Screening, selection and clonal propagation of *Amaranthus dubius* genotypes with different calcium and iron content

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January 2018

Screening, selection and clonal propagation of *Amaranthus dubius* genotypes with different calcium and iron content

by

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Sumbitted in fulfilment of the academic requirements for the degree of Master of Science in the School of Life Science, University of KwaZulu–Natal, Durban, South Africa.

January 2018

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

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ABSTRACT

Decontamination of *Amaranthus dubius* field-derived nodal explants was achieved in a 10 min soak with 1% (v/v) NaOCl and 2 drops of Tween 20® followed by three rinses in sterile distilled water, immersion in an antibiotic solution (¼ strength Murashige and Skoog basal salt medium, 50 µg l^{-1} rifampicin, 100 µg l^{-1} streptomycin/penicillin), at 1500 rpm for 5 h. Of the tested plant growth regulators, MS media supplemented with 2 mg l^{-1} benzylaminopurine (BAP) + 0.5 mg l^{-1} indole-3-acetic acid (IAA), and 0.1 mg l^{-1} IAA, respectively, gave the best *in vitro* responses of 4 shoots/nodal explant and 100% rooting. Plantlets were acclimatised over 21 days (d) in soil (S) and 1soil:1vermiculite (v/v) (1S:1V) substrates; a significant increase in the number of leaves occurred up to 21 d (6.8 to 16.2 in S and 6.3 to 13.1 in (v/v) 1S:1V). At 21 d in (v/v) 1S:1V, there were more leaves than in S, in contrast longer plant height and root length were observed in S than in (v/v) 1S:1V. The post-acclimatisation yield was 2 plants/nodal explant.

The variation in calcium (Ca) and iron (Fe) content within a population of greenhouse-germinated *A. dubius* seedlings was then evaluated, and specific genotypes were selected to investigate the effects of micropropagation, acclimatisation in S and (v/v) 1S:1V and physiological age (time) on their growth and Ca and Fe accumulation. After 60 d, using inductively coupled plasma-optical emission spectrometry, the content of leaf Ca ranged from 246.3 to 765.3 mg 100 g^{-1} dry mass (DM) and the Fe from 5.3 to 26.7 mg 100 g^{-1} DM. Based on the significant differences of these levels amongst the parent genotypes seven were selected and were 'ranked' as G47 > G45 > G11 > G41 = G8 > G39 > G15 for Ca and as G47 = G45 > G39 = G41 > G8 > G15 > G11 for Fe.

Nodal explants of the selected parent genotypes were subjected to the established micropropagation protocol (using S and (v/v) 1S:1V during acclimatisation). The post–acclimatisation yield was 2 to 4 plants/nodal explant. Over the 21 d of acclimatisation in the two substrates, there were clear genotypic effects on all the tested growth parameters in S. There were significant increases in S–grown plants in the number of leaves of G39 (7.0 to 10.6) and G47 (7.0 to 13.3), the plant height of G11 (5.6 to 12.3 cm) and the root lengths of G8 (6.6 to 17.3 cm) and G41 (10.0 to 16.6 cm). When grown in (v/v) 1S:1V, the plant height significantly increased from d 0 to 21 for G8 (7.0 to 12.3 cm) and G47 (6.3 to 10.6 cm). With regards to the effect of substrate, only the clones of G8 preferred nutrient-poor soil to produce more leaves (8) than when grown in S at d 21 (5). After transferring the clones to the greenhouse for 90 d, no significant differences in the root:shoot dry masses amongst the clones

were observed on each substrate, and the substrate had no effect on the root:shoot dry mass for each genotype.

After acclimatisation and transfer of the clones into the greenhouse it was observed that both physiological age (time, i.e. 15, 30, 60, 80 and 90 d) and substrate influenced their accumulation of Ca and Fe. Over time, in both substrates, the Ca content increased while Fe content decreased. Significant interactions were found between the genotype and substrate for both Ca and Fe, and between physiological age (time) and genotype for Fe only. In S, the clones of all the parent genotypes matched the Ca content of their parents at 15 d while for Fe that of five of the seven selected genotype clones were similar in Fe content to their parents at 60 d. Clones of four of the seven selected parent genotypes accumulated higher Ca and Fe levels when grown in (v/v) 1S:1V than in S at certain time intervals. In S, the Ca 'rankings' of all the clones did not match their respective parent genotypes at any time interval while in the case of Fe, the clones of two genotypes in S (G47 and G11) and one genotype in (v/v) 1S:1V (G47) matched their respective parents between 60 to 90 d of growth in the greenhouse.

In conclusion, nodal explants of the selected *A. dubius* genotypes with varying Ca and Fe contents, were clonally propagated *in vitro* using BAP and IAA and the yield after acclimatisation was 2 to 4 plants/nodal explant. The physiological age (time) and substrate affected the number of leaves of the cloned genotypes, whilst in the case of Ca and Fe, these levels were influenced by micropropagation, physiological age and substrate type. Phenotypic plasticity can be further evaluated by exposing the clones of the selected genotypes to varying water, salinity and heat stresses. Additionally, investigations to understand the clones' ability to accumulate Ca and Fe would be valuable, in this regard, quantifying inhibitory factors and exploring the effects of substrate properties such as pH and porosity are suggested.

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DECLARATION 1-PLAGIARISM

I, **Phindile Dladla,** declare that

1. The research reported in this thesis, except where otherwise indicated, is my own research.

2. This thesis has not been submitted for any degree or examination at any other university.

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PREFACE

The experimental work described in this thesis was conducted at the University of KwaZulu–Natal, School of Life Sciences under the supervision of Professor M. P. Watt and Dr. S. Shaik.

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.

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ACKNOWLEDGEMENTS

I wish to acknowledge and express my sincere appreciation to the following individuals and institution:

1. My supervisors Dr. Shakira Shaik and Prof. Paula Watt for their support, guidance and advice throughout this journey and even when I struggled with the work, they were patient and guided me through.

2. The staff at the School of Chemistry, Mrs Anita and Ms uNathi for their help and guidance with the calcium and iron analysis.

3. To my friends Nomphu, Pamela, Khetha and Njabulo for their constant support throughout this journey, through words of encouragement and listening patiently to all my frustrations.

4. My family, oMpembe, oZibula kaDele, oGadlela, oDwala abagabadela izikundla zwakwaBulawayo baphemba uzwathi. maDwala ami amMlophe ngiyabonga ngakho konke. KuBaba noMa, uSiphiwe, benoNtombizodwa, osisi bami, uSibongile, benoSamkelisiwe nezithandwa zami oMelokuhle beno Uminathi ngiyabonga, kwande.

5. To my Lord and saviour Christ Jesus who has carried me throughout all the difficult times in this journey. Praise be to God, He is worthy to be praised.

6. Finally, I would like to thank the National Research Foundation of South Africa for their financial support in the form of a scholarship.

Ngimubonile umMhlengi wami engilwelwa, udumo malube kuye uKristu Jesu

LIST OF ABBREVIATIONS

%	percent
°C	degrees Celcius
µmol m^{-2} s^{-1}	micro moles per square meter per second
1S:1V	1soil:1vermiculite (v/v)
ALV	African leafy vegetable
ANOVA	Analysis of Variance
BAP/BA	benzylaminopurine
Ca	calcium
cm	centimeter
d	day(s)
Fe	iron
g l^{-1}	grams per litre
h	hour(s)
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ICP–OES	inductively coupled plasma-optical emission spectrometry
KZN	KwaZulu–Natal
min	minute(s)
mg 100 g^{-1}	milligrams per 100 gram
ml	milliliter
MS	Murashige and Skoog
NAA	naphthaleneacetic acid
NaOCl	sodium hypochlorite
PGRs	plant growth regulators

S	soil
s	second(s)
t	time

Chapter 1. Introduction and rationale for the study

Approximately 800 million people are suffering from hunger in the world with 204 million cases occurring in Sub–Saharan Africa alone (Bain et al., 2013). Malnutrition or hidden hunger is a broad term used to describe undernutrition which is the insufficient dietary intake of nutrient rich foods (Bain et al., 2013; Ismail and Suffla, 2013). Children under the age of five are often the subjects of malnutrition, with many of them suffering from micronutrient deficiencies that result from undiversified diets that are cereal based and often lacking in iodine, vitamin A, iron (Fe), calcium (Ca), zinc (Zn) and other micronutrients (Lopriore and Muehlhoff, 2003; Faber, 2005; Faber and Wenhold, 2007; Oyedeji et al., 2014; Mayekiso et al., 2017). Majority of these children experience birth defects such as blindness, infection, anaemia, impaired physical and mental growth, and premature death as a result (MacIntyre and Labadarios 1999; Bonti–Ankomah, 2001; Lopriore and Muehlhoff, 2003).

Micronutrient deficiencies in many communities are mainly the outcome of physiological, pathological and socio–economic conditions (Vorster et al., 2007; Mavengahama et al., 2014), which can be addressed through fortification. Several initiatives in the form of food fortification and supplementation have been attempted to mitigate the effects of malnutrition such as government school feeding schemes and the encouragement of the one home one garden initiative (Kuyper et al., 2013). Fortified foods are enriched with micronutrients through genetic engineering or agronomy practices (Mavengahama et al., 2014). However, fortified foods are generally more expensive than unfortified foods and may be inaccessible to the majority of the populations in rural communities (Mavengahama et al., 2014). Green leafy vegetables have been identified as alternative food sources rich in essential micronutrients such as Ca and Fe, vitamin A and riboflavin (Modi et al., 2006; Achigan–Dako et al., 2014). Calcium for example, is a necessary nutrient for bodily functions such as nerve functions, it prevents blood clotting and most important is its role in building strong bones and maintaining strong teeth. Even though Ca is the most abundant micronutrient in our bodies we must continuously consume foods rich in Ca to obtain the required amount for metabolic processes as we cannot produce it on our own (Michaelsen et al., 2009; Sambrook, 2017). Similar to that of Ca, the consumption of Fe rich foods is of great importance, primarily because Fe is required for the red blood cells to transport oxygen in the body. Often the results of a diet that is Fe-poor are Fe deficiencies such as anaemia, a deficiency that can be fatal in infants and pregnant mothers (Martin, 2016).

As a result, research interests in underutilised crops and dark leafy vegetables have increased with the hope that these vegetables can aid in alleviating the effects of malnutrition in rural communities (Nesamvuni et al., 2001; Uusiku et al., 2010; Faber et al., 2010; van Rensburg et al., 2014; Mabhaudhi et al., 2017). These vegetables are referred to as African leafy vegetables (ALVs), a group of leafy green vegetables that are inexpensive and high in micronutrient content (Gockowski et al., 2003; Modi et al., 2006; Odhav et al., 2007). Although these vegetables are considered to be underutilised and their full potential in alleviating malnutrition remains unexploited (Kumar et al., 2014; Ebert, 2014; Njume et al., 2014), several of these species have been cultivated as vegetables and grain sources in some countries and are included in the diets of many communities in Africa, Bangladesh, Caribbean, China, Greece, India, Nepal and South Pacific Islands (Svirskis, 2003). However, of recent, the consumption of these vegetables has decreased because they are labelled as 'poor man's food' and knowledge associated with them as backwards thinking, therefore, the promotion of ALVs is essential in the effort of mitigating malnutrition (Smith and Eyzaguirre, 2005; Odhav et al., 2007; Dweba and Mearns, 2011). In South Africa alone, a wide variety of leafy vegetables can be found *viz. Amaranthus cruentus, Cleome gyandra, Corchorus olitorius, Cucumis melo, Cucurbita pepo, Momordica balsamina, Solanum retroflexum* and *Vigna unguiculata* to mention a few of the popular species (van Rensburg et al., 2007). It is speculated that these vegetables have several advantages over their cultivated varieties e.g. micro–macronutritional levels, medicinal properties, high concentrations of secondary metabolites such as antioxidants, high seed production rates, short growth period and resistance to abiotic and biotic environmental stresses (Svirskis, 2003; Mwai et al., 2007; Odhav et al 2007; Uusiku et al., 2010; Rastogi and Shukla, 2013; Achigan–Dako et al., 2014; Mabhaudhi et al., 2017). As a result, several studies have assessed and determined the micronutrient contents, vitamins and anti – nutritional contents of some ALVs (Wehmeyer, 1986; Aletor and Adeogun, 1995; Khader and Rama, 1998; Kruger et al., 1998; Frieberger et al., 1998; Nesamvuni et al., 2001; Steyn et al., 2001; Aletor et al., 2002; Gupta et al., 2005; Odhav et al., 2007; Yang and Keding, 2009; Molina et al., 2011; Schönfeldt and Prestorius, 2011; Muriuki et al., 2014). Others have also investigated the effects of different environmental conditions on the micronutrient content of ALVs by exposing them to altering water regimes, drought tolerance, seasonal changes and geographical locations (Odhav et al., 2007; Priya et al., 2007; Molina et al., 2011; Edwards et al., 2016; Riebeiro et al., 2017).

