that the *in vitro* shoots were able to root on a range of concentrations of IBA, IAA and other auxins (Hassanein and Soltan, 2000; Kolar *et al.,* 2008; Bhat *et al.,* 2010; Pandhure *et al.,* 2010; Sundari *et al.,* 2010; Sridhar and Naidu, 2011a, b). The findings of the current study and those of the other studies on *S. nigrum* suggest that the *in vitro* shoots had sufficient endogenous growth hormones to facilitate root morphogenesis. After 2 weeks acclimatisation in soil, 93-100 % and 19-59 % of the clones regenerated from seed- and field-derived leaf explants, respectively, survived acclimatisation and subsequently adapted well to the *ex vitro* environment and showed no morphological abnormalities.

Leaf segments from seedling plants were chosen for all the subsequent investigations because they produced a higher post-acclimatisation yield range over a shorter period of micropropagation (25 ± 0.5, 30 ± 0.6 and 28 ± 0.6 plants/explant after rooting on hormone-free MS medium, 1 mg l^{-1} IAA and 0.5 mg l^{-1} IBA, respectively, after 10 weeks of micropropagation) than field-grown plants (1 ± 0.3, 2 ± 0.3 and 2 ± 0.3 plants/explant after rooting on hormone-free MS medium, 1 mg l^{-1} IAA and 0.5 mg l^{-1} IBA, respectively, after 14 weeks of micropropagation) (Table 9). The main reasons for field-grown plants producing a low post-acclimatisation yield range (1-2 plants/explant) were the losses of leaf explants due to necrosis (40 ± 5.1 %) during shoot multiplication and the low survival percentages of the plantlets previously rooted on hormone-free MS medium (19 ± 8.1 %), 1 mg l^{-1} IAA (46 ± 8.1 %) and 0.5 mg l^{-1} IBA (59 ± 12.4 %). Padmapriya *et al.* (2011), micropropagated nodal explants of the same species via direct organogenesis, reported a post-acclimatisation yield of 39 plants/explant. This result was higher than those of the present study for the leaf explants from both seedling (25-30 plants/explant) and field (1-2 plants/explant) plants. The differences were likely due to using different types of explants and PGR concentrations (Mutlu and Turker, 2008; Sharma and Nautiyal, 2009; Charaya *et al*., 2011; Grozeva and Velkov, 2014). For a closely related species, *Solanum americanum*, Connor-Sánchez *et al.* (2010) reported that the leaves of seedling plants (micropropagated using direct organogenesis) produced a post-acclimatisation yield of approximately 20 plants/explant, i.e. lower than that obtained for seedling-derived leaf explants in the current study. The protocol used to micropropagate the seedling-derived leaf explants in the present study was consequently considered adequate for the main purpose of this study. In conclusion, the established protocol was to decontaminate leaf explants using 1 % (v/v) NaOCl with Tween 20® for 10 min followed by three 30 s rinses in sterile distilled water. They were then cultured onto 3 mg l^{-1} BAP for 4 weeks to induce and multiply shoots, followed by a week on 0.1 mg l^{-1} BAP to elongate them, then left for 3 weeks on hormone-free MS medium for rooting. However, the plantlets were acclimatised in 1 soil : 2 vermiculite (1S : 2V) instead of soil for 2 weeks in a growth room with a 16 h light (200 µmol m^{-2} s^{-1})/ 8 h dark photoperiod, at 25 and 23 °C, respectively.

Eight genotypes (i.e. G5, G6, G15, G20, G35, G43, G45 and G50) were selected for further investigations based on determinations of the leaf Ca and Fe levels of a population of fifty seedling plants (Figures 8 and 9). Leaf segments of the selected genotypes were micropropagated using the established direct organogenesis protocol to determine its effect on their *in vitro* growth responses. According to Hartmann *et al.* (1997) and George *et al.* (2008),

the genotype is a key factor that influences the response of an explant to *in vitro* morphogenesis. In the current study, this was evident in the post-acclimatisation yield (i.e. number of plants/explant) of the clones of the selected genotypes- that of G43 (32 ± 3.6) was significantly higher than those of G5 (13 ± 2.1), G6 (16 ± 1.8), G20 (16 ± 2.0), G35 (11 ± 1.6) and G50 (14 ± 1.6). The significant differences identified in the percentage of explants with shoots and number of shoots/explant amongst the selected genotypes during the shoot multiplication stage were the main reasons for the differences in their post-acclimatisation yield (Table 11). For example, after 4 weeks on the shoot multiplication medium, the G50 explants had significantly the lowest percentage of explants with shoots (25 %). Even though the percentage of explants with shoots was not statistically different amongst the other tested genotypes, 75 % of the G5, G35, and G45 explants, 83 % of the G15, G20 and G43 explants and 92 % of the G6 explants produced shoots. Regarding the number of shoots/explant, that of G43 (51 ± 5.8) was significantly the highest; those of the other tested genotypes ranged from 22-45 shoots. In agreement with the findings of the present study, the effect of genotype on *in vitro* morphogenesis has been reported by other researchers for vegetables such as *Brassica oleracea* (Pua *et al.*, 1999; Cosic *et al.*, 2015), *Cucumis sativum* (Grozeva and Velkov, 2014), *Lactuca sativa* (Xinrun and Conner, 1992; Ampomah-Dwamena *et al.*, 1997; Mohebodini *et al*., 2011; Latif *et al*., 2014), *Lycopersicon esculentum* (Gubis *et al*., 2003; Jabeen *et al.*, 2005; Grozeva *et al*., 2006; Aazami *et al.*, 2010; Liza *et al*., 2013), *Raphanus sativus* (Takahata *et al.*, 1996), *Solanum melongena* (Sharma and Rajam, 1995; Dhatt *et al.*, 2011; Kaur *et al.*, 2015; Muktadir *et al*., 2016), *Spinacia oleracea* (Shojaei *et al.*, 2010) and *Telfairia occidentalis* (Adesoye *et al.*, 2012).

Since plants are sessile, they have to adapt to their ever-changing environment in order to acquire resources (i.e. water and nutrients) (Gratani, 2014). Phenotypic plasticity occurs when a genotype expresses different phenotypes in response to its environment and is one of many strategies that a plant uses to cope with environmental changes (Gratani, 2014). In the present study, some growth measurements (number of branches and leaves, shoot and root lengths and, shoot and root fresh and dry masses) of the clones of the selected genotypes (i.e. G5, G6, G15, G20, G35, G43, G45 and G50) were recorded at the start of acclimatisation (w_0) and after 6 weeks *ex vitro* (w_6) growth (which included 2 weeks acclimatisation) in soil or in 1S : 2V to assess the effect of growth period and substrate on their *ex vitro* growth. In both the tested substrates, the shoot and root lengths, and fresh and dry masses of clones of most of the selected genotypes were affected by growth period (Tables 13-15). The exceptions were the number of

branches and leaves and root:shoot dry masses, i.e. there were no significant increases with regards to these parameters over time (Tables 12 and 16). Bvenura and Afolayan (2014c) assessed the growth responses (shoot length, number of leaves and root:shoot) of *S. nigrum* seedlings to organic and/or inorganic fertilisers when grown under field conditions and found that the growth increased with advancing plant age.

Substrate type is one of the most important factors (apart from genotype) that affects plant growth and development (Jankauskienė *et al.*, 2015) and many researchers have reported significant effects of substrate type on the growth of vegetables (Ayalew, 2014 [*Zea mays* and *Phaseolus vulgaris*]; Jankauskienė *et al.*, 2015 [*L. esculentum*]; Zucco *et al.*, 2015 [*Lycopersicon esculentum*]; Ohshiro *et al.*, 2016 [*Amaranthus* sp.]). In the present study, the shoot growth of only two (i.e. G5 [length and fresh mass] and G20 [number of leaves, and fresh and dry masses]) of the eight cloned genotypes was significantly affected by substrate type, i.e. greater in 1S : 2V than in soil (Tables 12-15).

Regarding the root parameters, those of seven of the eight cloned genotypes were affected by substrate type. This was possibly due to root growth having more phenotypic plasticity than the shoot growth because the roots needed to branch out in the substrate to acquire nutrients and water (Topp, 2016). The root lengths of the G5, G20, G35, G43, G45 and G50 clones were significantly greater in 1S : 2V (391 ± 46.1, 359 ± 52.5, 322 ± 45.3, 290 ± 23.0, 273 ± 18.7 and 294 ± 14.2 mm, respectively) than in soil (204 ± 30.7, 221 ± 30.5, 192 ± 21.8, 190 ± 33.0, 208 ± 13.6 and 213 ± 32.9 mm, respectively). The same trend was found in the root fresh masses of the clones of G5 (0.6 ± 0.2 g in soil and 4.4 ± 0.8 g in 1S : 2V) and G20 (2.2 ± 0.6 g in soil and 6.3 ± 0.8 g in 1S : 2V) and root dry masses of the G5 clones (0.4 ± 0.1 g in soil and 0.7 ± 0.1 g in 1S : 2V). The opposite trend was found in the dry masses of the G6, G43 and G45 clones (Table 15). The above findings were similar to that of Zucco *et al.* (2015) in which the root dry mass of *Lycopersicon esculentum* was significantly affected by soil type and vermicompost applications. Although the shoot and root parameters of the clones of some of the selected genotypes were affected by substrate type, the root:shoot dry masses of only the G6 clones were significantly affected by substrate type (i.e. 3.2 ± 0.7 in soil and 0.7 ± 0.2 in 1S : 2V), i.e. more growth was invested in the roots of the clones grown in soil than those in 1S : 2V.

5.2 Comparing the levels of leaf Ca and Fe of the selected genotypes with those of their clones and the effect of substrate type on the leaf Ca and Fe contents of the clones

Micropropagation is a method of mass multiplying plants with desired characteristics (Hartmann *et al.,* 1997; George *et al.,* 2008; Singh *et al.* 2013; Ncube *et al.,* 2015). In the present study, genotypes that were chosen on the basis of them having high Ca (G5 and G20), high Fe (G6 and G15), low Ca (G43 and G45) and low Fe (G35 and G50) were micropropagated using the established protocol. To determine if the desired characteristics (i.e. high or low Ca and Fe) of the parent plants were maintained by their clones, the leaf Ca and Fe levels of the 6 week-old clones (including 2 weeks acclimatisation) of G5, G6, G15, G20, G35, G43, G45 and G50, grown in soil, were compared with those of their 6 week-old seedling parents also grown in soil. The occurrence of somaclonal variation in the clones of the selected genotypes was not tested. However, precautions were taken to ensure that somaclonal variation did not occur in the clones, i.e. the selected genotypes were micropropagated using direct organogenesis and, only one PGR (*viz.* BAP) at low concentrations (i.e. 0.1 and 3 mg l^{-1} BAP) was used.

The results showed that only the Ca content of G43 (331 ± 29 mg 100 g^{-1} DM) and the Fe level of the G35 (3.0 ± 0.3 mg 100 g^{-1} DM) parents and clones (543 ± 147 and 5 ± 1.1 mg 100 g^{-1} DM, respectively) were the same (Figure 10). The Ca levels of two (G5 and G20) of the four tested genotypes (i.e. G5, G20, G43 and G45) were significantly lower than those of their respective parents, while that of only one genotype (G45) was significantly higher than its parent. A similar trend was found regarding leaf Fe content: significantly lower leaf Fe levels were found in the G6 and G15 clones than in those of their parents whereas the opposite was found for G50.

As with the present study, differences in tested traits between conventionally grown and micropropagated plants have been reported by other researchers (Ferdausi *et al.,* 2009; Radhakrishnan and Kumari, 2009; Fadel *et al.,* 2014; Makowczynska *et al.,* 2015), who proposed that their findings were possibly due to the influence of the environmental conditions of the stages of micropropagation (e.g. the PGR used and medium composition). In a study using two genotypes of *Solanum melongena*, Ferdausi *et al.* (2009) found that the levels of Fe of the micropropagated plants of each genotype (0.5531 and 0.5007 ppm) were two times higher than those of seedling plants of the two tested genotypes (0.2015 and 0.2509 ppm).

Radhakrishnan and Kumari (2009), compared the levels of protein in the leaves, roots, stems and fruits of conventionally grown (30.7, 21.2, 42.7 and 46.8 mg g^{-1} DM, respectively) and cloned (40.3, 22.7, 32.4 and 33.7 mg g^{-1} DM, respectively) soybean plants (*Glycine max*) (a herbaceous annual). They found that the leaves and roots of the micropropagated plants produced more protein than those of the conventionally grown plants, whereas the opposite was true for the stems and fruit.

In addition to growing the clones of the selected genotypes in soil, they were also grown in 1 soil : 2 vermiculite (1S : 2V) to determine if they exhibited the same levels of Ca and Fe as their parents when kept in a low nutrient substrate. Six of the eight cloned genotypes had similar or significantly higher levels of Ca or Fe than their parents (Figure 10). There were similar amounts of Ca between the G5 and G20 parents and their clones. However, significantly higher Ca levels were found in the G43 (768 ± 16 mg 100 g^{-1} DM) and G45 (619 ± 67 mg 100 g^{-1} DM) clones compared to their respective parents (331 ± 29 mg 100 g^{-1} DM for G43 and 333 ± 4 mg 100 g^{-1} DM for G45). With regards to Fe content, this was true for the clones of G35 and G50. In the context of the present study, the results suggested that the G5, G20, G35, G43, G45 and G50 clones grew better in 1S : 2V than in soil.

Subsequent to these comparisons, four of the tested genotypes (*viz.* G15, G20, G35 and G43) were chosen to investigate the effect of physiological age on their leaf Ca and Fe contents when grown in soil. After comparisons between the 6 week-old parents and their clones at each tested physiological age (4, 6 and 8 weeks of *ex vitro* growth [which included 2 weeks acclimatisation]), similarities were only found with regard to Ca content (Table 25), i.e. at all tested physiological ages, the Ca levels of the G20 clones (843 ± 68, 675 ± 57 and 674 ± 74 mg 100 g^{-1} DM, respectively) and its parent (864 ± 5 mg 100 g^{-1} DM) were the same. At 4 weeks, this was true for the Ca content of the G43 parent (331 ± 29 mg 100 g^{-1} DM) and its clones (591±74 mg 100 g^{-1} DM).

The significant differences in the Ca (G20>G43) and Fe (G15>G35) contents of the 6 week-old parent genotypes were evident (Figures 8 and 9). These were compared with the 'rankings' of their clones that were established at 4, 6 and 8 weeks *ex vitro* in soil (Table 25). There were differences in the Fe 'ranking' between the parents (G15 and G35) and their clones at all tested physiological ages (Table 27). This was likely due to the effect of micropropagation on Fe levels of the cloned genotypes. With regards to Ca content, a similarity (i.e. G20>G43) in the

'ranking', was only found at 4 weeks *ex vitro*. This suggests that, in addition to micropropagation, physiological age also had an effect on the Ca 'rankings' (Table 27).

Substrate type is known to affect the micronutrient contents of plants (Makus and Lester, 2002 [*Brassica junc*ea]; He *et al.*, 2003 [*Cucumis sativus* L.]; Nurzyński, 2013 [*Lycopersicon. esculentum*]; Ohshiro *et al.,* 2016 [*Amaranthus sp.*]). This is due to differences in the availability of micronutrients in the different substrates, differences in the ability of the roots to absorb them from the substrate and differences in the metabolism of micronutrients within the plant (Makus and Lester, 2002, Morgan and Connolly, 2013; Shukla *et al.,* 2014; Afolayan and Bvenura, 2016). The present study set out to determine the effect of two substrates (i.e. soil and 1S : 2V) on the leaf Ca and Fe levels of the 6 week-old clones of the selected genotypes (i.e. G5, G6, G15, G20, G35, G43, G45 and G50) and it was found that those of the clones of five of the eight selected genotypes (i.e. G6, G15 G35, G43 and G45) were not affected by substrate type (Figure 10). The clones of G5 and G20 grown in 1S : 2V (811 ± 66 and 875 ± 52 mg 100 g^{-1} DM, respectively) had significantly higher Ca levels than those kept in soil (437 ± 113 and 332 ± 62 mg 100 g^{-1} DM, respectively). This trend was also found for the shoot lengths (90 ± 10.4 mm in soil and 143 ± 8.5 mm in 1S : 2V) and shoot fresh masses (0.8 ± 0.2 g in soil and 1.7 ± 0.1 g in 1S : 2V) of the G5 clones and the number of leaves (12 ± 1.0 in soil and 22 ± 2.1 in 1S : 2V) and, shoot fresh (1.2 ± 0.3 g in soil and 2.7 ± 0.2 g in 1S : 2V) and dry masses (0.2 ± 0.0 g in soil and 0.5 ± 0.0 g in 1S : 2V) of the G20 clones. The clones of G50 had a significantly higher concentration of Fe in soil (10 ± 1.5 mg 100 g^{-1} DM) than in 1S : 2V (5 ± 1.0 mg 100 g^{-1} DM). These comparisons showed that the accumulation of both Ca and Fe of the different genotypes was affected by substrate type differently.

Subsequent to the above comparisons, four of the selected genotypes (*viz.* G15, G20, G35 and G43) were chosen for another study to determine the effect of substrate type on their Ca and Fe levels at different physiological ages (i.e. at 4, 6 and 8 weeks *ex vitro*). At all tested physiological ages, the Ca contents of the G20 and G43 clones were not significantly affected by substrate type (Table 25). Regarding Fe content, this was true for the clones of G35. The Fe levels of the G15 clones were significantly affected by substrate type at 8 weeks *ex vitro* only (4.1 ± 0.2 mg 100 g^{-1} DM in soil and 5.2 ± 0.3 mg 100 g^{-1} DM in 1S : 2V). This was also observed at 8 weeks *ex vitro* for the root:shoot dry masses of the clones of G15 (0.6 ± 0.0 in soil and 0.9 ± 0.1 in 1S : 2 V). Due to the G15 clones accumulating a greater root:shoot dry mass in 1S : 2V than in soil at 8 weeks *ex vitro*, the roots of the clones grown in 1S : 2V were

able to absorb more Fe for accumulation in the leaves. The interaction between genotype and substrate type had no significant effects on the Ca and Fe levels of the clones of the selected genotypes (i.e. G15, G20, G35 and G43), even though their shoot growth was significantly affected by this interaction (Tables 19-21). Hence, the clones of these genotypes exhibited less plasticity in their Ca and Fe contents than in their shoot growth. According to Bhargava *et al.* (2005), Bhargava *et al.* (2008) and Edugbo *et al.* (2015), it is important to select plants that show little-to-no variation for a desired trait when grown in different environments (in this case different substrates) because they can prove to be useful to plant breeders due to their consistency for a given trait. In this regard, the some of the selected genotypes (such as G15, G35 and G43) can prove to be of value in this research programme and further investigations must be done to evaluate the effect of different environmental conditions on the Ca and Fe levels of their clones. This can be done by growing the clones under different *ex vitro* conditions (greenhouse, shadehouse, glasshouse and in the field) and testing the effect of different light intensities, watering regimes, fertiliser treatments and soil pH on the growth and Ca and Fe contents of the clones.

5.3 The effect of physiological age on the growth and leaf Ca and Fe contents of the clones of the selected genotypes

Solanum nigrum is usually consumed when the plant reaches maturity at approximately 6 weeks (Edmonds and Chweya, 1997; Kremer and Lotz, 1998; Jimoh *et al.*, 2010), but information on the levels of leaf Ca and Fe as the plant ages is limited (Chweya, 1997; Bvenura and Afolayan, 2014a, b). In the present study, the effect of physiological age on some growth parameters (number of leaves, shoot and root fresh and dry masses and root:shoot) and, leaf Ca and Fe contents were investigated using clones of G15 (high Fe), G20 (high Ca), G35 (low Fe) and G43 (low Ca) grown in both soil and 1 soil : 2 vermiculite (1S : 2V).

In a study by Bvenura and Afolayan (2014c), the effects of organic and/or inorganic fertilisers on the growth responses (shoot length, number of leaves and root:shoot) of *S. nigrum* were assessed when seedlings were grown under field conditions over 12 weeks. They found that, as expected, the growth of the seedlings increased with advancing plant age. In the present study, the growth of each tested parameter (i.e. number of leaves, shoot and root fresh and dry masses and root:shoot dry mass) was affected differently by genotype (Tables 19-23). For example, when the G15 clones were grown in both the tested substrates, the number of leaves

and their shoot fresh masses were not affected by advancing plant age but their shoot dry masses and root fresh and dry masses significantly increased with increasing physiological age. For the clones of G20, when grown in soil, the number of leaves of this genotype remained the same over time but the shoot and root fresh and dry masses increased significantly. When the G20 clones were grown in 1S : 2V, significant increases in the number of leaves and, root fresh and dry masses were found over time while no significant differences over time were noted for the shoot fresh and dry masses. According to Harris (1992), a reduction in the root:shoot is an indication of favourable growing conditions. In the present study, this was only found in the root:shoot dry mass of the G15 (1.1 ± 0.1 at 4, 0.7 ± 0.1 at 6 and 0.6 ± 0.0 at 8 weeks *ex vitro*) and G20 (1.5 ± 0.0 at 4, 0.7 ± 0.1 at 6 and 0.9 ± 0.2 at 8 weeks *ex vitro*) clones when grown in soil. The root:shoot dry masses of the G15 and G20 clones in 1S : 2V and, G35 and G43 clones in both the tested substrates remained constant with advancing plant age (Table 24).

The effects of genotype, substrate type and physiological age on the shoot and root growth of the cloned genotypes were not independent of each other. Rather, they interacted with each other to elicit synergistic effects on the growth of the clones of the different genotypes. The number of leaves and, shoot and root fresh and dry masses of the clones of the selected genotypes were influenced by the genotype x substrate type interaction. This indicates that there will be plasticity in the growth (in terms of the tested shoot and root parameters) of the clones of each genotype when kept in different types of substrates. The number of leaves and the fresh masses of the shoots and roots of the clones, were significantly affected by the interaction between each genotype and the tested physiological ages. The combined interaction of the genotype, substrate type and physiological age significantly affected only the shoot fresh and dry masses and root fresh masses of the clones.