In this context, *Amaranthus* species in particular *Amaranthus dubius* was of interest in the current study as it is widespread and easily available as an alternative food source due to the significant amount of Ca and Fe content exhibited by *Amaranthus* species in their leaves and shoots (Guarino, 1997; Modi, 2007; Odhav et al., 2007; Priya et al., 2007; Yang and Keding, 2009; van Rensburg et al., 2007; Molina et al., 2011). The genus *Amaranthus* consists of an approximate of 60 – 70 species with edible leaves and stems, the species are categorised as vegetables, weeds, grains or ornamentals (Brenner and Widrlechner, 1998; Das, 2012) and their uses differ in different communities. *Amaranthus dubius,* for example, is considered as a weed and/or a vegetable harvested for its leaves and stems in some Asian and African countries (Costea et al., 2004; Ebert, 2014). As a result, the cultivation of *Amaranthus* has received considerable attention, especially grain and leafy *Amaranthus* species. However, due to the genotypic variations that occur amongst a natural seedling population regarding several traits such as in their micronutrient levels, protein content, secondary metabolites and morphological traits to mention a few (Singh et al., 2009a; Lymanskaya, 2012; Andini et al., 2013a; Golabadi et al., 2013; Pereira et al., 2014; Mukamuhirwa et al., 2015; Štefúnovà et al., 2015), there is a need to screen populations of *A. dubius* to select genotypes with desired traits. Although ALVs are said to be resistant to abiotic and biotic stresses, several factors apart from the genotype can influence the resultant morphogenic responses (plant height, number of leaves, yield), micro–macro nutrient content (Ca, Fe, and P), secondary metabolites (phenolics, flavanoids), anti–nutritional compounds (oxates, phytate, tannins), species type, geographical location and soil substrate can all alter the above responses of the plants (Srivastava, 2015). In this regard, it is important to select genotypes with specific desired traits such as Ca and Fe content for their selection and mass propagation to rear out into communities plagued by micronutrient malnutrition (Dlamini et al., 2010).

Conventional and biotechnological tools have been exploited to propagate ALVs. Conventional methods of cultivation are mainly through seed and vegetative (cuttings) growth but these methods often produce low output yields (Nuugulu, 2013). In this regard, *in vitro* techniques have been identified as an alternative method for the propagation, conservation and delivery of large quantities of genotypes of interest (Dubois, 2009). *In vitro* culture techniques are one of the most basic biotechnological tools used for the rapid production of clonal plants selected for traits of interest such as high micronutrients (Dubois, 2009). Micropropagation makes use of single explants for *in vitro* vegetative multiplication through the application of plant growth regulators (PGRs) in the culture medium (George, 1993). This often results in the

production of genetically identical plants, making *in vitro* propagation ideal for the multiplication and conservation of selected genotypes and of ALV species (Dubois, 2009). Several authors (Flores et al., 1982; Bennici et al., 1992; van Le et al., 1998; Pannu et al., 2013) have successfully established micropropagation protocols for *Amaranthus* through organogenesis, where plants arise directly from the meristem or indirectly from undifferentiated cell masses (callus) (George et al., 2008). These protocols were successfully established by the use of different explants (leaves, stems, hypocotyls and nodes) and the incorporation of PGRs in the media, which if successful can result in the establishment of *in vitro* shoots and roots in culture and thereafter, result in whole plants.

The effect of micropropagation on the micronutrient content of *in vitro* grown *Amaranthus* genotypes has not been investigated. Further, little has been done to ascertain the optimum stage of maturity for harvest of genotypes in relation to their Ca and Fe levels, which can be affected by several factors as mentioned above such as the genotype, species type, physiological age of the plant, the amount of anti–nutritional levels found in the plant (phytate, oxalic acid, tannins etc), the environmental conditions and substrate type to mention a few. Studies by Khader and Rama (1998), Khader and Rama (2003), Modi (2007), Odhav et al. (2007), Molina et al. (2011) and Ribeiro et al. (2017) have reported that some of the above factors have influenced the Ca and Fe levels exhibited by *Amaranthus* species. For this reason, this study set out to establish a micropropagation protocol from nodal explants of *A. dubius*, screen a natural population of *A. dubius* seedlings and select genotypes with desired the Ca and Fe levels and thereafter, determine the effects of micropropagation, physiological age and substrate type on the Ca and Fe content of the selected genotypes.

The objectives of this study were as listed below.

1) To establish a micropropagation protocol for *A. dubius*.

The efficacy of different combinations of sterilants to eliminate contaminants from nodal explants in culture was established. Then the most appropriate concentrations and combinations of plant growth regulators were selected to multiply nodal explants that would produce *in vitro* shoots and roots. Plants were then acclimatised on two substrates, soil (S, i.e. nutrient–rich) and soil:vermiculite (v/v) (1S:1V, i.e. nutrient–poor). Since all the pot sizes were identical in experiments comparing results from S to 1S:1V, the 1S:1V substrate was considered nutrient-poor because the amount of soil in the substrate was half that in the substrate containing S only.

2) To screen seedlings of *A. dubius* for the selection of genotypes with desired Ca and Fe content.

Seeds were germinated in S and after 60 days (d) leaves were harvested for micronutrient content (Ca and Fe) analysis using ICP–OES (Inductively Coupled Plasma–Optimal Emission Spectrometry, Perkin-Elmer, Germany). Seven genotypes (referred to as parent genotypes) were selected based on their Ca and Fe content for micropropagation studies. These were G8, G41, G45 and G47 for high Ca and Fe content, G15 for low Ca and Fe levels and G39 for high Fe and low Ca content and G11 for high Ca and low Fe.

3) To determine the effects of micropropagation, physiological age (time) and substrate type on the Ca and Fe content in the clones of the selected genotypes.

The clonally propagated parent genotypes were thereafter analysed for their Ca and Fe content while in the greenhouse, in S, to determine if they exhibited similar Ca and Fe levels to those of their respective parent genotypes at 60 d, following acclimatisation during 90 d of growth in the greenhouse.

Additionally, the leaves from the selected cloned genotypes were harvested at d 15, 30, 60, 80 and 90 and analysed by ICP–OES when grown in both substrates (S, i.e. nutrient–rich or (v/v) 1S:1V, i.e. nutrient–poor) to determine the effect of physiological age. Furthermore, the effect of substrates was observed to determine which substrate produced clones with higher Ca and Fe levels when compared to each other at each time interval.

Chapter 2. Literature Review

2.1 Background and importance of African leafy vegetables

Malnutrition is divided into two categories, overnutrition and undernutrition, which are both common in South Africa (de Klerk et al., 2004; Bain et al., 2013). Overnutrition is due to the high dietary intake of energy and macronutrients whereas undernutrition is the opposite (Bain et al., 2013). Several deficiencies such as iron (Fe), vitamin B–1, B–3, B–9, D and calcium (Ca) are the result of malnutrition and are detrimental to both young children and adults. These deficiencies are often prevalent in South African households that are financially struggling which leads to a lack of accesses of attaining sufficient quantities and varieties of high quality, micronutrient–rich and affordable foods such as green leafy vegetables, red meat, and fruits (Steyn and Herselman, 2005; Charlton and Kalula, 2015).

In South Africa, malnutrition is perpetuated by drought, flooding, and political conflicts and these factors contribute to food shortages resulting in instabilities in food security (White and Broadley, 2009). Although South Africa is a food self–sufficient country due to the commercial production and exportation of agricultural produce, it is equally challenged with food insecurity especially among poor households in rural areas (Bonti–Ankomah, 2001; de Klerk et al., 2004). More than 14 million people, approximately 35% of the South African population, are estimated to be vulnerable to food insecurity, with 1, 5 million children under the age 6 being stunted as a result of micronutrient malnutrition (Michaelsen et al., 2009). In a study by Steyn and Herselman (2005), the authors determined which deficiencies were common in KwaZulu–Natal (KZN) and found four deficiencies that frequently occurred amongst a rural community *viz*. vitamin A, Fe, zinc (Zn) and iodine (I).

To reduce the effects of micronutrient malnutrition several strategies in the form of fortification, feeding schemes in schools and the promotion of planting vegetables in home gardens amongst other initiatives have been attempted (Freedman, 2015). White and Broadley (2009); Bouis and Welch (2010), Thavarajah et al. (2011) and Mavengahama et al. (2014) agree that there is no single method of combating malnutrition but that a combination of methods must be used to increase and/or add micronutrients into the daily diets of the rural and poor. Food fortification, genetic biofortification, agronomic biofortification and dietary diversification are some of the methods in practice. Food fortification is the enrichment of foods by adding necessary micronutrients to crops, but, this is expensive and inaccessible to

most rural communities (Mavengahama et al., 2014). Agronomic biofortification involves the supplementation of minerals directly to vegetables, fruits and crop species through foliar applications or indirectly through the application of fertilisers and the improvement of mobilisation of minerals in the soil (White and Broadley, 2009; Bouis and Welch, 2010). The application of fertilisers to increase micronutrient availability in crops can be assisted by the selection and breeding of crops that have an increased ability to accumulate micronutrients from the soil and translocate them to the leaves for readily consumption (White and Broadley, 2009, Bouis and Welch, 2010). Genetic biofortification is mainly in the experimental stages, but uses plant breeding techniques for the selection of genotypes with desired traits mainly through marker assisted selection or genetic engineering (Mavengahama et al., 2014). Dietary supplementation is the ingestion of a product intended to add further nutritional value, either as single component or in combination with vitamins and mineral (Mavengahama et al., 2014). Martorell et al. (2015) investigated the effectiveness of fortified food in relation to anaemia in women and children in Costa Rica. Those authors' study consisted of fortifying wheat with ferrous fumarate, an Fe source and they found that the prevalence of anemia in children and women dropped significantly at a national level in the country. Evidence that fortified foods can mitigate certain forms of deficiencies if consumed appropriately. In South Africa, food fortification has made considerable strides, especially in staple foods, such as maize and wheat (Pretorius and Schönfeldt, 2012). In particular fortification in these staples aims to verify Vitamin A deficiency in the diets of South Africans. However, the inclusion of vitamin A has been said to influence the cost of maize, tripling the cost. Costs is increased because of the cost of vitamin B, extra equipment needed for mixing and quality control to ensure constant presence of vitamin B through quantitative analysis (Pretorius and Schönfeldt, 2012).

In addition to the fortification of foods, African leafy vegetables (ALVs) have been identified as alternative micronutrient rich food sources that are easily available for consumption of the majority of rural inhabitants and for the supplementation of diets of communities suffering from malnutrition (Andini et al., 2013b; Mayekiso et al., 2017). African leafy vegetables are a group of vegetables with high micronutrient content and can be used to help alleviate problems related to malnutrition (Smith and Eyzaguirre, 2005; van Rensburg et al., 2007; Mwaura et al., 2013; Njume et al., 2014; van Jaarsveld et al., 2014; Yang and Keding, 2009; Kamga et al., 2013). Research on ALVs has focused primarily on methods of cultivation, morphology and taxonomy, geographical distribution and micropropagation (Flores et al., 1982; Bennici et al., 1992; Modi et al., 2006; Achigan–Dako et al., 2014). Unlike commonly,

cultivated vegetables that plant breeders have selected and improved, research on the selection and cultivation for traits of interest among ALVs is limited (Gockowski et al., 2003).

Although, different tribes or groups of people around the world have collected and consumed leafy vegetables from the wild for many years, preserving seed for traits such as palatability and yield to be planted in the following seasons (Anonymous, 2010). Of recent, the consumption of ALVs has decreased due to the introduction and preference of western diets, also some people view ALVs as 'poor man's food' and associate knowledge of ALVs as backward knowledge (Dweba and Mearns, 2011; Taleni et al., 2012). Of recent, the micronutrient content of ALVs has become the main area of research interest with the aim of exploring their nutritional levels for the possible contribution in alleviating malnutrition in many rural communities. African leafy vegetables have been found to be good sources of protein, amino acids, micronutrients (Ca, Fe and magnesium [Mg] etc.), macronutrient phosphorus (P), anti-nutritional properties (tannins, oxalic acid etc.), beta-carotene and energy levels (Frieberger et al., 1998; Nesamvuni et al., 2001; van Rensburg et al., 2007; Venter et al., 2007; Nnami et al., 2009; Yang and Keding, 2009; Bian et al., 2014; van Jaarsveld et al., 2014).