At all tested physiological ages (i.e. at 4, 6 and 8 weeks *ex vitro*) and in both the tested substrates (*viz.* soil and 1S : 2V), the levels of leaf Ca of the G20 and G43 clones did not change significantly (Table 25). This trend was similar to those of the root:shoot dry masses of the G20 clones in 1S : 2V and the G43 clones in both tested substrates (Table 24). In soil, the consistency in the Ca content of the G20 clones over time was independent of their root:shoot which decreased over time (1.5 ± 0.0 at 4, 0.7 ± 0.1 at 6 and 0.9 ± 0.2 at 8 weeks). The trend found for the Ca levels of the G20 and G43 clones was in contrast to the findings of Chweya (1997) and Bvenura and Afolayan (2014a). The former reported that leaf Ca levels increased with physiological age in *S. nigrum* from weeks 3 (245.3 mg 100 g^{-1} DM) to 11 (446.7 mg 100

g^{-1} DM). This trend was also found by the latter researchers when *S. nigrum* plants were grown for 9 weeks in a glasshouse and 12 weeks in the field. Chweya (1997) only reported on the results found for the nutrient content of *S. nigrum* leaves and did not supply information on the conditions that the plants were grown under, i.e. whether they were grown in the field, greenhouse or glasshouse and the substrate used to grow them nor did they give explanations as to why Ca content increased over time. However, Bvenura and Afolayan (2014a) attributed their findings to the immobile nature of Ca and its inability to translocate within a plant as it grows (Loneragen, 1968; Khader and Rama, 2003). It should be noted that the present study was carried out using micropropagated plants that were housed in a greenhouse as opposed to that of Bvenura and Afolayan (2014a), who grew *S. nigrum* seedling plants in the field and glasshouse, which may account for the differing results. Apart from the G20 and G43 clones having similar or higher levels of Ca to their parents (Table 25), the trend found for the Ca contents in the clones of these two genotypes in the current study suggests that their leaves can be consumed at any point between 4 and 8 weeks of growth for the benefit of high Ca.

In terms of leaf Fe content, a significant decrease was recorded with increasing physiological age of the G15 clones in both the substrates and the clones of G35 in 1S : 2V. This trend must have resulted due to Fe being redistributed to newly forming plant parts (dilution effect) in preparation for flowering and fruit bearing (Khader and Rama, 1998, Makus and Lester, 2002). On the other hand, in soil, the Fe level of the G35 clones did not change significantly over time even though its root:shoot dry mass significantly decreased with advancing plant age (1.1 ± 0.1 at 4, 0.7 ± 0.1 at 6 and 0.6 ± 0.0 at 8 weeks). The trends found for the root:shoot dry masses of the G15 clones in 1S : 2V and the G35 clones in both the tested substrates (i.e. no significant changes in root:shoot over time) contrasted those found for their Fe levels over time. This suggests that, in both the tested substrates, the Fe concentrations of the G15 and G35 clones were independent of their root:shoot dry masses. With regards to the level of leaf Fe in *S. nigrum*, Chweya (1997) reported that it decreased from weeks 3 (54.4 mg 100 g^{-1} DM) to 7 (46.4 mg 100 g^{-1} DM), before significantly increasing up to week 11 (161.7 mg 100 g^{-1} DM). The reported decrease in the level of Fe from weeks 3 to 7 was consistent with the findings of this study. Bvenura and Afolayan (2014b) determined the leaf Fe contents of *S. nigrum* seedling plants every week for 9 weeks (in the glasshouse) and 12 weeks (in the field). They found no significant changes in the leaf Fe content of the glasshouse grown plants (426-435 mg kg^{-1}) over time. However, with regards to the field grown plants, they reported that Fe content decreased from 430 to 327 mg kg^{-1} (from weeks 3 to 8) prior to a significant increase (433 mg

kg^{-1} at 9 weeks). It is to be noted that the present study was carried out in a greenhouse, which may account for the different trend to those obtained by Bvenura and Afolayan (2014b). This can be a result of the different intensities of light, relative humidity and temperatures that the plants were exposed to when grown under field, greenhouse and/or glasshouse conditions. More work must be done to assess the trends (increase or decrease) of leaf Ca and Fe in different genotypes of *S. nigrum* after 8 weeks of *ex vitro* growth under different types of environmental conditions (mentioned in section 5.2) to determine the ideal growing period and conditions for the benefit of deriving high Ca and/or Fe.

5.4 Minimal growth storage of selected *Solanum nigrum* genotypes

Minimal growth storage, also known as 'slow growth', is a useful method that can be used to ensure that aseptic germplasm of genotypes of interest are readily available for micropropagation (Negash *et al.*, 2001; Rao, 2004; Bekheet, 2007; Engelmann, 2011; Joshi and Jadhav, 2013). For such a protocol to be considered efficient, the germplasm (such as shoots and calli) recovered after storage must maintain its regeneration capacity when transferred to shoot multiplication medium (Engelmann and Engels, 2002; Rao, 2004; Engelmann, 2011; Pérez-Molphe-Balch *et al.*, 2012; Cruz-Cruz *et al.*, 2013; Joshi and Jadhav, 2013). Roca *et al.* (1983) investigated the effect of various factors on the minimal growth storage of Cassava germplasm and found that storing the plants for 8 to 9 months on medium containing activated charcoal and a low osmotic concentration was able to limit growth and still ensure a high viability of shoots after this period. Tyagi *et al*. (2009) who tested the effect of various components (i.e. MS strength and concentration of agar, PGRs and sucrose) in the growth medium on minimal growth storage of *Elettaria cardamomum* (a herbaceous perennial) shoots. They found that, regardless of MS strength and sucrose concentration, the shoots retrieved after 18 months of storage regenerated (in terms of shoot and root production) within 4 weeks of being on shoot multiplication medium.

As *S. nigrum* is a short-lived seasonal plant, a constant supply of explant material from the parent plants of the selected genotypes will not always obtainable for additional studies. Hence, minimal growth storage is a useful method to ensure their availability. According to Engelman (1991), Engelman and Engels (2002) and Engelman (2011), storing germplasm under such conditions will decrease the cost of their maintenance *in vitro* by increasing the period between sub-cultures. In the present study, minimal growth was investigated to ensure a continuous

supply of aseptic germplasm of the selected genotypes (i.e. G5, G6, G15, G20, G35, G43, G45 and G50) for future studies in this research programme. As there are no published reports on the *in vitro* conservation of *S. nigrum*, the *in vitro* shoots of the selected genotypes from the shoot multiplication stage were cultured on ¼ Murashige and Skoog (MS) (1962) + 5 g l^{-1} sucrose or ⅓ MS + 10 g l^{-1} sucrose, because reducing the concentrations of the MS basal salt and sucrose in the growth medium are considered simple ways of limiting growth (Engelman, 1991; Rao, 2004; Ahmed and Anjum, 2010; da Silva and Scherwinski-Pereira, 2011; Pérez-Molphe-Balch *et al.*, 2012; Joshi and Jadhav, 2013). The shoots were stored on the two tested media for 8 weeks in a growth room (with a 16 h light (200 µmol $m^{-2} s^{-1}$)/ 8 h dark photoperiod, at 25 and 23 °C, respectively) to ensure their survival after this period.

The medium containing ¼ MS + 5 g l^{-1} sucrose was adequate to store the germplasm of the tested genotypes for 8 weeks because all shoots that were recovered were able to regenerate to form new plants. After the storage period, the retrieved shoots were successfully micropropagated. However, more work needs to be done to determine if the *in vitro* shoots of the different genotypes can be stored under the same conditions for longer than 8 weeks as this will be useful for future studies. Furthermore, as the main objectives of minimal growth storage using *in vitro* shoots and micropropagation via direct organogenesis are to produce true-to-type clones, any phenotypic variation in the clones must be investigated. This can be achieved by performing molecular analyses of the selected genotypes, the stored germplasm and the micropropagated plants using amplified fragment length polymorphism (AFLP) (Matasyoh *et al.*, 2015), inter sample sequence repeats (ISSR) (Das and Borah, 2015) or random amplification of polymorphic DNA (RAPD) (Das and Borah, 2015) analyses.

In the present study, storing the germplasm of the clones of the selected genotypes for 8 weeks using minimal growth storage eliminated the need for one sub-culture onto fresh shoot multiplication medium. Since many genotypes of *S. nigrum* were being investigated in this research programme, using minimal growth conditions to store their germplasm will decrease the cost of consumables and labour in the research laboratory.

5.5 Conclusions

1. Established *in vitro* protocol

- The established protocol involved decontamination of the leaves of 6-week old seedling plants of *Solanum nigrum* with 1 % (v/v) NaOCl with Tween 20® (10 min) followed by three 30 s rinses in sterile distilled water, then culturing on shoot multiplication medium containing 3 mg l^{-1} BAP for 4 weeks, elongation on medium containing 0.1 mg l^{-1} BAP for a week, rooting on hormone-free MS medium for 3 weeks. Acclimatisation should be performed in covered (with a polybag) pots containing 1 soil : 2 vermiculite (1S : 2V) in a growth room for 2 weeks, followed by transfer into bigger pots containing soil or 1S : 2V in a greenhouse.
- Using this protocol the post-acclimatisation yield obtained was 25 plants/explant.

2. Genotypic effects on *in vitro* and *ex vitro* growth responses of the clones of the selected genotypes

- In the *in vitro* environment, there were differences amongst the clones of the selected genotypes with regards to percentage explants with shoots and number of shoots/explant. These differences affected the post-acclimatisation yield of the clones. Amongst the clones of the selected genotypes, this protocol gave a yield range of 11-32 plants/explant.
- When grown in 1S : 2V for 6 weeks in the *ex vitro* environment, there were some significant differences in the shoot and root dry masses and the root:shoot dry masses amongst the clones of the selected genotypes. The G20 clones had significantly the highest shoot and root dry masses than the other tested genotypes. Regarding the root:shoot dry mass, that of the G15 clones was significantly higher than those of the clones of G6, G43, G45 and G50.
- In soil, there were no significant differences amongst the clones of the selected genotypes with regards to root dry masses and root:shoot dry masses. The G50 clones had a significantly higher shoot dry mass than the G5 clones.

3. Genotypic differences in the leaf Ca and Fe levels of a seedling population and comparisons of the Ca and Fe contents of the 6 week-old selected genotypes with those of their 6 week-old clones when grown in soil

- There was genotypic variation amongst a screened population of fifty 6-week old plants, regarding Ca (331.05-916.30 mg 100 g^{-1} dry mass [DM]) and Fe (0.64-14.95 mg 100 g^{-1} DM).
- When the selected parent genotypes (G5, G6, G15, G20, G35, G43 and G45) were compared to their clones, only G43 and G35 had similar Ca and Fe levels, respectively, to those of their parents.
- Hence, micropropagation affected the Ca and Fe contents of six (G5, G20 and G45 for Ca and G6, G15 and G50 for Fe) of the eight tested genotypes.

4. The effect of substrate type on the leaf Ca and Fe contents of the clones of the selected genotypes

- At 6 weeks *ex vitro*, the Ca and Fe levels of the clones of five (i.e. G6, G15 G35, G43 and G45) of the eight selected genotypes were not affected by substrate type.
- Higher amounts of Ca were found in the clones of G5 and G20 when grown in 1S : 2V than in soil. Regarding Fe, the opposite was true for the G50 clones.

5. The effect of physiological age (i.e. 4, 6 and 8 weeks *ex vitro*) on the leaf Ca and Fe contents of the clones of four (*viz.* G15, G20, G35 and G50) of the eight selected genotypes

- In both the tested substrates, the Ca contents of the G20 and G43 clones remained constant with advancing age. This was true for the Fe concentration of the G35 clones grown in soil. The Fe levels of the G15 clones in both substrates and the G35 clones in 1S : 2V decreased with increasing physiological age.