Table 1.1 elucidates the micronutrient content of popular ALVs as documented by van Rensburg et al. (2007). The range of attainable energy is 81 to 319 kJ, with the most energy attained from a 100 g serving of *Corchorus olitorius*. *Mormordica balsamina* had the highest protein, fibre, Ca and Fe content of 11.29 g dry mass (DM), 29 g DM, 941 mg 100 g^{-1} DM and 60.3 mg 100 g^{-1} DM, respectively). *Cleome gyandra* had the highest amounts of beta-carotene, folate and vitamin C of 9.22 mg, 417.6 µg and 37 mg, respectively (Table 1). *Amaranthus dubius*, the species of interest in this study was found to have three different micronutrient contents from studies by Odhav et al. (2007), Yang and Keding (2009) and Molina et al. (2011). Odhav et al. (2007) found that a 100 g^{-1} serving of *A. dubius* to contained 205.16 kJ of energy, 4 g per 100 g^{-1} FM protein, 2.87 g 100 g^{-1} FM fibre, 1686 mg 100 g^{-1} DM Ca and 25 mg 100 g^{-1} DM Fe. In contrast, Yang and Keding (2009) reported 3.5 g per 100 g^{-1} FM of protein, 3.1 vitamin A, 582 mg 100 g^{-1} DM Ca and 3.4 mg 100 g^{-1} DM Fe. Furthermore, Molina et al. (2011) also recorded results that were different to those of the latter mentioned authors. They found that leaves of *A. dubius* contained 3014.65 mg 100 g^{-1} DM Ca and 96.15 mg 100 g^{-1} DM Fe. The differences in the Ca and Fe levels amongst these studies probably differ as a result of geographical location (seasonal differences), genotypes/cultivars, anti-nutritional contents exhibited by each genotype and environmental conditions (light intensity, water regimes,

fertilisers) (Keller and Hrazdina, 1998; Walters, 2005; Kopsell and Kopsell, 2008; Pérez-Balibrea et al., 2008; Mou, 2009).

2.2 *Amaranthus*

Amaranthus originated in America and is one of the oldest food crops in the world, with cultivation dating as far back as 6700 BCE (Anonymous, 2010; Eshete et al., 2016). Populations of *Amaranthus* have spread to other continents and countries such as Africa, India, Mexico, Spain, and Nepal (Anonymous, 2010). The genus consists of varieties that are cultivated as leafy vegetables, grains or ornamental plants, with some of the species classified as weeds. *Amaranthus* is an annual crop with one growing season and is categorised as a C_4 photosynthetic plant with a polyploid level of 2n = 32, except for *A. dubius* which has a polyploid level of 2n = 64 (Das, 2012). These factors contribute to species resilience to drought, disease, pests, heat and its high micronutrient value in the seeds, stems and leaves (Priya et al., 2007; Achigan–Dako et al., 2014).

The environmental growth conditions in which the *Amaranthus* species can survive vary, as they are highly tolerant to altering temperatures and drought conditions. However, they are also well adapted to sub-tropical and tropical environments and grow well in summer (Anonymous, 2010). At temperatures between 18 °C and 25 °C seed germination is optimal but at temperatures below 18 °C growth discontinues (Anonymous, 2010). van Jaarsveld et al. (2014) reported *Amaranthus* to be one of the most popular leafy vegetables in Africa, with the most popular species being *A. hypochondriachus, A. tricolor*, *A. hybridus* and *A. blitum* that are consumed primarily because of their flavour, nutritional content and palatability.

2.2.1 Plant description and uses of *Amaranthus*

2.2.1.1 *Amaranthus* species description

Species from the genus *Amaranthus* are commonly known as “red herb” and/or “pig weed” in English, “imbuya” and/or “imifino” in isiZulu or isiXhosa and “morogo” in Vhenda or Sotho across the different communities and languages in South Africa (van Rensburg et al., 2007; van Jaarsveld et al., 2014). These vegetables grow spontaneously in many urban and rural areas, making them highly accessible and are inexpensive for the poor (Modi et al., 2006; Gido et al., 2017). The genus includes different varieties which grow as weeds, garden flowers, ornamentals or crops. The species can be classified into three categories, vegetable

Amaranthus, e.g. *A. tricolor*, grain *Amaranthus*, e.g. *A. cruentus* and weed *Amaranthus* e.g. *A. dubius* (Das, 2012).

2.2.1.2 Morphology and taxonomy

The genus *Amaranthus* consists of approximately 60 – 70 species that are C4 dicotyledonous herbaceous plants that have an erect growth habit (Xu and Sun, 2001; van Rensburg et al., 2007; Brenner et al., 2010; Oduwaye et al., 2014; Parra-Cota et al., 2014; van Rensburg et al., 2014; Ohshiro et al., 2016). The genus consists of at least 17 species with edible leaves and three grain (*A. caudatus, A. cruentus* and *A. hypochondriacus*) species that are grown for their seeds. Morphological differences within a genus can induce variations in the phenotype of each species. This evident in that *Amaranthus* species vary in their seed colour, leaf colour and shape, stem diameter and flower colour (Tisserat and Galletta, 1988; Shukla et al., 2010; Sogbohossou and Achigan– Dako, 2014; Gerrano et al., 2014; Gerrano et al., 2015). The height of the mature plants varies between 0.3 to 2 m depending on the variety, phenotype, environmental conditions and genetic variation (van Rensburg et al., 2007). Differences in basal branch length, inflorescence length, leaf width, petiole length, leaf number, terminal inflorescence, lateral length and number of branches per plant have been recorded in *Amaranthus* (Mwase et al., 2014).

The large and complex flowers that are arranged into clusters in auxiliary and terminal racemes are used to classify the grain *Amaranthus* species. Some varieties have green, crimson or red flowers, which are unisexual, sub-sessile with five sepals that are between 1 – 2 mm long, the seeds are obovoid, compressed, and have white, yellow or black colouration (Achigan–Dako et al., 2014). The leaves are categorised as broad leaves, they are variable in size, with the shape being lanceolate, alternate, simple with entire margins, green and red, orange or purple in colour with some having distinct markings. Grain *Amaranthus* can grow to heights between 1.52 m to 2 m when they are mature (Achigan–Dako et al., 2014). Vegetable *Amaranthus* are known by their inflorescence features in the form of short spikes, the origin of the flower bud from the leaf axil, its three tepal lobes, stamens, brownish black seed, and its unspecific growth habit (Achigan–Dako et al., 2014). They are believed to contain higher amounts of micronutrients and carotenoids (Mobina and Jagatpati, 2015).

Weedy *Amaranthus* have inflorescences which are spike-like or paniculate and glomerules that are isolated at the base of the inflorescence and clustered towards the apex (Achigan–Dako et al., 2014).

The leaves are ovate in shape, and have an alternate leaf arrangement; these traits are similar throughout the genus (Mwase et al., 2014). The female flowers have five sepals, and the fruit and the seeds are circular and black in colour (Achigan-Dako et al., 2014). One of the weedy species, *Amaranthus dubius*, has a purple stem and root colour, green inflorescence and the leaves have a trace of purple around the midrib. Grant (1959) identified *A. dubius* as the only known tetraploid species (n = 32) among weedy *Amaranthus*, with all others species being diploid (n = 16). Grant (1959) argued that ploidy levels play a major part in a plant's morphology, physiology and ecology, and is the reason some species succeed in different environments and can colonise new habitats therefore increasing their population size. Furthermore, weedy *Amaranthus* owes its wide genetic variability to their resistance to pesticides such as glyphosate that has led to the selection of highly resistant genotypes (Teaster and Hoagland, 2014).

Achigan-Dako et al. (2014) argued that monoecious weedy species belonging to the genus *Amaranthus* were superior in their ability to colonise new environments as they are self-compatible and self-pollinating which allows these species to adapt well in different environmental conditions. However, Sauer (1972) found weedy *Amaranthus* species which are dioecious, self-incompatible and widely distributed over different terrestrial environments to be as successful in colonisation as the monoecious species. Of the dioecious species *A. palmeri* is the most successful weed that inhabits areas of Europe, Mexico, Canada and different parts of USA (Grant, 1959; Costea et al., 2004; Das, 2012). This defines the advances weedy *Amaranthus* have as a superior genus capable of inhabiting different environments and adapting accordingly for survival.

Table 1.1: Nutrient content of commonly utilised African leafy vegetables (ALVs) in South Africa (adapted from van Rensburg et al., 2007)

ALV								Micronutrient content per 100 g^{-1} DM		
Botanical name	**Common name**	**Energy (kJ)**	**Protein (100 g^{-1} FM)**	**Fibre (100 g^{-1} FM)**	**Beta-carotene (mg)**	**Folate (µg)**	**Vit C (mg)**	**Ca (mg 100 g^{-1} DM)**	**Fe (mg 100 g^{-1} DM)**	**References**
Amaranthus cruentus	Pig weed	272	4.2	6.7	7.13	75	2	443	5.1	Yang and Keding (2009)
Brassica rapa	Chinese cabbage	120	2.5	2.2	3.59	92	8	152	1.4	van Jaarsveld et al. (2014)
Cleome gyandra	Spider flower	191	6.82	4.48	9.22	417.6	37	206	9.7	Nesamvuni et al. (2001)
Corchorus olitorius	Jews mallow	319	3.2	10.8	4.3	45	1	310	3.5	van Jaarsveld et al. (2014)
Cucumis melo	Melon	296	3.5	3.8	4.96	6.8	10	212	6.4	van Jaarsveld et al. (2014)
Cucurbita pepo	Pumpkin	222	2.9	3.0	4.25	47	2	177	9.2	van Jaarsveld et al. (2014)
Momordica balsamina	Balsam pear	222	11.29	29.0	–	–	4	941	60.3	Hanssan and Umar (2006)
Solanum retroflexum	Young nightshade	81	3.65	2.73	7.56	5.2	7.5	1.48	9.34	Nesamvuni et al. (2001)
Vigna unguiculata	Cowpea	280	4.7	5.8	7.03	105	2	177	9.2	van Rensburg et al. (2007)

DM: dry mass, kJ:Kilo jules, g: grams, mg: milligrams, µg: micrograms, vit C: vitamin C, Ca: calcium, Fe: iron

2.2.1.3 Medicinal uses

The medicinal uses of *Amaranthus* differ throughout tribal groups and communities globally. In Gabon, heated leaves are used to dress wounds, tumours, inflammations and as a diuretic (Achigan-Dako et al., 2014). Boiled roots with honey are used as a laxative for infants in Senegal. In Ethiopia *A. cruentus* boiled leaves are used as an expellant for tapeworms and in Southeast Asia, a decoction of the root is used to treat gonorrhoea and used as a vitamin and antibiotic (Achigan-Dako et al., 2014). In the Philippines, *Amaranthus* is used for its antiviral and anticancer properties (Ragasa et al., 2015). In Ghana, the water from soaked plants is used as a bath to treat pain in the limbs (Achigan-Dako et al., 2014). In Sudan, the ash from the stems is used as wound dressing (Achigan-Dako et al., 2014). In Southeast Asia and in Africa *Amaranthus* leaves and stems are used as a leafy vegetable that compete with spinach leaves in micronutrients and protein content (van Le et al., 1998). Seedlings, young leaves from mature plants, harvested at certain growth points are used to make stews or to add to curries (Achigan-Dako et al., 2014). In Tzaneen, South Africa, leaves and stems of *A. spinosis* are dried and ground for use as snuff (van Rensburg et al., 2007). In 1908 in some areas of Limpopo (South Africa), when salt was scarce, the whole plant of different *Amaranthus* species were dried out, burnt to produce ash, which was then dissolved in water and the precipitate of the filtrate of ash was used as salt (Fox and Young, 1983; van Rensburg et al., 2007). The oil found in *Amaranthus* seed helps people suffering from hypertension, cardiovascular disease, high blood pressure and cholesterol. Baral et al. (2011) also found the ground seed of *A. spinosus* to be useful as a dressing for broken bones, to treat internal bleeding, diarrhoea and excessive menstrual bleeding.

2.3 Nutritional value of *Amaranthus*

2.3.1 Ca and Fe levels in *Amaranthus*

Establishing the micronutrient content of *Amaranthus* species has been of interest to researchers from as early as 1986. Studies by Wehmeyer (1986), Aletor and Adeogun (1995), Freiberger et al. (1998), Kruger et al. (1998), Nesamvuni et al. (2001), Steyn et al. (2001), Aletor et al. (2002), Gupta et al. (2005), Odhav et al. (2007), Singh et al. (2009a), Yang and Keding (2009), Molina et al. (2011), Schönfeldt and Prestorius (2011) and Muriuki et al. (2014) have identified the micronutrient levels (Ca and Fe) which are beneficial to the human diet when incorporated regularly. A high amount of Ca and Fe is beneficial in the diets mainly because role they play in bodily functions. Fe is important for the transport of oxygen and has

a role in the functions of the brain, as a result, Fe deficiencies can cause mental retardation (Michaelson et al., 2009). Since dietary Fe is present in two forms, i.e. heme and nonheme Fe, both animal and plant sources are important to obtain the required Fe amounts. Of particular interest is nonheme Fe that uses the divalent metal transporter DMT1, which converts dietary ferric iron (Fe^{3+}) to (Fe^{2+}) before uptake. The absorption can be enhanced or inhibited by oxalic acid, polyphenols and phytates to mention a few (Michaelson et al., 2009).

Table 2 illustrates the Ca and Fe content (mg 100 g^{-1} DM) of *Amaranthus* species as documented by different authors. Amongst those studies, different methods for micronutrient analysis were used, with the most common being atomic absorption spectrometry (AAS) and inductively coupled plasma-optical emission spectrometry (ICP–OES). The former uses radiation to determine the micronutrient amount by reading the spectra produced when the sample is excited by radiation (Garcia and Bàez, 2012). The ICP–OES method is based on spontaneous emissions of photons from the sample that have been excited by radio frequency discharge (Hou and Jones, 2002). In Table 1.2 it can be seen that *Amaranthus* Ca content in the ranged from 0.94 mg 100 g^{-1} DM (*A. esculentus*, Nesamvuni et al., 2001) to 3931 mg 100 g^{-1} DM (*A. spinosus*, Odhav et al., 2007) and Fe content from 0.46 mg 100 g^{-1} DM (*A. esculentus*, Nesamvuni et al., 2001) to 96.15 mg 100 g^{-1} DM (*A. gradis*, Molina et al., 2011). This is evidence that amongst species and or genotypes/cultivars of the same species of vegetables, variations occur, in this case in the Ca and Fe levels (Singh et al., 2013; Oliveira et al., 2016; Wantanabe et al., 2016).

The species of interest to the present study, *A. dubius*, has been analysed by two methods, AAS and ICP–OES, to determine micronutrient content (Odhav et al., 2007; Yang and Keding, 2009; Molina et al., 2011). The Ca content from the leaves of *A. dubius* ranged from 582 to 1686 mg 100 g^{-1} DM and from 18.64 to 25 mg 100 g^{-1} DM (Odhav et al., 2007). In the study by Yang and Keding (2009), comparing the Ca, Zn and vitamin C content from five *Amaranthus* species, *A. dubius* was found to have the highest Ca (582 mg 100 g^{-1} FM), zinc (1.5 mg 100 g^{-1} FM) and vitamin C (78 mg 100 g^{-1}) levels than those exhibited by *A. blitum, A. cruentus, A. tricolor* and *A. viridis*. However, in a study by Molina et al. (2011) done in Venezuela, the authors investigated the effects of seasonal changes on the Ca and Fe levels of *A. dubius* found that the Ca content was 3014.65 and 3161.75 mg 100 g^{-1} DM, respectively, for the wet and dry season. The Ca and Fe documented by Molina et al. (2011) were considerably higher than those documented by Odhav et al. (2007) and Yang and Keding (2009). This is an indication of genetic variation amongst *A. dubius*, furthermore location seems to be a factor.

Due to the popularity of *Amaranthus* species, there are several studies that have quantified their micronutrient contents and either compared them to each other or with other ALV species. Nesamvuni et al. (2001) investigated the use and micronutrient contents of popular ALVs grown in Venda and found that *A. esculentus* was a poor source of Ca and Fe (0.94 and 0.46 mg 100 g^{-1} DM, respectively) when compared to those of *A. hyvridus* (3 and 9.77 mg 100 g^{-1} DM, respectively) and *A. standleyanus* (2.66 and 4.78 mg 100 g^{-1} DM, respectively). Steyn et al. (2001) conducted a study on the ALVs in the Northern Province of South Africa to determine which leafy vegetables were popular in that area. They found that two of the leafy vegetables they found exhibited the highest Ca content of 2171 mg 100 g^{-1} DM which was exhibited by *Momordica balsamina* followed by that of 479 mg 100 g^{-1} DM exhibited by *A. spinosus*. In a similar study by Aletor et al. (2002), *A. hybridus* was found to have a significantly higher Ca and Mg levels (699 and 694 mg 100 g^{-1} DM) than those in *Vernonia amygdalina* (524 and 731 mg 100 g^{-1} DM) and *Telfaira occidentalis* (521 and 779 mg 100 g^{-1} DM). Schönfeldt and Prestorius (2011) investigated the micronutrient levels of five ALVs (*A. tricolor*, *Cucurbita maxima*, *Cleome gyandra*, *Vigna unguiculata* and *Corchorus olitorius*) consumed in South Africa and found that the raw leaves of these ALVs contained high amounts Ca of Fe than in the cooked leaves.

2.3.2 Other micronutrients in ALVs

As emphasised earlier, most ALVs have high micronutrient contents that are found in their leaves. Some of these ALVs have higher Ca and Fe levels than those of *A. dubius,* the species of interest for this study. In a study by Nesamvuni et al. (2001) the authors compared the micronutrient content of popular ALVs in Venda and found that *Cleome gyandra,* had the highest protein (6.8 mg 100 g^{-1}), Ca (206 mg 100 g^{-1}), folate (418 µg 100 g^{-1}), vitamin C (37 mg 100 g^{-1}) and beta-carotene (9.22 mg 100 g^{-1}) content amongst the species (*A. esculentus, A. hybridus, A. strandleyanus, Bidens pilosa, C. gyandra, C. monophylla, Cucumis maxima. M. foetida Solanum retrflexum*).

In a similar study, Odhav et al. (2007) evaluated the nutritional content of twenty traditional ALVs (*A. dubius, A. hybridus, A. spinosus, Asystasia gangetica, B. pilosa, Centella asiatica, Ceratotheca triloba, Chenopodium album, C. monophylla, Cucumis metuliferus, Emex australis, Galinsoga parviflora, Justicia flava, M. balsamina, Oxygonum sinuatum, Physalis viscosa, Portulaca oleracea, Senna occidentalis, Solanum nodiflorum* and *Wahlenbergia undulata*) collected in the KwaZulu–Natal region. Those authors found that the Ca content of

all the species was relatively high with values ranging from 160 mg 100 g^{-1} DM (*E. australi*) to 3931 mg 100 g^{-1} DM (*A. spinosus*) and were from 11 mg 100 g^{-1} DM (*S. occidentalis*) to 1317 mg 100 g^{-1} DM (*A. hybridus*) for their Fe contents. The authors concluded that a because of the vast biodiversity we have in South Africa, adding more leafy vegetables to the diets of the rural poor could address malnutrition challenges.

The results from the above studies are an indication that the variations in the micronutrient levels could have been influenced by different environmental conditions, species type and or genotype. Therefore, there is a need to know which are the contributing factors and how do they alter the Ca and Fe content exhibited by ALVs.

Table 1.2: Calcium and iron content (mg 100 g^{-1} DM) of common *Amaranthus* species from different provinces in South Africa and countries in Africa.

Species	**Method of analysis**	**Ca (mg 100 g^{-1} DM)**	**Fe (mg 100 g^{-1} DM)**	**References**
A. albus	AAS	235.19	11.42	Muriuki et al. (2014)
A. aspera	ICP–OES	142.97	4.7	Singh et al. (2009a)
A. blitum	AAS	270	3	Yang and Keding (2009)
A. cauditus	AAS	3348	23.2	Wehmeyer (1986)
A. cruentus	AAS	305	3.8	Yang and Keding (2009)
A. cruentus	AAS	222.69	11.61	Muriuki et al. (2014)
A. dubius	AAS	582	3.4	Yang and Keding (2009)
A. dubius	ICP–OES	1686	25	Odhav et al. (2007)
A. dubius	AAS	3014.68	96.15	Molina et al. (2011)
A. esculentus	AAS	336.47	18.64	Muriuki et al. (2014)
A. esculentus	AAS	0.94	0.46	Nesamvuni et al. (2001)
A. gradis	AAS	2350	82.5	Wehmeyer (1986)
A. graecizans	AAS	1850	9.8	Wehmeyer (1986)
A. hybridus	ICP–OES	2363	21	Odhav et al. (2007)
A. hybridus	AAS	210	9.65	Aletor and Adeogun (1995)
A. hybridus	AAS	334	10.8	Wehmeyer (1986)
A. hybridus	AAS	3	9.77	Nesamvuni et al. (2001)
A. hybridus	AAS	198.48	10.57	Muriuki et al. (2014)
A. hypochondriacus	AAS	131.06	9.55	Muriuki et al. (2014)
A. sessile	ICP–OES	83.97	8.57	Singh et al. (2009a)
A. spinosus	AAS	479	14.4	Steyn et al. (2001)
A. spinosus	AAS	479	14.4	Wehmeyer (1986)
A. spinosus	ICP–OES	3931	32	Odhav et al. (2007)
A. standleyanus	AAS	2.66	4.78	Nesamvuni et al. (2001)
A. thunbergii	AAS	288	12.5	Wehmeyer (1986)

Table 1.2 (continued)

Species	**Method of analysis**	**Ca (mg 100 g^{-1} DM)**	**Fe (mg 100 g^{-1} DM)**	**References**
A. thunbergii	AAS	288	12.5	Steyn et al. (2001)
A. tricolor	AAS	358	2.4	Yang and Keding (2009)
A. tricolor	Ca: Precipitated as CaC_2O_4, then titrated by KMnO4, Fe: colorimetrically by dipyridyl method	239	15.01	Gupta et al. (2005)
A. viridis	AAS	410	8.9	Yang and Keding (2009)
A. viridus	ICP–AES	274	6.87	Freiberger et al. (1998)
Amaranthus sp.	AAS	2378	21	Kruger et al. (1998)

ICP–AES/OES: inductively coupled plasma – atomic/optical emission spectrometry, AAS: atomic absorption spectroscopy

2.4 Factors affecting micronutrient content and availability of Ca and Fe in the leaves of ALVs

The ability of ALVs to absorb different micronutrients from the soil is influenced by anti-nutrient factors such as oxalates, tannins and dietary fibre, genotype, environmental growing conditions, i.e. substrate type, micro and macroclimate, plant physiology, maturity of plant and postharvest handling and storage (Cornforth et al., 1978; Rangarajan and Kelly, 1998; Frossard et al., 2000; Gupta et al., 2005; Bergquist et al., 2006; Modi, 2007; van der Walt et al., 2009; Mellem et al., 2012; Priya and Santhi, 2014; Ohshiro et al., 2016). Modi (2007) investigated the effect of temperature and plant age (20, 40 and 60 days [d]) on the micronutrient content in five *Amaranthus* species (*A. hybridus* var. *cruentus, A. hypochondriacus, A. tricolor, A. thunbergii* and *A. hybridus*). The author established that Ca and Fe content differed with varying temperature conditions and physiological age. In hot temperatures (33/27 °C, i.e. day/night) the Ca range was 51 – 64 mg 100 g^{-1} DM and the Fe range was 45 – 64 mg 100 g^{-1} DM. In addition, he observed that increases in the physiological age of all the *Amaranthus* species resulted in an increase in Ca and Fe between 20 to 60 d and 20 to 40 d for Ca and Fe, respectively. The author concluded that *Amaranthus* should be grown in warm temperature (27/21 °C, i.e. day/night) conditions and that juvenile leaves should be harvested for consumption as they are richer in Ca and Fe. Although, both Ca and Fe increased with physiological age, the author recommends the consumption of juvenile leaves as it is likely that older leaves will be less palatable and have high anti-nutrients that might interfere with the availability of the micronutrients. In a similar study, Khader and Rama (1998) investigated the effects of physiological age (15, 30 and 45 d of growth in the field) on commonly grown leafy vegetables in India (*Amaranthus blitum, Amaranthus gongeticus, Portulaca oleracea, Hibiscus subdariffe, Hibiscus cannabinus* and *Spineces oleracea*). They found that the Mg and Fe levels of all the species increased and that Zn and copper (Cu) decreased with physiological age. The authors concluded that the reason why different micronutrients differed within the ALVs, was the result of their ability to accumulate and access micronutrients from the soil. In addition to the research in 1998, Khader and Rama (2003) investigated the effect of physiological age on the macronutrient, phosphorus (P) and on the micronutrients, Ca and Mg of *Amaranthus* species (*A. blitum* and *A. gongeticus*), *Hibiscus* species, *Portulaca oleracea* and *Oleracea* at three different physiological stages (15 and 30 d) and found similar results. The Ca and Mg contents increased as *A. blitum* matured, but P decreased with maturity. The authors suggested

that the reason why high amounts of Ca are found in older parts of the plants were due to the immobility and failure of Ca translocate from older plant parts to juvenile parts.

In addition to the effects of plant physiological age, the presence of anti-nutritional components in ALVs have been reported to effect the accumulation of Ca and Fe (Aletor and Adeogun, 1995; Gupta et al., 2005; Agbaire, 2012; Caglarirmak and Hepcimen, 2013; Udousoro et al., 2013; Bian et al., 2014). Anti-nutrients have negative effects on human health, and if consumed in large amounts i.e the consumption of foods with high oxalate content can affect oxygen transportation in the body resulting in blue baby syndrome (Mnkeni et al., 2007). As reported by Modi (2007), *Amaranthus* plants with high fibre, oxalate and phytate contents are likely to have less Ca available in the leaves at harvest because these anti-nutrients interfere with Ca absorption and lower its bioavailability in the leaves. Furthermore, the amount of anti-nutrients also varies amongst ALVs an indication that there will be times in the plants growth that will be favourable for Ca and Fe or anti-nutrient accumulation (Gupta et al., 2005)

Since plants acquire Ca from the soil, their roots must balance the delivery of Ca to the xylem, which can then enable the shoots of the plant to accumulate Ca in the leaves (He, 2016). However, mechanisms such as the presence of the Casparian band that acts a barrier for the movement of solutes in the soil may affect the translocation of Ca from the soil to the plant (White, 2001). In addition, there are genotypic variations in the ability of a plant to absorb and translocate micronutrients, this may also be responsible for the increase or decrease of micronutrients exhibited by the plant. Furthermore, the soil properties such as the cation exchange capacity (CEC), porosity and pH, may influence the ability of the soil to supply micronutrients to the plants. A high CEC influences the ability of the substrate to hold on to more micronutrients, making them sufficient in Ca, Fe, Mg and other cations (Sonon et al., 2014). In addition, the pH of the soil has been reported to have significant effects on the availability of Ca and Fe for plant uptake (Morrissey and Guerinot, 2009). In this regard, in soils that are aerobic or with a pH greater than 7.8, Fe availability can be compromised because Fe is often available in its insoluble form (Fe^{3+}) (Frossard et al. 2000; Morrissey and Guerinot, 2009). However, at lower pH Fe is unbound from the Fe^{3+} ion and becomes available for root uptake (Morrissey and Guerinot, 2009). Therefore, obtaining suitable soil pH is also of importance when considering factors that affect Ca and Fe availability.