6. Storing germplasm of the selected genotypes under minimal growth conditions

- Using a medium containing ¼ MS + 5 g l^{-1} sucrose, *in vitro* shoots of the selected genotypes were stored under minimal growth conditions for 8 weeks and the recovered shoots were able to regenerate to form new plants. Hence, ¼ MS + 5 g l^{-1} sucrose was adequate to store the germplasm of the selected genotypes for future studies.

In conclusion, micropropagation affected the Ca and Fe levels of the clones of all eight selected genotypes. Physiological age had no significant affect on the Ca contents of the cloned selected genotypes but it did have an effect on their Fe levels. The interaction between genotype and physiological age affected both the Ca and Fe levels of the clones of the selected genotypes.

The Ca and Fe concentrations of the clones of most of the selected genotypes were not significantly affected by substrate type. In addition, there was no significant interaction between the genotypes and the substrate, indicating that the cloned *S. nigrum* genotypes can be grown in both soil and 1S : 2V for the benefit of high Ca and Fe.

6. References

Aazami, M.A., Torabi, M., Jalili, E., 2010. *In vitro* response of promising tomato genotypes for tolerance to osmotic stress. African Journal of Biotechnology 9, 4014-4017.

Acham, H., Oldewage-Theron, W., Egal, A.A., 2012. Dietary diversity, micronutrient intake and their variation among black women in informal settlements in South Africa: A cross-sectional study. International Journal of Nutrition and Metabolism 4, 24-39.

Achigan-Dako, E.G., Sogbohossou, O.E.D., Maundu, P., 2014. Current knowledge on Amaranthus spp.: research avenues for improved nutritional value and yield in leafy amaranths in sub-Saharan Africa. Euphytica 197, 303 – 317.

Achikanu, C.E., Eze-Steven, P.E., Ude, C.M., Ugwuokolie, O.C., 2013. Determination of vitamin and mineral composition of common leaf vegetables in south eastern Nigeria. International Journal of Current Microbiology and Applied Sciences 2, 347-353.

Acipa, A., Kamatenesi-Mugisha, M., Oryem-Origa, H., 2013. Nutritional profile of some selected food plants of Otwal and Ngai sub countries, Oyam Disctrict, Northern Uganda. African Journal of Food, Agriculture, Nutrition and Development 13, 7428-7448.

Acquaah, G., 2009. Principles of plant genetics and breeding, 2nd ed. John Wiley & Sons, UK, pp. 152-153.

Adesoye, A.I., Okooboh, G.O., Akande, S.R., Balogun, M.O., Odu, B.O., 2012. Effect of phytohormones and genotype on meristem and shoot tip culture of *Telfairia occidentalis* Hook F. Journal of Applied Biosciences 49, 3415-3424.

Afrasiab, H., Rashid, N., Akram, M., 2017. An efficient method for direct shoot regeneration from leaf explants of *Solanum nigrum* induced by thidiazuron. International Journal of Agriculture and Biology 19, 348-354.

Afolayan, A.J., Jimoh, F.O., 2008. Nutritional quality of some wild leafy vegetables in South Africa. International Journal of Food Science and Nutrition 26, 1-8.

Afolayan, A.J., Bvenura, C., 2016. Proximate and Phytate Accumulation in *Solanum nigrum* L. cultivated on fertiliser-amended soils. Communications in Soil Science and Plant Analysis 47, 1398-1416.

Agbo, A.E., Kouame, C., Anin, A.O.L., Soro, L.C., N'ZI J., Fondio, L., Gnakri, D., 2014. Seasonal variation in nutritional conpositions of spider plant (*Cleome gynandra* L.) in South Cote d'Ivoire. International Journal of Agricultural Policy and Research 2, 406-413.

Ahmed, M., Anjum, A.A., 2010. *In vitro* storage of some pear genotypes with the minimal growth technique. Turkey Journal of Agriculture 34, 25-32.

Akdemir, H., Kaya, E., Ozden, Y., 2010. *In vitro* proliferation and minimum growth storage of *Fraser photinia*: Influences of different medium, sugar combinations and culture vessels. Scientia Horticulturae 126, 268-275.

Akubugwo, I.E., Obasi, A.N., Ginika, S.C., 2007. Nutritional potential of the leaves and seeds of Black Nightshade- *Solanum nigrum* L. var *virginicum* from Afikpo-Nigeria. Pakistan Journal of Nutrition 6, 323-326.

Akubugwo, I.E., Obasi, A.N., Chinyere, G.C., Ugbogu, A.E., 2008. Mineral and phytochemical contents in leaves of *Amaranthus hybridus* L. and *Solanum nigrum* L. subjected to different processing methods. African Journal of Biochemistry Research 2, 40-44.

Aletor, V.A., Adeogun, O.A., 1994. Nutrient and antinutrient components of some tropical leafy vegetables. Food Chemistry 53, 375–379.

Ampomah-Dwamena, C., Conner, A.J., Fautrier, A.G., 1997. Genotypic response of lettuce cotyledons to regeneration *in vitro*. Scientia Horticulturae 71, 137-145.

Aphane, J., Chadha, M.L., Oluoch, M.O., 2003. Increasing the consumption of micronutrient-rich foods through production and promotion of indigenous foods, in: Kalb, T. (ed.), FAO-AVRDC International Workshop Proceedings. Arusha, Tanzania, pp. 5-8.

Ashrafudoulla, M., Bellah, S.F., Alam, F., Faisal, S.S., Kafi, M.A.H., Fuad, F., 2016. Phytochemical screening of *Solanum nigrum* L., *S. myriacanthus* Dunal, *Solanum melongena* and *Averrhoa bilimbi* in Bangladesh. Journal of Medicinal Plants 4, 35-38.

Ayalew, T., 2014. Effects of soil types and nutrient levels on early leaf development of maize, bean and sunflower crops. African Journal of Agricultural Research 9, 1970-1975.

Ayeni, L.S., Omole, T.O., Adeleye, E.O., Ojeniyi, S.O., 2010. Integrated application of poultry manure and NPK fertiliser on performance of tomato in derived savannah transition zone of Southwest Nigeria. Science and Nature 8, 50-54.

Bairu, M.W., Aremu, A.O., van Staden, J. 2011. Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation 63, 147-173.

Baligar, V.C., Fageria, N.K., He, Z.L., 2001. Nutrient use efficiency in plants. Communications in Soil Science and Plant Analysis 32, 921-950.

Baloyi, B.M., Ayodele, V.I., Addo-Bediako, A., 2013. Effects of leaf harvest on crude protein and mineral contents of selected early maturing lines of lablab (*Lablab purpureus*). African Journal of Agricultural Research 8, 449-453.

Barnea, A., Yom-Tov, Y., Friedman, J., 1990. Differential germination of two closely related species of *Solanum* in response to bird ingestion. Oikos 57, 222-228.

Barrett, S.C.H., 1982. Genetic variation in weeds, in: Charudattan, R., Walker, H. (Eds.), Biological control of weeds with plant pathogens. Wiley and Sons, New York, pp. 73-98.

Bassett, I.J., Munro, D.B., 1985. The biology of Canadian weeds *Solanum ptycanthum* Dun., *S. nigrum* L. and *S. sarrachoides* Sendt. Canadian Journal of Plant Science 65, 401-414.

Beebe, S., Gonzalez, A.V., Rengifo, J., 2000. Research on trace minerals in the common bean. Food and Nutrition Bulletin 21, 387-391.

Bekheet, S.A., 2000. *In vitro* preservation of *Asparagus officinalis*. Biologia Plantarum 43(2), 179-183.

Bekheet, S.A., 2007. *In vitro* preservation of globe artichoke germplasm. Plant Tissue Culture and Biotechnology 17, 1-9.

Berti, C., Faber, M., Smuts, C.M., 2014. Prevention and control of micronutrient deficiencies in developing countries: current perspectives. Nutrition and Dietary Supplements 6, 41-57.

Bertrand-Desbrunais, A., Noirot, M., Charrier, A., 1992. Slow growth *in vitro* conservation of coffee (*Coffea spp.*). Plant Cell, Tissue and Organ Culture 31, 105-110.

Bhan, M.K., Sommerfelt, H., Strand, T., 2001. Micronutrient deficiency in children. British Journal of Nutrition 85, 199-203.

Bhargava, A., Shukla, S., Ohri, D. 2005. Analysis of genotype x environment interaction for grain yield in *Chenopodium* spp. Czech Journal of Genetics and Plant Breeding 2, 64-72.

Bhargava, A., Shukla, S., Ohri, D. 2008. Genotype x environment interaction studies in *Chenopodium album* L.: an underutilised crop with promising potential. Communications in Biometry and Crop Science 3, 3-15.

Bhat, M.A., Mujib, A., Junaid, A., 2010. *In vitro* regeneration of *Solanum nigrum* with enhanced solasodine production. Biologia Plantarum 54, 757-760.

Blössner, M., de Onis, M., 2005. Malnutrition: Quantifying the health impact at national and local levels. World Health Organization, Geneva 12, 1-52.

Bouis, H.E., Welch, R.M., 2010. Biofortification- A sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. Crop Science 50, 20-32.

Bukenya, Z.R., Hall, J.B., 1988. *Solanum (Solanaceae)* in Ghana. Bothalia 18, 79-88.

Bvenura, C., Afolayan, A.J., 2014a. Mineral uptake in *Solanum nigrum* L. cultivated on fertiliser amended soils of the Eastern Cape, South Africa. Journal of Applied Botany and Food Quality 87, 220-226.

Bvenura, C., Afolayan, A.J., 2014b. Maturity effects on mineral concentration and uptake in *Solanum nigrum* L. Acta Agriculturae Scandinavica, Section B- Soil and Plant Science 64, 657-665.

Bvenura, C., Afolayan, A.J., 2014c. Growth and physiological response of *Solanum nigrum* L. to organic and/or inorganic fertilisers. Journal of Applied Botany and Food Quality 87, 168-174.

Bvenura, C., Afolayan, A.J., 2015. The role of wild vegetables in household food security in South Africa: A review. Food Research International 76, 1001-1011.

Bwembya, G.C., Thwala, J.M., Otieno, D.A., 2014. Vitamin A and mineral content of some common vegetables consumed in Swaziland. Research Journal of Agriculture, Science and Technology 15, 9-17.

Byrnes, D.R., Dinssa, F.F., Weller, S.C. Simon, J.E., 2017. Elemental micronutrient content and horticultural performance of various vegetable amaranth genotypes. Journal of the American Society for Horticultural Science 142, 265-271.

Cantos, M., Zarate, R., Troncoso, A., 1998. *In vitro* germplasm conservation of *Atropa baetica* by cold-storage. Botanic Gardens Micropropagation News 2, 37-39.

Chakona, G., Shackleton, C., 2017. Minimum dietary diversity scores for women indicate micronutrient adequacy and food insecurity status in South African towns. Nutrients 9, 1-16.

Charaya, P., Sanghera, G.S., Gossal, S.S., 2011. Effect of genotype and explants on direct *in vitro* plant regeneration in brinjal (*Solanum melogena* L.). The Journal of Plant Science Research 27, 37-43.

Chauhan, R., Ruby, K.M., Shori, A., Dwivedi, J., 2012. *Solanum nigrum* with dynamic therapeutic role: a review. International Journal of Pharmaceutical Sciences, Review and Research 15, 65-71

Cha-um, S., Kirdmanee, C., 2007. Minimal growth *in vitro* culture for preservation of plant species. Fruit, Vegetable and Cereal Science and Biotechnology 1, 13-25.