It is apparent that the micronutrient content found in ALVs is affected by several factors *viz.* anti-nutrients, genotype, plant physiological age, geographical location, climatic conditions

(temperature) and soil pH to mention a few (Cornforth et al., 1978; Odhav et al., 2007; Kleiber et al., 2009; Agbaire, 2012; Pereira et al., 2014; van Jaarsveld et al., 2014; Mawoyo et al., 2017). Therefore, it is important to determine not only the micronutrient contents of ALVs, but also to investigate the role that the above mentioned factors have in reducing bioavailability of micronutrients (Ca and Fe).

2.5 Propagation of *Amaranthus*

2.5.1 Conventional propagation

Conventional propagation of *Amaranthus* is primarily through seeds which grow well in soil that is fertile and well-drained, and can be planted on other media including vermiculite (Flores et al., 1982; Olle et al., 2012). The seeds, which are fragile and small can be easily blocked from emergence if planted too deep or blocked by a thin crust of soil after some rain (Anonymous, 2010). In this regard, to germinate them the seeds must be placed on moist germination paper in plastic boxes at 2 – 5 °C for a month and thereafter moved to 20/30 °C growth chamber, where seeds germinate in 2 weeks (Brenner and Widrlechner, 1998). Most *Amaranthus* plants are day length sensitive and will grow to maturity, flower and produce seeds in less than 3 months if cultivated under a photoperiod of less than 12 hours (Brenner and Widrlechner, 1998).

2.5.2 Micropropagation of *Amaranthus*

African leafy vegetables are beneficial plants in terms of human nutrition because they supply vitamins, minerals, micronutrients and proteins to the human diet (Andini et al., 2013a). However, conventional propagation methods through seed prevent the selection, mass scale production of important genotypes and storage of large germplasms. The applications of *in vitro* culture techniques to ALVs have the potential to propagate large quantities of plants with traits of interest. The first successful culture of *Amaranthus* species was carried out by Flores et al. (1982), who developed *in vitro* culture systems for both vegetable and grain *Amaranthus* species. Those authors used leaf discs and hypocotyl segments that were two to three weeks old to produce callus which later produced abnormal roots on the leaf discs and shoots on hypocotyl derived callus. Since then various cultures of *Amaranthus* species have been established for micropropagation (Table 1.3). One of the major disadvantages of *in vitro* culture is the onset of contamination (bacterial or fungal) during culture (Bhojwani, 1990; Liefert and Waites, 1991).

Prior to obtaining plants with desired genotypes, a decontamination protocol must be established for the explant sources. Contaminants are the main reason for the loss of plants in culture and therefore need to be isolated and eliminated at an early stage. The propagation of disease free material through *in vitro* propagation may increase the ability of an explant to regenerate (Leifert and Waites, 1991; George, 1993). In this regards, studies on the elimination of contaminants through the incorporation of fungicides and antibiotics have been investigated (Kunneman and Faaj-Groenen, 1987; Niedz and Bausher, 2002; Mng'omba and Sileshi, 2012).

In this regards, several methods of limiting contaminants through the application of fungicides (de Oliveira et al., 2010; Orlikowska et al., 2017), the use of biocides (ethanol, calcium [CaOCl] or sodium hypoclorite [NaOCl]), and of mercuric chloride ($HgCl_2$) amongst surface sterilants (Leifert and Waites, 1991, Leifert and Cassells, 2001). In addition, soaking of explants in antibiotics (Phillips et al., 1981; Young et al., 1984; Falkiner 1997) or in 8-hydroxyqinoline-citrate (Orlikowska et al., 2017) have been found to be useful in eliminating bacterial contaminants. However, the effectiveness of decontamination treatments is dependent on the type of bacterial contaminant and ability of the antibiotic to penetrate the plant tissue (Frey–Klett et al., 2011). Environmental conditions, physical and chemical properties of the plant also influence the ability of the antibiotics to reduce bacterial contamination (Frey–Klett et al., 2011). In the case of using antibiotics, study by Haldeman et al. (1987) found that 10 µg cm^{-3} rifampicin and 1 g dm^{-3} benomyl were effective combinations in controlling bacterial and fungal contaminants in *Camelia* cultures. Reed et al. (1998), found streptomycin/gentamicin, timentin and gentamicin were effective in reducing the bacterial contaminants found in hazelnut cultures. de Oliveira et al. (2010), concluded that the use 500 mg L^{-1} cefotaxime effectively reduced the occurrence of bacterial contaminants in citrus cultures to less than 30%. Similarly, Rani and Dantu (2012) found that incorporating 100 mg l^{-1} cefotaxime reduced the bacterial contaminates in *Piper longum* cultures. Altan et al. (2010) discovered that 10 and 50 µg g^{-1} of benomyl in combination with nystatin were effective in reducing fungal contaminants in *Lilium* plants when explants were washed with these combinations prior to culture.

Successful control of contaminants in culture has been established for different explant with the use of antibiotics and fungicides incorporated in culture (Liefert and Waites, 1991; Reed and Tanprasent, 1995; Niedz and Bausher, 2002). Pannu et al. (2013) successfully surface sterilised leaves of *A. spinosus* with 0.5% (v/v) NaOCl for 15 – 20 min and had less than 10% contamination in culture. Flores et al. (1982) used 70% (v/v) ethanol for 2 min followed by

immersion in 10% (v/v) Clorox® for leaf explants of *A. cruentus*, *A. hypochondriacus* and *A. tricolor* and reported 0 – 5% contamination.

Several micropropagation protocols have been established for *Amaranthus* species, some are represented in Table 1.3. The authors of these studies have mainly used leaf explants to produce callus and then shoots followed by rooting. In the current study, nodal explants have been chosen for micropropagation studies, and will use the established protocols as guidelines. This has been accomplished by the use of plant growth regulators (PGRs) in different concentrations and ratios to manipulate the explants (George, 1993). In *Amaranthus* species, as in all species, the manipulation of the concentrations and ratios of auxins and cytokinins in the medium result in either callus induction, shoot multiplication and/or rooting. Hence, in direct organogenesis shoot multiplication occurs when both auxin:cytokinin are present in the medium, whereas root formation occurs in the presence of auxin alone (George, 1993; George et al., 2008). Indirect organogenesis induces the dedifferentiated tissue in the presence of auxins to form callus prior to shoot proliferation (George, 1993). *Amaranthus* plants cultured in media with a low cytokinin ratio induces callus, high cytokinin and low auxin ratios induce shoots through organogenesis and high auxin and low cytokinin induces *in vitro* roots.

Bennici et al. (1992) and Bennici et al. (1997), used indirect organogenesis as a means of propagation of different *Amaranthus* species. They suggested that the multiplication of explants in culture can produce superior genotypes that are resistant to abiotic and biotic stress and superior in specific amino acids. Explants from *A. caudatus, A. cruentus,* and *A. hypochondriacus* produced callus in 2.3 μM 2,4-D + 2.3 μM kinetin and NAA (1 – naphthaleneacetic acid) + BA (benzylaminopurine). However, a combination of NAA plus BA did not induce callus in *A. caudatus.* Shoot multiplication on medium supplemented with 1 mg l^{-1} IAA (indole-3-acetic acid) + 1 mg l^{-1} BAP resulted in a yield of 10 shoots/leaf explant. In this regard, the authors reported that regeneration of callus in *Amaranthus* explants is dependent on physiological age of explant, PGR combination and genotype. Those authors found the most suitable basal salt medium for shoot regeneration was MS medium supplemented with kinetin and IAA or BAP and IAA, or B5 medium supplemented with kinetin and NAA or MS medium supplemented with NAA or BAP. *In vitro* rooting was best on half strength MS with IBA (indole butyric acid).

Pannu et al. (2013) compared the antimicrobial activity in *in vivo* and *in vitro* grown *A. spinosus* plants. Shoot regeneration from callus of leaf explants and nodal explants from media

containing different PGR concentration of NAA, BA and IAA was investigated. The leaf explants produced 8.66 shoots/leaf explant and 93.3% shoot regeneration was observed in cultured in medium containing 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA. Shoot regeneration of nodal explants also resulted in 93.3% shoot regenerated in 0.5 mg l^{-1} BA + 0.1 mg l^{-1} NAA but no recording of the number of shoots/nodal explant were determined. Thereafter, root regeneration in medium containing 0.2 mg l^{-1} IBA resulted in 66.6% root induction.

Although different concentrations and combinations produce varied *in vitro* responses, it is evident from the studies above that *Amaranthus* species can regenerate shoots and roots at low concentrations of cytokinins and auxins (Bennici et al., 1992). The differences in species response is also dependent on the morphogenic route of propagation.

Table 1.3: Examples of studies utilising different morphogenic routes for *Amaranthus* micropropagation

Species	Explant type	Morphogenesis route	Basal salt	PGR	Results	References
A. cruentus, hypochondriacus, tricolor	Leaves	Indirect organogenesis	Gamborg et al. (1968)	0.1 mg l^{-1} NAA + 0.1 mg l^{-1} Zeatin	Callus produced shoots	Flores et al. (1982)
A. cruentus, hybidus, hypochondriacus	Stem segments	Indirect organogenesis	Murashige and Skoog (1962) with vitamins	3 mg l^{-1} Kinetin + 0.3 mg l^{-1} IAA	Callus produced shoots	Bennici et al. (1992)
	Stem segments	Indirect organogenesis	B5 medium	0.5 mg l^{-1} Kinetin + 0.1 mg l^{-1} NAA	Callus produced shoots	Bennici et al. (1992)
	Stem segments	Indirect organogenesis	Murashige and Skoog (1962) with vitamins	2 mg l^{-1} 2iP + 0.5 mg l^{-1} NAA OR 0.5 mg l^{-1} NAA + 1 mg l^{-1} BAP	Callus produced shoots	Bennici et al. (1992)
A. edulis	Thin cell layers	Somatic embryogenesis	Murashige and Skoog (1962)	2 µM TDZ and 10 µM CPPU	Callus produces buds then shoots	van Le et al. (1998)
A. spinosus	Leaves	Indirect organogenesis	Murashige and Skoog (1962) with vitamins	0.2 mg l^{-1}BA + 0.5 mg l^{-1} NAA	Callus produced shoots	Pannu et al. (2013)
	Nodal	Direct organogenesis	Murashige and Skoog (1962) with vitamins	0.5 mg l^{-1}BA +0.1 mg l^{-1} NAA	Shoots	Pannu et al. (2013)

PGR: plant growth regulator: NAA: naphthaleneacetic acid, BA:6-benzylaminopurine, IBA:indol-3-butyric acid, TDZ: thidiazuron, CPPU: forchlorfenuron, SE: somatic embryogenesis

Chapter 3. Materials and methods

3.1 Plant material

Whole plants of *Amaranthus dubius* (25 – 30 cm stem height) were collected from Randles' Nursery, Durban, South Africa (29° 49`` S, 30° 58`` E) (Fig. 3.1). Thereafter, the plants were transplanted into flower pots (17.5 cm diameter, 20.5 cm height) (Grovida, South Africa) in soil (Fig. 3.2) (Grovida, South Africa) and kept in a shade house at the School of Life Sciences, University of KwaZulu–Natal, Durban, South Africa (29° 52`` S, 30° 59`` E; 25°C day (d)/18°C night). Plants were watered three times daily for 3 min each with municipal water through an overhead irrigation system (Grovida, South Africa). Plants were sprayed with fungicides bi-weekly and fertilisers on a weekly basis (Table 3.1). The fungicides used were mixtures of 2 g l^{-1} Dithane (Efeckto, South Africa) and 1 ml l^{-1} Supremo (Grovida, South Africa) applied as a foliar spray and a mixture of 1 ml l^{-1} Chronos (Makhteshin-Agan, South Africa) and 1.25 ml l^{-1} Orius (Makhteshin-Agan, South Africa) applied to the soil. The fertilisers used were 1 ml l^{-1} of the trace element solution Trelmix (21.3 g l^{-1} Fe, 3 g l^{-1} Cu, 2.7 g l^{-1} Mn, 2.3 g l^{-1} Zn, 1.0 g l^{-1} B, 0.3 g l^{-1} Mo and 0.3 g l^{-1} Mg) (Hubers, South Africa) and 2.5 g l^{-1} Multifeed® (190 g kg^{-1} N, 82 g kg^{-1} P, 158 g kg^{-1} K, 6.1 g kg^{-1} S, 0.9 g kg^{-1} Mg, 0.35 g kg^{-1} Zn, 1 g kg^{-1} B, 0.07 g kg^{-1} Mo, 0.75 g kg^{-1} Fe, 0.3g kg^{-1} Mn and 0.08 g l^{-1} Cu) (Nulandis®, South Africa) applied as a foliar spray every alternate week. Manual removal of flowering buds and cutting back of older shoots were performed weekly to maintain the plants in a juvenile state.