Cheema, J., Yadav, K., Sharma, N., Saini, I., Aggarwal, A., 2017. Nutritional quality characteristics of different wild and underutilized fruits of Terai region, Uttarakhand (India). International Journal of Fruit Science 17, 72-81.

Choudhary, A.K., Barman, P., Subramaniam, S., Pathi, K.M., Kaushik, S., 2014. *In vitro* culture of economically important plant: *Solanum nigrum*. The Science and Technology Journal 1, 23-25.

Chweya, J.A., 1997. Genetic enhancement of indigenous vegetables in Kenya. Traditional African Vegetables International Plant Genetic Resources Institute (IPGRI), Rome, pp. 56-95.

Ciarka, D., Gawronski, S.W., 1996. Biology and control of black nightshade resistant to triazine herbicides. Ochrona Roslin 40, 3-4.

Clark, R.B., 1983. Plant genotype differences in the uptake, translocation, accumulation and use of mineral elements required for plant growth. Plant and Soil 72, 175-196.

Connor-Sánchez, A., Domínguez-May, A.V., Keb-Llanes, M.A., González-Estrada1, T.A., Peña–Ramírez, Y.J., 2010. Efficient plant regeneration from leaf explants of *Solanum americanum*. African Journal of Biotechnology 9, 5830-5835.

Cosic, T., Motyka, V., Raspor, M., Savic, J., Cingel, A., Vinterhalter, B., Vinterhalter, D., Travnivkova, A., Dobrev, P.I., Bohanec, B., Ninkovic, S., 2015. *In vitro* shoot organogenesis and comparative analysis of endogenous phytohormones in kohlrabi (*Brassica oleracea* var. *gongylodes*): effects of genotype, explant type and applied cytokinins. Plant Cell, Tissue Organ Culture 121, 741-760.

Crowther, P., 2008. The association between household food security and mortality in children under 5 years of age in Agincourt, Limpopo Province. MSc Thesis. University of Witwatersrand.

Cruz-Cruz, C.A., Gonzalez-Arnao, M.T., Engelmann, F., 2013. Biotechnology and conservation of plant biodiversity. Resources 2, 73-95.

D' Arcy, W.G., 1991. The *Solanaceae* since 1976, with the review of its biogeography, in: Hawkes, J.G., Lester, L.N., Nee, M., Estrada, N. (Eds.) *Solanaceae* III: taxonomy, chemistry, evolution. Royal Botanic Gardens, UK, pp. 75-137.

Das, A.K., Borah, S.P., 2015. Assessment of genetic diversity among some important wild species of non-tuberous *Solanum* using RAPD and ISSR markers. International Journal of Pharma and Biosciences 6(3), 1029-1042.

da Silva, T.L., Scherwinski-Pereira, J.E., 2011. *In vitro* conservation of *Piper aduncum* and *Piper hispidinervum* under slow-growth conditions. Pesquisa Agropecáuria Brasileira 46, 384-389.

Dehmer, K.J., Hammer, K., 2004. Taxonomic status and geographic provenance of germplasm accessions in the *Solanum nigrum* L. complex: AFLP data. Genetic Resources and Crop Evolution 51, 551-558.

Del Monte, J.P., Tarquis, A.M., 1997. The role of temperature in the seed germination of two species of the *Solanum nigrum* complex. Journal of Experimental Botany 48, 2087-2093.

Dhatt, A.S., Mir, K.A., Sandhu, J.S., Sidhu A.S., 2011. Effect of genotype, explant and culture medium on organogenesis in brinjal. Indian Journal of Horticulture 68, 332-335.

Dubois, T., 2009. Importance of tissue culture for orphan crops. African Technology Development Forum 6,48-52.

du Toit, D.C., 2011. Food security. Department of Agriculture, Forestry and Fisheries. [accessed: 27 October 2016 from: http://www.nda.agric.za/docs/genreports/foodsecurity.pdf].

Dweba, T.P., Mearns, M.A., 2011. Conserving indigenous knowledge as the key to the current and future use of traditional vegetables. International Journal of Information Management 31, 564-571.

Ebert, A.W., 2014. Potential of underutilised traditional vegetables and legume crops to contribute to food and nutritional security, income and more sustainable production systems. Sustainability 6, 319-335.

Edugbo, R.E., Nwofia, G.E., Fayeun, L.S. 2015. An assessment of soybean (*Glycine max*, l. Merrill) grain yield in different environments using AMMI and GGE biplot models in Humidorest Fringes of Southeast Nigeria. Agricultura Tropica Et Subtropica 48, 82-90.

Edmonds, J.M., 1977. Taxonomic studies on *Solanum* section *solanum* (Maurella). Botanical Journal of the Linnean Society 75, 141-178.

Edmonds, J.M., Chweya, J.A., 1997. Black nightshades *(Solanum nigrum L.*) and related species. Promoting the conservation and use of underutilised and neglected crops. Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute, Rome 15, 7-89.

Engelmann, F., 1991. *In vitro* conservation of tropical plant germplasm- A review. Euphytica 57, 227-243.

Engelmann, F., 2004. Plant Cryopreservation: Progress and Prospects. *In vitro* Cellular and Developmental Biology 40, 427-433.

Engelmann, F., 2011. Use of biotechnologies for the conservation of plant biodiversity. *In vitro* Cellular and Developmental Biology 47, 5-16.

Engelmann, F., Engels, J.M.M., 2002. Technologies and strategies for *ex situ* conservation, in: Engels, J.M.M., Rao, V.R., Brown, A.H.D., Jackson, M.T. (Eds.), Managing Plant Genetic Diversity. CABI Publishing., New York, pp. 89-103.

Ewais, E.A., Desouky, S.A., Eshazly, E.H., 2015. Studies on callus induction, phytochemical constituents and antimicrobial activity of *Solanum nigrum* L. (*Solanaceae*). Nature and Science 13, 133-138.

Ewulo, B.S., Ojeniyi, S.O., Akanni, D.A., 2008. Effect of poultry manure on selected soil physical and chemical properties, growth, yield and nutrient status of tomato. African Journal of Agricultural Research 3, 612-616.

Faber, M., Wenhold, F., 2007. Nutrition in contemporary South Africa. Water South Africa 33, 393-400.

Faber, M., Oelofse, A., van Jaarsveld, P.J., Wenhold, F.A.M., Jansen van Rensburg, W.S., 2010. African leafy vegetables consumed by households in the Limpopo and KwaZulu-Natal provinces in South Africa. South African Journal of Clinical Nutrition 23, 30-38.

Fadel, D., Kintzios, S., Economou, A.S., Moschopoulou, G., Constantinidou, H.A., 2014. Increased antioxidant activity, despite reduced rosminaric acid accumulation in methanolic extracts, of spearmint (*Mentha spicata* L.) plantlets regenerated *in vitro*. The Open Horticulture Journal 7, 1-5.

Fah, W.K. 2013. Tissue culture studies, secondary metabolites and pigment extraction from *Allamanda cathartica* L. MSc. Thesis. University of Malaysia, Kuala Lumpur.

Food and Agriculture Organisation, 2010. The state of food insecurity in the world: Addressing food insecurity in protracted crises, Rome, pp. 4-49.

Ferdausi, A., Nath, U.K., Das, B.L., Alam, M.S., 2009. *In vitro* regeneration system in brinjal (*Solanum melongena* L.) for stress tolerant somaclone selection. Journal of the Bangladesh Agricultural University 7, 253-258.

Fong, J.D.M., Masunaga, T., Sato, K., 2015. Control of micronutrients availability in soil and concentration in rice grain through field water management. Journal of Agricultural Science 7, 163-174.

Gashi, B., Abdullai, K., Sota, V., Kongjika, E., 2015. Micropropagation and *in vitro* conservation of the rare and threatened plants *Ramonda serbica* and Ram*onda nathaliae.* Physiology and Molecular Biology of Plants 21, 123-136.

Geetha, G., Harathi, K., Naidu, C.V., 2016. Role of silver nitrate on *in vitro* flowering and shoot regeneration of *Solanum nigrum* (L.)- An important multipurpose medicinal plant. American Journal of Plant Sciences 7, 1021-1032.

Genc, Y., Humphries, J.M., Lyons, G.H., Graham, R.D., 2005. Exploiting genotypic variability in plant nutrient accumulation to alleviate micronutrient deficiency in populations. Journal of Trace Elements in Medicine and Biology 18, 319-324.

George, E.F., Hall, M.A., de Klerk, G.J., 2008. Plant propagation by tissue culture- The background. Springer, third ed. Springer, Netherlands, pp. 175-204.

Gibson, R.S., 2005. Principles of nutritional assessment, second edition. Oxford University Press, Oxford, pp. 908.

Ghani, A., Ali, Z., Islam, T., Sanaullah, S., Saeed S., 2014. Nutrient evaluation and elemental analysis of four selected medicinal plants of soon valley Khushab, Punjab, Pakistan. Pakistan Journal of Pharmaceutical Science 27, 597-600.

Givelberg, A., Horowitz, M., Poljakoff-Mayber, A., 1984. Germination behaviour of *Solanum nigrum* seeds. Journal of Experimental Botany 35, 588-598.

Glew, R.S., Vanderjagt, D.J., Bosse, R., Huang, Y.S., Chuang, L.T., Glew, R.H., 2005. The nutrient content of three edible plants of the Republic of Niger. Journal of Food Composition and Analysis 18, 15-27.

Gogoi, P., Islam, M., 2014. Comparative phenological study of medicinal plants with special reference to *Solanum nigrum* L. and *S. myriacanthus* Dunal of upper Assam, India. International Journal of Medicinal and Aromatic Plants 4, 26-33.

Gotor, E., Irungu, C., 2010. The impact of biodiversity international's African leafy vegetables programme in Kenya. Impact Assessment and Project Appraisal 28, 41-55.

Govender, L., Pillay, K., Siwela, M., Modi, A., Mabhaudhi, T., 2017. Food and nutrition insecurity in selected rural communities of KwaZulu-Natal, South Africa- linking human nutrition and agriculture. International Journal of Environmental Research and Public Health 14, 1-21.

Gratani, L., 2014. Plant phenotypic plasticity in response to environmental factors. Advances in Botany 2014, 1-17.

Grozeva, S., Velkov, N., 2014. *In vitro* plant regeneration of two cucumber (*Cucumis sativus* L.) genotypes: Effects of explant types and culture medium. Genetika 46, 485-493.

Grozeva, S., Rodev, V., Danailov, Z., 2006. Effect of genotype, explant type and culture medium on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro*. Bulgarian Journal of Agricultural Science 12, 435-439.

Gubis, J., Lajchova, Z., Farago, J., Jurakova, Z., 2003. Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro*. Czech Journal of Genetics and Plant Breeding 39, 9-14.

Hammond, R., Buah, J.N., Asare, P.A., Acheampong, S., 2014. Optimizing sterilization condition for the initiation of sweet potato (*Ipomoea batatas*) culture *in vitro*. Asian Journal of Biotechnology 6, 25-37.

Harn, C., 1972. Production of plants from anthers of *Solanum nigrum* cultured *in vitro*. Caryologia 25, 429-437.

Harris, R.W., 1992. Root-Shoot ratio. Journal of Arboriculture 18.