Figure 3.1: *A. dubius* plant at Randles Nursery site (29° 49`` S, 30° 58`` E), bar = 9 cm.

Figure 3.2: *A. dubius* plant in a flower pot located in the shade house at University of KwaZulu–Natal, Durban, bar = 9 cm

Table 3.1: Fungicide/fertiliser regimes performed weekly on *A. dubius* plants located in the shade house.

Day	**Fungicide/ fertiliser treatment (leaves/ soils)**
Monday	Dithane 2 g l^{-1} and Supremo 1 ml l^{-1} (foliar spray)
Wednesday	Multifeed 2.5 g l^{-1} OR Trelimix 1 ml l^{-1} (foliar spray)
Friday	Chronos 1 ml l^{-1} and Orius 1.25 ml l^{-1} (soil application)

3.2 Micropropagation of *A. dubius* field-derived nodal explants

3.2.1 Surface sterilisation and culture initiation

3.2.1.1 Initial surface sterilisation treatment

Nodal explants (1 cm height) of *A. dubius* were used to establish a surface sterilisation protocol. Ten explants were rinsed with 1% (v/v) sodium hypochlorite (NaOCl) with two drops of Tween 20® for 10 min followed by three rinses in sterile distilled water, aseptically excised and cultured into shoot multiplication media in culture tubes as described in section 3.2.2. This resulted in high levels of bacterial contamination after 3 d in culture (>50%, data not shown.

3.2.1.2 Effect of antibiotics against bacterial contamination

A sterile loop was used to isolate two visually different bacterial colonies from the contaminated cultures of nodal explants of *A. dubius.* The two bacterial isolates were then dispensed separately into test tubes (1.4 cm diameter) already containing 5 ml Luria broth (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, and 10 g l^{-1} NaCl) adapted from Gerhardt et al. (1994) and Sambrook and Russell (2001) and placed on a mechanical shaker at 1500 rpms, in a growth room (25°C d/18°C night; 16 h light (200 µmol m^{-2} s^{-1})/8 h dark photoperiod) to incubate overnight. Then, 1 ml of each of the cultures was spread onto the surface of the Luria broth agar (as above with the addition of 10 g l^{-1} agar) plates using a sterile glass rod. Thereafter, three holes were punched on the agar using a sterile leaf disc cutter of 1.2 cm diameter. Punched holes were impregnated with sterile distilled water (control), 50 or 100 µg l^{-1} ampicillin (pH 7) (Sigma, South Africa), rifampicin (pH 7.3) (Sigma, South Africa) or streptomycin/penicillin (pH 6.0 – 6.7) (Sigma, South Africa). The Petri dishes were then incubated at 24°C for 72 h in a laboratory oven. The diameter of inhibition was recorded daily, over three d.

3.2.1.3 Final decontamination protocol

Nodal explants from the shadehouse (field-derived) plants were decontaminated with 1% (v/v) NaOCl with two drops of Tween 20® for 10 min followed by two 2 min rinses in sterile distilled water and aseptically trimmed to remove dead ends. Subsequently, explants were placed into culture bottles (5 cm diameter, 10 cm height) containing an antibiotic solution of 50 µg l^{-1} rifampicin (pH 7.3) and 100 µg l^{-1} streptomycin/penicillin (pH 6.0 – 6.7) and then placed on a mechanical shaker for 5 h at 1500 rpm. Thereafter, the nodal explants were cultured in shoot multiplication medium with media composition as described in section 3.2.2.1.

3.2.2 *In vitro* clonal propagation of *A. dubius*

3.2.2.1 Basal media composition and growth room conditions

All media contained full strength Murashige and Skoog (MS) salts with vitamins (Murashige and Skoog, 1962; Highveld Biological, South Africa), 1 ml l^{-1} Previcur® (Propamocarb 600 g l^{-1}) (Bayer, South Africa) and 30 g l^{-1} sucrose, the pH was adjusted to 5.6 – 5.8 and the media then autoclaved at 121 °C at 1 KPa for 20 min. In addition, semi-solid media contained 10 g l^{-1} agar (Sigma, South Africa) and liquid media contained 2 ml methylene blue (Sigma, South Africa). Other components are listed in the ensuing sections. Media were dispensed into 50 ml culture tubes (2.5 cm diameter, 10 cm height) for semi-solid media and McCartney bottles (2

cm diameter, 7 cm height) for liquid media with one explant per vessel. Cultures were kept in a growth room 24°C d/18°C night, 16 h fluorescent light (200 μmol m^{-2} s^{-1})/8 h dark photoperiod).

3.2.2.2 Shoot multiplication

Nodal explants were cultured into both media types described in section 3.2.2.1 which were supplemented with five combinations of two PGRs (Table 3.2). In the liquid media the explants were placed upright in the culture vessel. Cultures were kept in the growth room at the same conditions as in section 3.2.2.1. Shoot number, shoot length, percentage contamination and percentage explants producing shoots were recorded after a 14 – d period.

Table 3.2: Combinations of BAP (6-benzylaminopurine) (Sigma, South Africa) and IAA (indole-3-acetic acid) (Sigma, South Africa) PGRs to induce shoot multiplication in *A. dubius* nodal explants after a 14-d culture period.

Media Type	**Plant growth regulators**	
	BAP (mg l^{-1})	**IAA (mg l^{-1})**
Semi-solid	1	0.5
	2	0.5
	1	0.1
	1	1
	0	0
Liquid	1	0.5
	2	0. 5
	1	0.1
	1	1
	0	0

3.2.2.3 Elongation

After two weeks in multiplication media, only shoots with a shoot length > 2 cm from the semi-solid medium containing 2 mg l^{-1} BAP and 0.5 mg l^{-1} IAA, were cultured individually in culture tubes into elongation medium which contained the basal medium (section 3.2.2.1) and supplemented with 0.1 mg l^{-1} BAP and 0.1 mg l^{-1} IAA.

3.2.2.4 Rooting

After 2 weeks in elongation medium, *in vitro* grown shoots were transferred into three rooting media: basal media supplemented with 0 (hormone free), 0.05, and 0.1 mg l^{-1} IAA to determine the percentage rooting and kept in the growth room (as described in section 3.2.2.1).

3.2.2.5 Acclimatisation and transfer to the greenhouse

All rooted plantlets > 3 cm in shoot length were selected for the acclimatisation period of 21 d. Roots were washed in distilled water to remove any remaining media. One plantlet was placed into each pot (10 cm diameter, 8 cm height) consisting of two different substrate types, i.e. (S, i.e. nutrient-rich) or soil:vermiculite (v/v) (1S:1V, i.e. nutrient-poor). Thereafter, each pot was enclosed in a plastic bag to regulate humidity. Plants were retained in the growth room (section 3.2.2.1) and after 7 d, holes were made in the plastic bags to decrease humidity. They were left for another 7 d then moved to the mist tent (85 – 94% humidity) in the greenhouse for a further 7 d (the greenhouse was not a controlled environment). Each plant was then transplanted into a larger pot (14.5 cm height, 17.5 cm diameter) and transferred into the greenhouse (27 – 39˚C, 85 – 90% humidity and 500 – 1100 μmol s^{-2} m^{-1}). Acclimatisation data recorded included the number of leaves, plant height and root length every 7 d for 21 d.

pH measurement of *ex vitro* substrates

The pH of both substrates were measured by soaking each to field capacity (water content held in soil after excess water has drained away). Thereafter, the substrates were watered five times at 10 min intervals with 500 ml of water and the excess liquid collected and measured for pH (n = 5) value. The pH values were 5.92 and 6.26 for S and (v/v) 1S:1V, respectively, and there was no significant difference between the substrates.

3.3 Screening and selection of *A. dubius* genotypes

Seed germination

Seeds of *A. dubius* were collected randomly from a field population of plants at Randles' Nursery and stored at 4 °C in a 50 ml sterile plastic tube on activated silica gel until needed. Fifty seeds were germinated in polystyrene seedling trays (68 × 34.5 cm) in soil, irrigated three

times daily for 3 min via an overhead irrigation system inside the mist tent (25°C d/18°C night and 84 – 94% RH) for 60 d.

3.4 Digestion and micronutrient analysis of *A. dubius* dried samples

Fifty seedlings (30 cm in height) with ± 25 – 30 leaves were used to screen and select genotypes (selected parent genotypes) of interest on the basis of Ca and Fe content. Leaves (5 – 6) from the top of the plant were harvested and an average fresh mass of 0.24 g recorded before the leaves were oven-dried at 80 °C for 48 h.

For sample digestion, 0.15 g of leaf dry mass from each genotype was digested over a hot plate in 5 ml of nitric acid for approximately 3 min (Nayar et al., 1975). Following digestion, samples were transferred into 25 ml volumetric flasks to which Millipore water was filled to the mark. Samples were then filtered through 0.22 µm sterile filters (Pall Corporation, USA) into 15 ml sterile plastic vials.

Leaf samples were evaluated for Ca and Fe content using ICP–OES. Known concentrations of Ca and Fe standards (1000 ppm) were used to prepare stock solutions ranging from 1 – 150 ppm for Ca and 0.1 – 5 ppm for Fe. These concentrations were used to generate calibration curves in order to determine the Ca and Fe content in leaf samples. The amount of Ca and Fe in each leaf sample was determined using the formula below. All analyses were performed in triplicate and the leaf Ca and Fe content was expressed in mg 100 g^{-1} using the calculations below.

$$\text{Concentration (mg kg}^{-1}) = \frac{\text{concentration of standard (mg l}^{-1}) \times \text{volume of sample (ml)}}{\text{mass of sample (g)}}$$

$$\text{mg 100 g}^{-1} = \frac{\text{mg kg}^{-1}}{10}$$

3.5 Clonal propagation of parent genotypes of *A. dubius*

Seven genotypes were selected on the basis of their Ca and Fe content for micropropagation following micronutrient analysis. Genotypes G8, G41, G45 and G47 were chosen for high Ca and Fe content, G15 for low Ca and Fe levels, G39 for high Fe and low Ca content and G11

for high Ca and low Fe levels. Nodal explants of the selected parent genotypes were multiplied, elongated, and rooted as described in sections 3.2.2.1 – 3.2.2.4.

Roots of the plantlets of the selected parent genotypes were washed in distilled water to remove any excess medium. Plants were then acclimatised and maintained in the growth room as described in section 3.2.2.5. Data recorded included leaf number, plant height and root length over 21 d of acclimatisation using non-destructive techniques. Following acclimatisation and transfer to the greenhouse, leaves from selected parent genotypes were harvested over different time intervals (15, 30, 60, 80 and 90 d) to determine their Ca and Fe levels. Leaf samples were prepared and analysed as described in section 3.4. At d 90, fresh and dry masses of shoots and roots and root:shoot dry mass were determined.

3.6 Statistical analyses

All data were analysed using Genstat statistical package 17th edition (VSN International, Hernal Hempstead, UK). Prior to analysis data were tested for normality using D'Agostino-Pearson test ($p < 0.05$). Data were then analysed with a One Way Analysis of Variance (ANOVA) and means were compared using Tukey's test at 95% confidence interval. Differences among treatments were represented by assigning different alphabets to the means, values that did not share an alphabet were recognised as being significantly different. Comparisons between substrates were determined by a paired t – test at 95% confidence interval.

Chapter 4. Results

4.1 Establishment of micropropagation protocols for field-derived nodal explants of *Amaranthus dubius*

4.1.1 Establishment of decontamination protocols

Nodal explants were subjected to three different treatments in order to obtain an effective decontamination protocol (Table 4.1). Bacterial and fungal contaminants were observed after three days (d) in culture when protocol A was used. To eradicate them, 1 ml l^{-1} Previcur® with or without 1 ml l^{-1} methylene blue (Table 4.1) was incorporated in the media. The combination was effective in eliminating only fungal contaminants. There were significant differences amongst the tested treatments (Table 4.1). Using treatment C, a significantly higher percentage survival and lower percentage contamination were observed when compared to those from treatments A and B. After a further culture period of 14 d, the contamination increased from 18.7% to 50% after using protocol C.

Table 4.1: Percentage contamination and survival of field nodal explants on multiplication medium (full strength MS, 2 mg l^{-1} BAP, 0.5 mg l^{-1} IAA, 1 ml l^{-1} Previcur® (B, C), methylene blue (C) after 3 d in culture using protocol A and after 14 d using protocols B and C. Dissimilar alphabet characters denote statistical differences; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 10, mean ± SE; a – c: comparison within each column.

Treatments	%Contamination	%Survival
A) 1% NaOCl (v/v) + 70% ethanol (v/v) + with two drops of Tween 20®	89.3 ± 2.01^{a} (fungal and bacterial)	10.7 ± 2.01^{c}
B) 1% NaOCl (v/v) + 70% ethanol (v/v) + 1 ml l^{-1} Previcur® (v/v) + with two drops of Tween 20®	49.3 ± 2.57^{b} (bacterial)	50.6 ± 2.57^{b}
C) 1% NaOCl (v/v) + 1 ml l^{-1} Previcur® (v/v) + 1 ml l^{-1} Methylene blue (v/v) + with two drops of Tween 20®	18.7 ± 2.01^{c} (bacterial)	81.3 ± 2.01^{a}

Two bacterial strains were visually identified from the contaminated cultures and isolated as described in section 3.2.1.2. Then, different antibiotics (ampicillin, rifampicin, streptomycin/ penicillin) were tested at different concentrations (0, 50, 100 μg l^{-1}) to determine the diameter of the zone of inhibition for each bacterial strain (Table 4.2). For bacterial strain 1, combinations of streptomycin/penicillin at 50 and 100 μg l^{-1} inhibited bacterial growth by 2.63 ± 0.05 and 3.07 ± 0.03 cm, respectively. For the same strain, there were no significant differences in the zone of inhibition between the antibiotic concentrations (Table 4.2). For bacterial strain 2, streptomycin/penicillin at 100 μg l^{-1} and rifampicin at 50 μg l^{-1} and at 100 μg l^{-1} significantly inhibited bacterial growth by 1.1 ± 0.0 cm, 0.53 ± 0.0 cm and 0.76 ± 0.0 cm, respectively (Table 4.2). A t-test analysis revealed no significant differences between the 50 and 100 μg l^{-1} rifampicin concentrations, so the 50 μg l^{-1} concentration was chosen. Ampicillin did not inhibit the bacterial growth of either strain. Therefore, rifampicin at 50 μg l^{-1} and streptomycin/penicillin at 100 μg l^{-1} were incorporated into the decontamination protocol. The final decontamination protocol used in subsequent studies reduced contamination from 89.3 ± 2.01% (Table 4.1) to 5.0 ± 0.8% (Table 4.5).

Table 4.2: The diameter of the zone of inhibition (cm) measured after 3 d for the two bacterial strains isolated from contaminated cultures. Dissimilar alphabet characters denote statistical differences; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, $n = 9$, mean ± SE; a – b, comparison within each column.

		Zone of inhibition (cm)	
Antibiotic	**Concentration (μg l^{-1})**	**Bacterial strain 1**	**Bacterial strain 2**
Ampicillin	0	0	0
	50	0	0
	100	0	0
Streptomycin/Penicillin	0	0	0
	50	2.63 ± 0.05^{a}	0
	100	3.07 ± 0.03^{a}	1.1 ± 0.0^{a}
Rifampicin	0	0	0
	50	0	0.53 ± 0.0^{b}
	100	0	0.76 ± 0.0^{a}

It was concluded that bacterial contaminants were eliminated by the surface sterilisation of nodal explants in 1% (v/v) NaOCl + 1 ml l^{-1} (v/v) Previcur® + two drops of Tween 20® coupled with their immersion in an antibiotic solution (¼ MS, 50 µg l^{-1} rifampicin and 100 µg l^{-1} streptomycin/penicillin) for 5 h on a mechanical shaker at 1500 rpm (Table 4.2) before culturing in shoot multiplication media with 1 ml l^{-1} Previcur®.

4.1.2 Direct organogenesis from field nodal explants

4.1.2.1 Shoot multiplication

To determine the best plant growth regulator (PGR) combination for shoot multiplication of the field-derived nodal explants, permutations of indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP) in semi-solid and liquid media were investigated (Table 4.3). Regardless of the media type and PGR combination, shoots developed from the field nodal explants after 14 d in culture (Table 4.3).

The extent of bacterial contaminants observed following the adopted decontamination protocol varied amongst media types. The percentage bacterial contamination in liquid media was 87 ± 0.3% to 90 ± 0.3% with no significant difference amongst the tested PGR combinations (Table 4.3). In semi-solid media bacterial contamination (30 ± 0.1% to 40 ± 0.3%, Table 4.3) was lower than in liquid media (Table 4.3). Also, significantly more explants produced shoots in semi-solid (60 ± 0.3% to 70 ± 0.3%) than in liquid (10 ± 0.3% to 12 ± 0.4%) media (Table 4.3).

The number of shoots/nodal explant produced was 2 ± 0.2 to 4 ± 0.2 in semi-solid media and 1 ± 0.0 to 3 ± 0.1 in liquid media, amongst the tested PGR combinations (Table 4.3). There was a significant difference between the number of shoots/nodal explant in media with 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA and those in 1 mg l^{-1} BAP + 1 mg l^{-1} IAA than those in the other tested PGR combinations. Amongst the semi-solid media tested, the highest number of shoots/nodal explant was recorded in media with 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA (4 ± 0.2) and with 1 mg l^{-1} BAP + 1 mg l^{-1} IAA (4 ± 0.4). Both these media produced significantly more shoots than liquid media with the same PGR combinations and concentrations (Table 4.3).

The highest shoot lengths of 2.70 ± 0.1 cm and 2.1 ± 0.1 cm were recorded in the media with 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA and 1 mg l^{-1} BAP + 0.5 mg l^{-1} IAA, respectively (Table 4.3). In the former, the shoot lengths were significantly longer than those in the other tested PGR combinations in semi-solid and in liquid media.

Table 4.3: The effect of PGR combinations and media type on the percentage bacterial contamination, percentage explants producing shoots, number of shoots/nodal explant and shoot length of nodal explants. Dissimilar alphabet characters denote statistical differences; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 10, mean ± SE; a – e: comparison within each column for semi-solid media and x – y for liquid media.

	PGR					
Media type	**BAP (mg l^{-1})**	**IAA (mg l^{-1})**	**% Bacterial contamination**	**% Explants producing shoots**	**Number of shoots/nodal explant**	**Shoot length (cm)**
Semi-solid media	1	0.5	40 ± 0.3^{a}	60 ± 0.3^{b}	3.0 ± 0.2^{b}	2.1 ± 0.1^{b}
	2	0.5	31 ± 0.3^{b}	69 ± 0.3^{a}	4.0 ± 0.2^{a}	2.7 ± 0.1^{a}
	1	0.1	32 ± 0.5^{b}	67 ± 0.5^{a}	2.0 ± 0.1^{b}	1.1 ± 0.1^{c}
	1	1	30 ± 0.3^{b}	70 ± 0.3^{a}	4.0 ± 0.4^{a}	1.3 ± 0.1^{c}
	1	0	30 ± 0.1^{b}	70 ± 0.1^{a}	2.0 ± 0.3^{b}	2.0 ± 0.2^{c}
Liquid media	1	0.5	90 ± 0.3^{x}	10 ± 0.3^{x}	1.8 ± 0.3^{yz}	0.9 ± 0.1^{x}
	2	0.5	88 ± 0.4^{x}	12 ± 0.4^{x}	3.0 ± 0.1^{x}	0.7 ± 0.0^{xy}
	1	0.1	87 ± 0.3^{x}	11 ± 0.3^{x}	1.0 ± 0.0^{y}	0.8 ± 0.1^{x}
	1	1	88 ± 0.3^{x}	12 ± 0.3^{x}	1.7 ± 0.3^{y}	1.2 ± 0.2^{x}
	1	0	89 ± 0.3^{x}	11 ± 0.3^{x}	1.0 ± 0.0^{yz}	0.5 ± 0.0^{xy}

4.1.2.2 Rooting

For the purpose of rooting, shoots from the best shoot-producing semi-solid medium (2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA) were elongated in 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} IAA for 14 d. Once shoot length was > 3 cm, the shoots were transferred into different rooting media containing 0, 0.05 and 0.1 mg l^{-1} IAA (Table 4.4). The hormone-free rooting medium and that supplemented with 0.05 mg l^{-1} IAA promoted *in vitro* rooting in 40% of the shoots. There was 100% rooting in the medium with 0.1 mg l^{-1} IAA, which was significantly higher than those in 0 and 0.05 mg l^{-1} IAA (Table 4.4). The yield (plant/nodal explant) was determined for the field-derived explant and averaged at 2 ± 0.2 for the three tested rooting media.

Table 4.4: The effect of three different IAA combinations on rooting of shoots multiplied in full strength MS, 2 mg l^{-1} BAP, 0.5 mg l^{-1} IAA and 1 ml l^{-1} Previcur® and the yield from each nodal explant. Dissimilar alphabet characters denote statistical differences; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 10, mean ± SE; a – b: comparison within each column.

IAA (mg l^{-1})	% Explants with *in vitro* roots	Yield (plant/nodal explant)
0	40 ± 0.3^{b}	2 ± 0.1^{a}
0.05	40 ± 0.3^{b}	2 ± 0.2^{a}
0.1	100 ± 0.4^{a}	2 ± 0.2^{a}

In conclusion, the best PGR combination for shoot multiplication from field nodes was in semi-solid medium containing 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA which produced 4 shoots/nodal explant. The best PGR concentration for rooting of *in vitro* shoots was 0.1 mg l^{-1} IAA producing 100% *in vitro* roots.

4.1.3 Acclimatisation of in vitro regenerated plants in soil and soil:vermiculite (1:1)

Plants regenerated in culture were acclimatised, as described in section 3.2.2.5. In both soil (S) and soil:vermiculite (1:1) (v/v) (1S:1V), the percentage survival of acclimatised plants was 80% (data not shown). The number of leaves, plant height and root length were measured at 7 d intervals over 21 d during acclimatisation in the two different substrates (Fig. 4.1).

The number of leaves of the regenerated plants significantly increased from d 7 to 14 in S (Fig. 4.1). In (v/v) 1S:1V, the leaf number significantly increased from d 0 (6 ± 0.35) to 7 (16 ± 1.54), decreased from d 7 (16 ± 1.54) to 14 (10 ± 0.71) and significantly increased from d 14 (10 ± 0.74) to 21 (13 ± 0.54). At d 7, the number of leaves of the regenerated plants was significantly higher in (v/v) 1S:1V (16 ± 1.54) than in S (8 ± 0.35), but there were no significant differences between the substrates at d 0, 14 and 21 (Fig. 4.1A).

The plant height of the regenerated plants significantly increased from d 7 (10 ± 0.71) to 14 (17.67 ± 1.63) in S but in (v/v) 1S:1V there were no significant differences over time. The effect of substrate was observed only at d 14 and 21 for the plant height where plants in S (17.67 ± 1.63 and 19.6 ± 1.47), respectively, were significantly longer than those acclimatised in (v/v) 1S:1V (8 ± 0.51 and 13 ± 1.51), respectively (Fig. 4.1B).

The root length of the regenerated plants significantly increased over time from d 7 (9 ± 0.62) to 14 (22.67 ± 0.97) in S. There were no significant differences in the root length for the regenerated plants grown in (v/v) 1S:1V from d 0 to 21. At d 14 (22.67 ± 0.97) and 21 (25.42 ± 1.07), the root lengths of the regenerated plants grown in S were significantly longer than those grown in (v/v) 1S:1V (13 ± 1.01 and 10 ± 0.61), respectively. However, no significant differences in the root length were observed between plants grown in S and in (v/v) 1S:1V at d 0 and 7 (Fig. 4.1C).

In conclusion, all the growth parameters significantly increased when the plants were grown in S from d 7 to 14. Whereas in (v/v) 1S:1V, the number of leaves was the only parameter to increase significantly from d 0 to 7 and d 14 to 21. The substrate effect on the number of leaves was shown only at d 7 with a higher number of leaves in (v/v) 1S:1V than in S. Also, an effect of the substrate on both the plant height and root length was observed at d 14 and 21 with S causing longer lengths for both parameters.