Hartmann, H.T., Kester, D.E., Davies, F.T., Geneve, R.L., 1997. Plant propagation: Principles and practices, sixth ed. Prentice Hall International, London, UK, pp. 639-679.

Hassanein, A.M., Soltan, D.M., 2000. *Solanum nigrum* is a model system in plant tissue and protoplast cultures. Biologia Plantarum 43, 501-509.

He, M., Chen, Z., Sakurai, K., Iwasaki, K., Shen, Y., Zhou, J., 2003. Effect of differences in substrate formulations on cucumber growth under soilless bag culture in greenhouse. Soil Science and Plant Nutrition 49, 763-767.

Jabeen, N., Chaudhry, Z., Rashid., H., Mirza, B., 2005. Effect of genotype and explant type on *in vitro* shoot regeneration of tomato (*Lycopersicon esculentum* mill.). Pakistan Journal of Botany 37, 899-903.

Jacoby, A., Labuschagne, M.T., Viljoen, C.D., 2003. Genetic relationships between southern African *Solanum retroflexum* Dun. and other related species measured by morphological and DNA markers. Euphytica 132, 109-113.

Jagatheeswari, D., Bharathi, T., Ali, H.S.J., 2013. Black Nightshade (*Solanum nigrum* L.)- An updated overview. International Journal of Pharmaceutical and Biological Archives 4, 288-295.

Jankauskienė, J., Brazaitytė, A., Viškelis, P., 2015. Effect of different growing substrates on physiological processes, productivity and quality of tomato in soilless culture. in: Asaduzzaman, M.D. (Ed.), Soilless Culture-Use of Substrates for the Production of Quality Horticultural Crops. InTech Open Science, pp. 100-124.

Jansen van Rensburg, W.S., Venter, S.L., Netshiluvhi, T.R., van den Heever, E., Vorster, H.J., de Ronde, J.A., Bornman, C.H., 2004. Role of indigenous leafy vegetables in combating hunger and malnutrition. South African Journal of Botany 70, 52-59.

Jansen van Rensburg, W.S., van Averbeke, W., Slabbert R., Faber, M., van Jaarsveld, P., van Heerden, I., Wenhold, F., Oleofse, A., 2007. African leafy vegetables in South Africa. Water South Africa 33, 317-326.

Jimoh, F.O., Adedapo, A.A., Afolayan, A.J., 2010. Comparison of the nutritional value and biological activities of the acetone, methanol and water extracts of the leaves of *Solanum nigrum* and *Leonotis leonnorus*. Food and Chemical Toxicology 48, 964-971.

Joshi, V., Jadhav, S.K., 2013. Effect of temperature and media supplements on slow growth conservation of medicinal plant *Spilanthes acmella*. Botanica Serbica 37, 155 160.

Kachiguma, N.A., Mwase, W., Maliro, M., Damaliphetsa, A., 2015. Chemical and mineral composition of amaranth (*Amaranthus* L.) species collected from central Malawi. Journal of Food Research 4, 92-102.

Kadlecek, P., Ticha, I., Haisel, D., Capkova, V., Schäfer, C., 2001. Importance of *in vitro* pretreatment for *ex vitro* acclimatisation and growth. Plant Sciences 161, 695-701.

Kareem, A., Radhakrishnan, D., Sondhi, Y., Aiyaz, M., Roy, M.V., Sugimoto, K., Prasad, K., 2016. De novo assembly of plant body plan: A step ahead of Deadpool. Regeneration 3, 182-197.

Kaur, M., Dhatt, A.S., Sandhu, J.S., Gosal, S.S., 2015. Effect of genotype, explant and culture media on direct plant regeneration in eggplant. Indian Journal of Horticulture 72, 232-238.

Kandimalla, R., Kolita, S., Choudhary, B., Kotoky, J., 2015. A review on anti-diabetic potential of genus *Solanum* (Solanaceae). Journal of Drug Delivery and Therapeutics 5, 24-27.

Kannan, T.M., Nagarajan, S.M., Kulothungan, S., 2006. Micropropagation of *Solanum nigrum* L.- A medicinal herb. Plant Archives 6, 97-99.

Karp, A., 1994. Origins, causes and uses of variation in plant tissue cultures. in: Plant cell and tissue culture. Vasil, I.K., Thorpe, T.A (eds). Kluwer Academic Publishers, Dordrecht. pp. 139-152.

Kavitha, M.S., Wesely, E.G., Mehalingam, P., 2012. Direct multiple shoot regeneration from shoot tip and nodal explants of *Solanum nigrum* L.- A medicinal herb. Journal of Ornamental and Horticultural Plants 2, 65-72.

Kazi, N.A., 2015. Polyploidy in *Solanaceous* crops. Asian Journal of Multidisciplinary Studies 3, 69-73.

Keeley, P.E., Thullen, R.J., 1983. Influence of planting date on the growth of black nightshade (*Solanum nigrum*). Weed Science 31, 180-184.

Keller, E.R.J., Senula, A., Leunufna, S., Grube, M., 2006. Slow growth storage and cryopreservation-tools to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. International Journal of Refrigeration 29, 411-417.

Kevers, C., Franck, T., Strasser, R.J., Dommes., J., Gaspar, T., 2004. Hyperhydricity of micropropagated shoots: A typically stress-induced change of physiological state. Plant Cell, Tissue and Organ Culture 77, 181-191.

Khader, V., Rama, S., 1998. Selected mineral content of common leafy vegetables consumed in India at different stages of maturity. Plant Foods for Human Nutrition 53, 71-81.

Khader, V., Rama, S., 2003. Effect of maturity on macromineral content of selected leafy vegetables. Asia Pacific Journal of Clinical Nutrition 12, 45-49.

Khattak, J.Z.K., Anwar, Z., Aftab, S., Afzal, M., 2012. *Solanum nigrum* as potent therapy: A review. British Journal of Pharmacology and Toxicology 3, 185-189.

Kilcher, M.R., 1981. Plant development, stage of maturity and nutrient composition. Journal of Range Management 34, 363-364.

Knapp, S., 2008. Typification of *Solanum (Solanaceae)* sp. described by Martin de Sesse y Lacasta and Jose' Mariano Mocino. Anales del Jardin Botanico de Madrid 65, 7-23.

Kolar, A.B., Vivekanandan, L., Ghouse, B.M., 2008. *In vitro* regeneration and flower induction on *Solanum nigrum* L. from Pachamalai Hills of Eastern Ghats. Plant Tissue Culture and Biotechnology 18, 43-48.

Kopsell, D.E., Kopsell, D.A., Lefsrud, M.G., Curran-Celentano, J., 2005. Variability in elemental accumulations among leafy *Brassica oleracea* cultivars and selections. Journal of Plant Nutrition 27, 1813-1826.

Kremer, E., Lotz, L.A.P., 1998. Germination and emergence characteristics of triazine-susceptible and triazine-resistant biotypes of *Solanum nigrum*. Journal of Applied Ecology 35, 302-310.

Krstic', L.N., Merkulov, L.S., Boza, P.P., 2002. The variability of leaf anatomical characteristics of *Solanum nigrum* L. (Solanales, Solanaceae) from different habitats. Proceedings for Natural Sciences, Matica Srpska Novi Sad 102, 59-70.

Kubota, C., Kozai, T., 1994. Low-temperature storage for quality preservation and growth suppression of broccoli plantlets cultured *in vitro*. American Society for Horticultural Science 29, 1191-1194.

Kunitake, H., Mii, M., 1998. Somatic embryogenesis and its application for breeding and micropropagation in asparagus (*Asparagus officinalis* L.). Plant Biotechnology 15, 51-61.

Labadarios, D., 2005. Malnutrition in the developing world: The triple burden. South African Journal of Clinical Nutrition 18, 119-121.

Labadarios, D., Steyn, N., Maunder, E., Macintyre, U., Swart, R., Gericke, G., Huskisson, J., Dannhauser, A., Voster, H.H., Nesamvuni, A.E., 2000. The National Food Consumption Survey (NFCS): Children Aged 1-9 years, South Africa 1999. South African Journal of Clinical Nutrition 14, 16-19.

Labadarios, D., Kruger, S.H., Swart, R., Maunder, E.M.W., Gericke, G.J., Kuzwayo, P.M.N., Ntsie, P.R., Steyn, N.P., Schloss, I., Dhansay, M.A., Jooste, P.L., 2008. National food

consumption survey-fortification baselin (NFCS-FB-I). South African Journal of Clinical Nutrition 21, 275-300.

Latif, B., Javaran, M.J., Alizadeh, H., Memari, H.R., Mohammadi, R., 2014. Interactions of genotype and plant growth regulators affecting direct shoot regeneration of lettuce (*Lactuca sativa* L.). International Journal of Biosciences 5, 315-322.

Lewu, F.B., Lewu, M.N., Mavengahama, S., 2012. Influence of genotype and harvesting age on the mineral dynamics of *Brassica oleracea* var. *acephala* cultivated in South Africa. Journal of Food, Agriculture and Environment 10, 563-566.

Linnaeus, C., 1753. Species plantarum. Impensis GC Nauk 1, 1-1072.

Liza, L.N., Nasar, A.N.M., Zinnah, K.M.A., Chowdhury, A.N., Ashrafuzzaman, M., 2013. *In vitro* growth media effect for regeneration of tomato (*Lycopersicon esculentum*) and evaluation of the salt tolerance activity of callus. Journal of Agriculture and Sustainability 3, 132-143.

Loneragen, J.F., 1968. Nutrient requirements of plants. Nature 220, 1307-1308.

Mahmoud, S.N., Al-Ani, N.K., 2016. Effect of different methods on contamination and viability of nodal segments of *Cestrum nocturnum* L. International Journal of Research in Biosciences 4, 4-9.

Makowczynska, J., Grzegorczyk-Karolak, I., Wysokinska, H., 2015. Antioxidant activity of tissue culture-raised *Ballota nigra* L. plants grown *ex vitro*. Acta Poloniae Pharmaceutica 72, 769-775.

Makus, D.J., Lester, G., 2002. Effect of soil type, light intensity, and cultivar on leaf nutrients in mustard greens. Subtropical Plant Science 54, 23-28.

Manako, M.L.K., van den Berg, R.G., Feron, R.M.C., van der Weerden, G M., Mariani, C., 2007. Genetic diversity of the African hexaploid species *Solanum scabrum* Mill. and *Solanum nigrum* L. (Solanaceae). Genetic Resources and Crop Evolution 55, 409-418.

Matasyoh, L.G., Bosire, A.N.A., 2016. The *Solanum nigrum* complex (Black Nightshade) grown in the Rift Valley, Western and Nyanza Provinces of Kenya. Journal of Life Sciences 10, 228-232.

Matasyoh, L.G., Abel, S., Budahn, H., Klocke, E., 2015. Characterisation of the *Solanum nigrum* complex of Kenya by AFLP markers. International Journal of Agricultural Science and Technology 3, 8-14.

Mchiza, Z.J., Steyn, N.P., Hill, J., Kruger, A., Schönfeldt, H., Nel, J., Wentzel-Viljoen, E., 2015. A review of dietary surveys in the adult South African population from 2000 to 2015. Nutrients 7, 8227-8250.

Misselhorn, A.A., 2005. What drives food insecurity in southern Africa? A meta-analysis of household economy studies. Global Environmental Change 15, 33-43.

Modi, A.T., 2007. Growth, temperature and plant age influence on nutritional quality of *Amaranthus* leaves and seed germination capacity. Water South Africa 33, 369-376.

Mohebodini, M., Javaran, M.J., Mahboudi, F., Alizadeh, H., 2011. Effects of genotype, explant age and growth regulators on callus induction and direct shoot regeneration of lettuce (*Lactuca sativa* L.). Australian Journal of Crop Science 5, 92-95.

Morgan, J.B., Connolly, E.L., 2013. Plant-soil interactions: Nutrient uptake. Nature Education Knowledge 4, 2-3.

Muktadir, M., Habib, M.A., Mian, A.K., Akhond, A.Y., 2016. Regeneration efficiency based on genotype, culture condition and growth regulators of eggplant (*Solanum melongena* L.). Agriculture and Natural Resources 50, 38-42.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

Murungi, L.K., Salifu, D., Masinde, P., Wesonga, J., Nyende, A., Knapp, M., 2014. The effects of the invasive tomato red spider mite (Acari: Tetranychidae) on growth and leaf yield of African nightshades. Crop Protection 59, 57-62.

Musa, A., 2012. Influence of plant leaf locations on the bioaccumulations of phytotoxins and nutrients in *Corchorus olitorius* at market maturity. International Journal of Biology 4, 130-139.

Musa, A., Ogbadoyi, E.O., 2012. Effect of plant leaf positions on some micronutrients, anti-nutrients and toxic substances in *Telfairia occidentalis* at the vegetative phase. American Journal of Experimental Agriculture 2, 219-232.

Musa, A., Ezenwa, M.I., Oladiran, J.A., Akanya, H.O., Ogbadoyi, E.O., 2010. Effect of soil nitrogen levels on some micronutrients, antinutrients and toxic substances in *Corchorus olitorius* grown in Minna, Nigeria. African Journal of Agricultural Research 5, 3075-3081.

Mutlu, E.C., Turker, A.U., 2008. Efficient plant regeneration of bittersweet (*Solanum dulcamara* L.), a medicinal plant. Acta Societatis Botanicorum Poloniae 77, 275-280.

Mwafusi, C.N., 1992. Effects of propagation method and deflowering on vegetative growth, leaf yield, phenolic and glycoalkaloid contents of three black nightshade selections used as vegetable in Kenya. MSc Thesis. University of Nairobi.

Mwai, G.N., Onyango, J.C., Abukusta-Onyango, O., 2007. Taxon identification and characterisation of African nightshades (*Solanum* L. section *Solanum*). African Journal of Food, Agriculture, Nutrition and Development 7, 1-13.

Nandhini, T., Paramaguru, P., 2014. Evaluating the taxonomic status of *Solanum nigrum* L. using flow cytometry and DNA barcoding technique. Journal of Spices and Aromatic Crops 23, 233-242.

Nayar, P.K., Misra, K., Patnaik, S., 1975. Rapid determination of silicon in rice plant. Plant and Soil 42, 491-494.

Ncube, B., Baskaran, P., van Staden, J., 2015. Transition from *in vitro* to an *ex vitro* environment: Is the metabolism altered? *In vitro* Cellular and Developmental Biology- Plant 51, 166-173.

Negash, A., Krens, F., Schaart, J., Visser, B., 2001. *In vitro* conservation of enset under slow-growth conditions. Plant Cell, Tissue and Organ Culture 66, 107-111.

Ngoroyemoto, N., Aremu, A.O., Kulkarni, M.G., Finnie, J.F., Van Staden, J., 2017. A preliminary survey of commonly-consumed indigenous fruits and vegetables in four informal/rural settlements in KwaZulu-Natal. South African Journal of Botany 109, 360-

Nicolle, C., Simon, G., Rock, E., Amouroux, P., Remesy, C., 2004. Genetic variability influences carotenoid, vitamin, phenolic and mineral content in white, yellow, purple, orange and dark-orange carrot cultivars. Journal of American Society for Horticultural Science 129, 523-529.

Njume, C., Goduka, N.I., George, G., 2014. Indigenous leafy vegetables (*imifino, morogo, muhuro*) in South Africa: A rich and unexplored source of nutrients and antioxidants. African Journal of Biotechnology 13, 1933-1942.

Nupo, S.S., Onigbogi, I.O., Akinlotan, J.V., Ilori, O.A., 2013. Effect of different processing methods on the nutrients and antinutrient composition of *Senecio biafrae*, *Crassocephalum crepidiodes* and *Solanum nigrum* consumed in south west Nigeria. American Journal of Food and Nutrition 3, 147-154.

Nurzyński, J., 2013. Effect of substrates on nutrient content in root zone and leaves of greenhouse tomato. Acta Scientiarum Polonorum Horticulture 12, 169-178.

Nyeem, M.A.B., Rashid, A.M.U., Nowrose, M., Hossain, M.A., 2017. *Solanum nigrum* (Maku): A review of pharmacological activities and clinical effects. International Journal of Applied Research, 3, 12-17.

Ochieng, J., Afari-Sefa, V., Karanja, D., Rajendran, S., Silvest, S., Kessy, R., 2016. Promoting consumption of traditional African vegetables and its effect on food and nutrition security in

Tanzania. in: African Association of Agricultural Economists Fifth International Conference, September 23-26, 2016, Addis Ababa, Ethiopia, pp. 2-20.

Odhav, B., Beekrum, S., Naidoo, N., Baijnath, H., 2007. Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. Journal of Food Composition and Analysis 20, 430-435.

Oduse, K.A., Idowu, M.A., Adegbite, A.A., 2012. Chemical and phytochemical profile of some uncommon green leafy vegetables consumed in South West, Nigeria. Journal of Environmental Science, Toxicology and Food Technology 1, 22-26.

Oelberg, K., 1956. Factors affecting the nutritive value of range forest. Journal of Range Management 9, 220-225.

Ogg, A.G.Jr., Rogers, B.S., Schilling, E.E., 1981. Characterisation of black nightshade (*Solanum nigrum*) and related species in the United States. Weed Science 29, 27-32.

Ohshiro, M., Hossain, A., Nakamura, I., Akamine, H., Tamaki, M., Bhowmik P.C., Nose, A., 2016. Effects of soil types and fertilisers on growth, yield, and quality of edible *Amaranthus tricolor* lines in Okinawa, Japan. Plant Production Science 19, 61-72.

Ojiewo, C.O., Murakami, K., Masinde, P.W., Agong, S.G., 2007. Polyploidy breeding of African Nightshade (*Solanum* section *Solanum*). International Journal of Plant Breeding 1, 10-21.

Oniang'o, R.K., Mutuku, J.M., Malaba, S.J., 2004. Contemporary African food habits and their nutritional and health implications. Asia Pacific Journal of Clinical Nutrition 12, 231-236.

Onyango, C.M., Ontita, E.G., Onwong'a, R.N., Nyamongo, D., Gapusi, J.R., 2016. Status and production practices of vegetable African nightshade (*Solanum nigrum* L.) in selected communities of Kenya. American Journal of Experimental Agriculture 13, 1-12.

Oyekunle, S.M., Adebayo, O.L., Olajide, A.K., Olufunmilayo, O.B., Temitayo, O.O., 2014. Partitioning and distribution of random amplified polymorphic DNA (RAPD) variation among

eggplant *Solanum* L. in Southwest Nigeria. International Journal of Genetics and Molecular Biology 6, 1-7.

Padmapriya, H., Karthikeyan, A.V.P., Hussain, J.G., Karthi, C., Velayutham, P., 2011. An efficient protocol for *in vitro* propagation of *Solanum nigrum* L. from nodal explants. Journal of Agricultural Technology 7, 1063-1073.

Padmashree, A., Sharma, G.K., Semwal, A.D., Mahesh, C., 2014. Antioxygenic activity of *Solanum nigrum* L. leaves in sunflower oil model system and its thermal stability. Food and Nutrition Sciences 5, 1022-1029.

Pandhure, N., Bansode, R., Kothekar, V., 2010. *In vitro* multiplication of important medicinal plant *Solanum nigrum* L. Recent Research in Science and Technology 2, 33-35.

Pandino, G., Lombardo, S., Mauromicale, G., 2011. Mineral profile in globe artichoke as affected by genotype, head part, and environment. Journal of the Science and Food Agriculture 91, 302-308.

Pérez-Molphe-Balch, E., Pérez-Reyes, M.E., de Lourdes de La Rosa-Carrillo, M.A., 2012. *In vitro* conservation of *Turbinicarpus* (Cactaceae) under slow growth conditions. Haseltonia 17, 51-57.

Pérez-Tornero, O., Ortín-Párraga, F., Egea, J., Burgos, L., 1999. Medium-term storage of apricot shoot tips *in vitro* by minimal growth method. American Society for Horticultural Science 34, 1277-1278.

Poczai, P., Hyvoenen, J., 2011. On the origin of *Solanum nigrum*: Can networks help? Molecular Biology Report. 38, 1171-1185.

Pospisilova, J., Ticha, I., Kadlecek, P., Haisel, D., Plzakova, S., 1999. Acclimatisation of micropropagated to *ex vitro* conditions. Biology of Plants 42, 481-497.

Potawale, S.E., Sinha, S.D., Shroff, K.K., Dhalawat, H.J., Boraste, S.S., Gandhi, S.P., Tondare, A.D., 2008. *Solanum nigrum* Linn: A phytopharmacological review. Pharmacology Online 3, 140-163.

Pua, E., Deng, X., Koh, A.T., 1999. Genotypic variability of de novo shoot morphogenesis of *Brassica oleracea in vitro* in response to ethylene inhibitors and putrescine. Journal of Plant Physiology 155, 598-605.

Radhakrishnan, R., Kumari, B.D.R., 2009. Changes in protein content in micropropagated and conventional soybean plants (*Glycine max* (L.) Merr.). World Journal of Agricultural Sciences 5, 186-189.

Rao, N.K., 2004. Plant genetic resources: Advancing conservation and use through biotechnology. African Journal of Biotechnology 3, 136-145.

Rathore, V., Gupta, D., 2013. *In vitro* clonal propagation of *Solanum nigrum*. Research Journal of Chemical and Environmental Sciences 1, 32-33.

Reed, B.M., 1999. *In vitro* storage conditions for mint germplasm. American Society for Horticultural Science 34, 350-352.

Roca, W.M., Reyes, R., Beltran, J., 1983. Effect of various factors on minimal growth in tissue culture storage of cassava germplasm. in: Symposium of The International Society for Tropical Root Crops 6, 441-446.

Rogers, B.S., Ogg, A.G., 1981. Biology of weeds of the *Solanum nigrum* complex (*Solanum* sections S*olanum*) in North America. Agricultural Reviews and Manuals 23, 1-30.

Ronoh, R., Ekhuya, N.A., Linde, M., Winkelmann, T., Abukutsa-Onyango, M., Dinssa, F.F., Debener, T., 2017. African nightshades: Genetic, biochemical and metabolite diversity of an underutilised indigenous leafy vegetable and its potential for plant breeding. The Journal of Horticultural Science and Biotechnology 1, 1-9.

Rout, G.R., Sahoo, S., 2015. Role of iron in plant growth and metabolism. Reviews in Agricultural Science 3, 1-24.

Safaa, A.M., Abd El Fattah, M.S., 2007. Effect of nitrogen forms on nitrate contents and mineral composition in lettuce plants in sandy and calcareous soils. Journal of Applied Sciences Research 3, 1630-1636.

Saher, S., Piqueras, A., Hellin, E., Olmos, E., 2003. Hyperhydricity in micropropagated carnation shoots: The role of oxidative stress. Physiologia Plantarum 120, 152-161.

Sarethy, I.P., Kashyap, A., Bahal, U., Sejwal, N., Gabrani, R., 2014. Study of liquid culture system for micropropagation of the medicinal plant *Solanum nigrum* L. and its effect on antioxidant property. Acta Physiologiae Plantarum 36, 2863-2870.

Sarma, H., Sarma, A., 2011. *Solanum nigrum* L., a nutraceutical enriched herb or invasive weed?. International Conference on Environment and BioScience, Singapore 21, 105-109.

Seeiso, M.T., Materechera, S.A., 2014. Biomass yields and crude protein content of two African indigenous leafy vegetables in response to kraal manure application and leaf cutting management. African Journal of Agricultural Research 9, 397-406.

Sharma, P., Rajam, M.V., 1995. Genotype, explant and position effects on organogenesis and somatic embryogenesis in eggplant (*Solanum melongena L.*). Journal of Experimental Botany 46, 135-141.

Sharma, G., Nautiyal, A.R., 2009. Influence of explants type and plant growth regulators on *in vitro* multiple shoots regeneration of a laurel from Himalaya. Nature and Science 7, 1-7.

Sheela, K., Nath, K.G., Vijayalakshmi, D., Yankanchi, G.M., Patil, R.B., 2004. Proximate composition of underutilised green leafy vegetables in Southern Karnataka. Journal of Human Ecology 15, 227-229.

Shojaei, T.R., Salari, V., Ramazan, D., Ehyaei, M., Gharechahi, J., Chaleshtori, R.M., 2010. The effect of plant growth regulators, explants and cultivars on spinach (*Spinacia oleracea* L.) tissue culture. African Journal of Biotechnology 9, 4179-4185.

Shukla, R., Sharma, Y.K., Shukla, A.K., 2014. Molecular mechanism of nutrient uptake in plants. International Journal of Current Research and Academic Review 2, 142-154.

Shukla, S., Bhargava, A., Chatterjee, A., Srivastava, J., Singh, N., Singh, S.P., 2006. Mineral profile and variability in vegetable amaranth (*Amaranthus tricolor)*. Plant Foods for Human Nutrition 61, 23-28.

Singh, T.D., Singh, C.H., Nongalleima, K., Moirangthem, S., Devi, H.S., 2013. Analysis of growth, yield potential and horticultural performance of conventional vs. micropropagated plants of *Curcuma longa* var. Lakadong. African Journal of Biotechnology 12, 1604-1608.

Smith, F.I., Eyzaguirre, P., 2007. African leafy vegetables: Their role in the World Health Organisation's global fruit and vegetables initiative. African Journal of Food, Agriculture, Nutrition and Development 7, 1-18.

Soria, J.V., Heiser, C.B., 1961. A statistical study of relationships of certain species of the *Solanum nigrum* complex. Economic Botany 15, 245-255.

Sridhar, T.M., Naidu, C.V., 2011a. An efficient callus induction and plant regeneration of *Solanum nigrum* (L.)-An important antiulcer medicinal plant. Journal of Phytology 3, 23-28.

Sridhar, T.M., Naidu, C.V., 2011b. *In vitro* direct shoot organogenesis from leaf explants of *Solanum nigrum* (L.)-An important antiulcer medicinal plant. Journal of Phytology 3, 29-35.

Stamp, P., Messmer, R., Walter, A., 2012. Competitive underutilised crops will depend on the state funding of breeding programmes: An opinion on the example of Europe. Plant Breeding 131, 461-464.

Stankiewics, M., Gadamski, G., Gawronski, S.W., 2001. Genetic variation and phylogenetic relationships of triazine-resistant and triazine-susceptable biotypes of *Solanum nigrum*-analysis using RAPD markers. Weed Research 41, 287-300.

Stebbins, G.L., Paddock, E.F., 1949. The *Solanum nigrum* complex in Pacific North America. Madrono 10(3), 70-81.

Steyn, N.P., Olivier, J., Winter, P., Burger, S., Nesamvuni, C., 2001. A survey of wild, green, leafy vegetables and their potential in combating micronutrient deficiencies in rural populations. South African Journal of Science 97, 276-278.

Steyn, N., Eksteen, G., Senekal, M., 2016. Assessment of the dietary intake of schoolchildren in South Africa: 15 Years after the First National Study. Nutrients 8, 1-14.

Subramanian, R., Gayathri, S., Rathnavel, C., Raj. V., 2012. Analysis of mineral and heavy metals in some medicinal plants collected from local market. Asian Pacific Journal of Tropical Biomedicine 74-78.

Sundari, M.S., Benniamin, A., Manickam, V.S., 2010. Micropropagation and *in vitro* flowering in *Solanum nigrum* Linn. A medicinal plant. International Journal of Biological Technology 1, 29-32.

Suthar, A.C., Naik, V.R., Mulani, R.M., 2009. Seed and seed germination in *Solanum nigrum* Linn. American-Eurasian Journal of Agricultural and Environmental Science 5, 179-183.

Swain, S.S., Tripathy, T., Mohapatra, P.K., Chand, P.K., 2010. Photosynthetic and transpiration responses of *in vitro*-regenerated *Solanum nigrum* L. plants to *ex vitro* adaptation. *In vitro* Cellular and Developmental Biology-Plant 46, 134-141.

Symon, D.E., 1981. A revision of the genus *Solanum* in Australia. Journal of the Adelaide Botanic Gardens 4, 1-367.

Taab, A., Andersson, L., 2008. Seed dormancy dynamics and germination characteristics of *Solanum nigrum*. Weed Research 49, 490-498.

Taab, A., Andersson, L., 2009. Seasonal changes in seed dormancy of *Solanum nigrum* and *Solanum physalifolium*. Weed Research 49, 90-97.

Tadele, Z., Assefa K., 2012. Increasing food production in Africa by boosting the productivity of understudied crops. Agronomy 2, 240-283.

Takahata, Y., Komatsu, H., Kaizuma, N., 1996. Microspore culture of radish (*Raphanus sativus* L.): Influence of genotype and culture conditions on embryogenesis. Plant Cell Reports 16, 163-166.

Tayie, F.A.K., Asibey-Berko, E., 2001. Mineral contents of some indigenous vegetables of Ghana. Ghana Journal of Science 41, 49-54.

Topp, C.N., 2016. Hope in change: The role of root plasticity in crop yield stability. Plant Physiology 172, 5-6.

Tumwet, T.N., Mwangi, A.M., Kogi-Makau, W., Kangethe, E.K., 2013. Effect of different cooking methods on the nutritional value of some African indigenous leafy vegetables in Kenya. African Crop Science Conference Proceedings 11, 441-443.

Tyagi, R.K., Goswami, R., Sanayaima, R., Singh, R., Tandon, R., Agrawal, A., 2009. Micropropagation and slow growth conservation of cardamom (*Elettaria cardomomum* Maton). *In vitro* Cellular and Developmental Biology- Plant 45, 721-729.

Uusiku, N.P., Duodu, K.G., Bester, M.J., Faber, M., 2010. Nutritional value of leafy vegetables of sub-Saharan African and their potential contribution to human health. A review. Journal of Food Composition and Analysis 23, 499-509.

van der Hoeven, M., Faber, M., Osei, J., Kruger, A., Smuts, C.M., 2016. Effect of African leafy vegetables on the micronutrient status of mildly deficient farm-school children in South Africa: A randomized controlled study. Public Health Nutrition 19, 935-945.

Vats, A., Nagpal, K.L., 2013. Evaluation of mineral contents. International Journal of Research in Engineering and Applied Sciences 3, 63-72.

Venkateswarlu, J., Rao, M.K., 1972. Breeding system, crossabiliity relationships and isolating mechanisms in the *Solanum nigrum* complex. Cytologia 37, 317-326.

Verma, S.K., Rai, M.K., Asthana, P., Jaiswal, V.S., Jaiswal, U., 2010. *In vitro* plantlets from alginate-encapsulated shoot tips of *Solanum nigrum*. Scientia Horticulturae 124, 517-521.

Vorster, H.J., Jansen van Rensburg, W. S., van Zijl, J.J.B., van den Heever, E., 2002. Germplasm management of African leafy vegetables for the nutritional and food security needs of vulnerable groups in South Africa. Progress report. Pretoria: Agricultural Research Council -Vegetable and Ornamental Plants Institute, South Africa, pp. 130.

Vorster, H.J., Jansen van Rensburg, W.S., van Zijl, J.J.B., Venter, S.L., 2007. The importance of traditional leafy vegetables in South Africa. African Journal for Food, Agriculture, Nutrition and Development 7, 1-13.

Wahua, C., Olaleye, S.M., 2013. Comparative taxonomic studies on *Solanum aethiopicum* Linn and *Solanum nigrum* Linn (*Solanaceae).* Greener Journal of Agricultural Sciences 3, 849-854.

Wee, S.L., Alderson, P.G., Yap, W.S.P., 2015. Establishment of plantlet regeneration system from nodal, internodal and leaf explants of *Sauropus androgynous* (Sweet shoot). Asian Journal of Biotechnology 7, 46-59.

Weese, T.L., Bohs, L., 2007. A three-gene phylogeny of the genus *Solanum (Solanaceae).* Systematic Botany 32, 445-463.

World Health Organization (WHO)., 2003. Diet, nutrition and the prevention of chronic diseases. Report of a Joint FAO/WHO Expert Consultation, WHO Technical Report Series no. 916, Geneva.

Witomska, M., Lukaszewska, A., Tyzkiewicz, A., 2008. *In vitro* storage of *Hosta Tratt.* Cultures. Journal of Fruit and Ornamental Plant Research 16, 371-382.

Xinrun, Z., Conner, A.J., 1992. Genotypic effects on tissue culture response of lettuce cotyledons. Journal of Genetics and Breeding 46, 287-90.

Xu, K., Chang, Y., Liu, K., Wang, F., Liu, Z., Zhang, T., Li, T., Zhang, Y., Zhang, F., Zhang, J., Wang, Y., Niu, W., Jia, S., Xie, H., Tan, G., Li, C., 2014. Regeneration of *Solanum nigrum* by somatic embryogenesis, involving frog egg-like body, a novel structure. Public Library of Science 9, 1-8.

Yousaf, Z., Shinwari, Z.K., Khan, M.A., 2010. Phenetic analysis of medicinally important species of the genus *Solanum* from Pakistan. Pakistan Journal of Botany 42, 1827-1833.

Zhao, X.Y., Su, Y.H., Zhang, C.L., Wang, L., Li, X.G., Zhang, X.S., 2013. Differences in capacities of *in vitro* organ regeneration between two *Arabidopsis* ecotypes Wassilewskija and Columbia. Plant Cell, Tissue and Organ Culture 112, 65-74.

Zimmerman, M.B., Hurrell, R.F., 2002. Improving iron, zinc and vitamin A nutrition through plant biotechnology. Current Opinion in Biotechnology 13, 142-145.

Zucco, M.A., Walters, S.A., Chong, S., Klubek, B.P., Masabni, J.G., 2015. Effect of soil type and vermicompost applications on tomato growth. International Journal of Recycling of Organic Waste in Agriculture 4, 135–141.