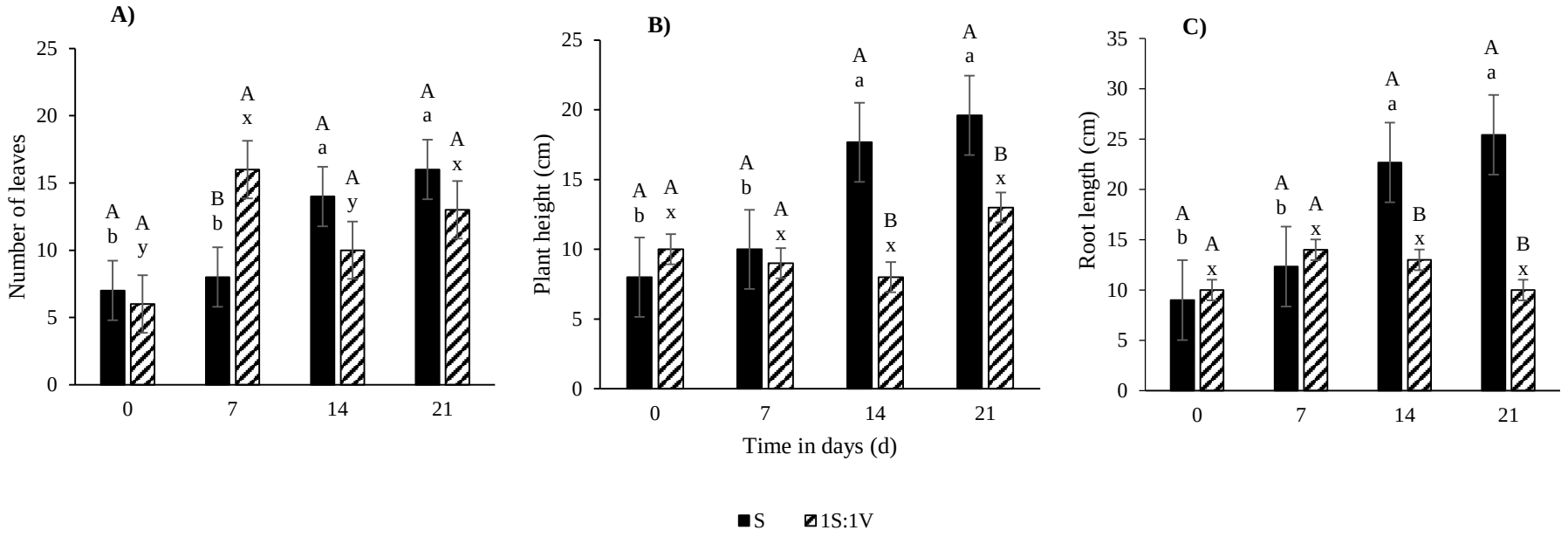

Figure 4.1: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length of plants regenerated in soil (S) or soil:vermiculite (1:1) (v/v) (1S:1V) during 21 d of acclimatisation. Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) or in (v/v) 1S:1V (x – y) over time; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 10, mean ± SE; t-test: comparison of each growth parameter at each time interval between substrates: A – B.

4.2 Screening and selection for Ca and Fe content from a seedling population and micropropagation of the selected parent genotypes

4.2.1 Ca and Fe content in the tested population

4.2.1.1 Ca content

The leaf Ca contents from a seedling population of 50 *A. dubius* genotypes were tested (Fig. 4.2). The Ca contents ranged from 246.3 ± 1.14 mg 100 g^{-1} dry mass (DM) (G36) to 765.3 ± 6.07 mg 100 g^{-1} DM (G47) for this study and were within the range of 582 mg 100 g^{-1} DM to 3014.68 mg 100 g^{-1} DM reported in the literature (Table 1.2). Seven genotypes (G8, G11, G15, G39, G41, G45 and G47) (hereafter referred to as parent genotypes) were selected based on their Ca content (Fig. 4.2) and 'ranked' from the highest to the lowest as G47 > G45 > G11 > G41 = G8 > G39 > G15. The parent genotypes G8, G11, G41, G45 and G47 were selected for their high Ca content and G15 and G39 were selected for their low Ca content. These genotypes were micropropagated according to the established protocol and thereafter the effects of micropropagation, substrate type and physiological age (time) on the growth and Ca content of their clones were investigated.

4.2.1.2 Fe content

The leaf materials harvested from a seedling population of 50 *A. dubius* genotypes were tested for their Fe content (Fig. 4.3). The Fe levels were found to be between 5.25 ± 0.06 mg 100 g^{-1} DM (G11) and 26.68 ± 0.10 mg 100 g^{-1} DM (G33) for the current study which were within the range of 3.4 mg 100 g^{-1} DM to 95.15 mg 100 g^{-1} DM reported in the literature (Table 1.2). Seven parent genotypes were selected based on their Fe levels (G8, G11, G15, G39, G41, G45 and G47) (Fig. 4.3) and 'ranked' from the highest to the lowest as G47 = G45 > G39 = G41 > G8 > G15 > G11. Genotypes G8, G39, G41, G45 and G47 were selected for their high Fe levels, G11 and G15 were selected for their low Fe levels and all these selected parent genotypes were subjected to the established micropropagation protocol. The effects of micropropagation, substrate type and physiological age (time) on the growth and Fe content of clones of the parent genotypes were then evaluated.

In conclusion, phenotypic variations in Ca and Fe content (Fig. 4.2, 4.3) within the tested natural breeding population were detected.

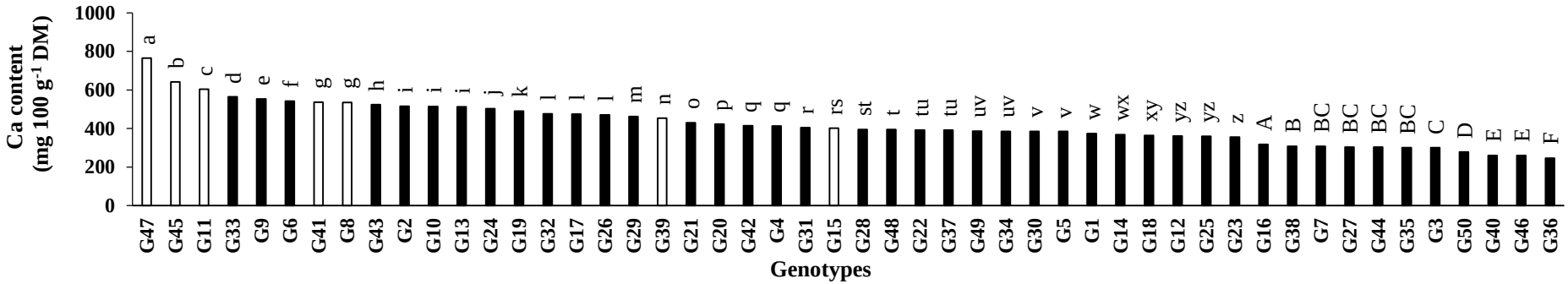

Figure 4.2: Calcium content from the leaves of *A. dubius* genotypes (G) (n = 50) harvested 60 d after germination. The selected parent genotypes G8, G11, G15, G39, G41, G45 and G47 are represented as clear bars across the 50 screened genotypes. Dissimilar alphabet characters denote statistical differences; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; a – F: comparison amongst the genotypes.

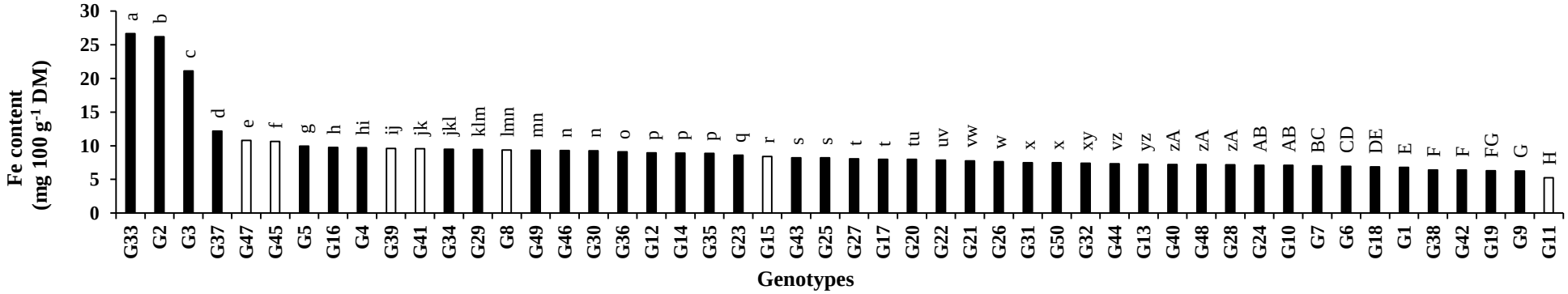

Figure 4.3: Iron content from 60 d old leaves of *A. dubius* genotypes (G) (n = 50). The selected parent genotypes G8, G11, G15, G39, G41, G45 and G47 are represented as clear bars across the 50 screened genotypes. Dissimilar alphabet characters denote statistical differences; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; a – H: comparison amongst the genotypes.

4.2.2 Micropropagation of the selected parent genotypes

Nodal explants from the selected parent genotypes (section 4.2.1) were micropropagated, as per the established protocol (section 4.1.1). Bacterial contamination ranged from 5.0 ± 0.8% to 12.5 ± 1.3% (Table 4.5). There were no significant differences in the percentage bacterial contamination between the G8 and G11 clones and amongst the G8, G15, G39, G41, G45 and G47 clones.

The percentage explants producing shoots ranged from 87.5 ± 4.3% to 95 ± 1.5% and there were no significant differences amongst the tested genotypes. The percentage explants producing shoots was higher by approximately 26% amongst the parent genotypes (Table 4.5) than those from the field-derived nodal explants (Table 4.3). The number of shoots produced amongst the genotypes (2 ± 0.6 to 5 ± 1.4; Table 4.5) were similar to those produced by the field-derived nodal explants (2 ± 0.1 to 4 ± 0.2; Table 4.3).

Significant differences in the shoot lengths were observed amongst G47, G45 and G39 and between G47 and G11 clones. Amongst the parent genotypes, the shoot length was between 0.6 ± 0.2 cm and 1.9 ± 0.1 cm (Table 4.5) and from the field-derived nodal explants were between 1.1 ± 0.1 cm and 2.7 ± 0.1 cm (Table 4.3).

The percentage explants producing roots was similar for field-derived nodal explants (100 ± 0.4%; Table 4.4) and amongst the parent genotypes (87 ± 1.45% to 95 ± 0.6%; Table 4.5).

The yield (plants/nodal explant) amongst the clones of the parent genotypes was 2 ± 0.3 to 4 ± 0.3 (Table 4.5). The yield from the G45 (4 ± 0.3) clone was significantly higher than those of the G8, G39 and G41 clones (Table 4.5). The yield from the field-derived plants was 2 ± 0.2 plants/nodal explant (Table 4.4) similar to those of G8, G39 and G41 clones (Table 4.5). The yield differences were due to the multiplication stage where different genotypes produced different numbers of shoots/nodal explant, an expression of phenotypic variation amongst the parent genotypes (Table 4.5).

It can be concluded that nodes from the parent genotypes (Table 4.5) were the explant of choice for use during multiplication than those from the field (Table 4.3) because the former produced up to 26% more shoots and had less contaminants in culture.

Table 4.5: The percentage bacterial contamination, percentage explants producing shoots, number of shoots/explant, shoot length, percentage explants producing roots and yield of the clones of the parent genotypes on full strength MS, 2 mg l^{-1} BAP, 0.5 mg l^{-1} IAA and 1 ml l^{-1} Previcur®. Dissimilar alphabet characters denote statistical differences within each column: a – b; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 10 – 15, mean ± SE.

Genotype	% Bacterial contamination	% Explants producing shoots	No. of shoots/nodal explant	Shoot length (cm)	% Explants producing roots	Yield (plant/nodal explant)
G8	7.5 ± 1.3^{ab}	92.5 ± 3.1^{a}	3 ± 0.3^{a}	1.7 ± 0.1^{ab}	93 ± 0.6^{a}	2 ± 0.3^{b}
G11	5.0 ± 0.8^{b}	95.0 ± 1.5^{a}	3 ± 1.5^{a}	0.6 ± 0.2^{c}	95 ± 0.6^{a}	3 ± 0.3^{ab}
G15	12.5 ± 0.8^{a}	87.5 ± 4.6^{a}	4 ± 0.8^{a}	1.1 ± 0.2^{abc}	87 ± 1.4^{a}	3 ± 0.3^{ab}
G39	12.5 ± 1.3^{a}	87.5 ± 4.3^{a}	3 ± 0.8^{a}	0.9 ± 0.2^{bc}	87 ± 2.7^{a}	2 ± 0.3^{b}
G41	12.5 ± 1.9^{a}	87.5 ± 7.4^{a}	2 ± 0.6^{a}	1.2 ± 0.3^{abc}	87 ± 4.8^{a}	2 ± 0.3^{b}
G45	12.5 ± 1.3^{a}	87.5 ± 7.1^{a}	5 ± 1.4^{a}	1.0 ± 0.2^{bc}	87 ± 3.4^{a}	4 ± 0.3^{a}
G47	12.5 ± 1.5^{a}	87.5 ± 4.6^{a}	4 ± 0.3^{a}	1.9 ± 0.1^{a}	87 ± 0.6^{a}	3 ± 0.3^{ab}

4.3 The effect of substrate and physiological age (time) on *ex vitro* growth parameters and on Ca and Fe content of the clones of the parent genotypes

4.3.1 The effect of substrate and time on the growth parameters of the clones of the parent genotypes during 21 d of acclimatisation and over 90 d after transfer to the greenhouse

Some growth parameters (number of leaves, plant height and root length) were measured over 21 d on the clones of the parent genotypes grown in soil (S) or soil:vermiculite (1:1) (v/v) (1S:1V). There were no significant differences in the growth parameters between substrates at each tested time interval for G11, G15, G41, G45 and G47 clones. However, after 21 d, the leaf number of the G8 clones (Fig. 4.4A) and the plant height of the G39 clones (Fig. 4.7B) were significantly different between substrates. After 21 d, a significantly higher number of leaves was observed for the G8 clones grown in (v/v) 1S:1V (12 ± 1.52) than those grown in S (7 ± 1.51) (Fig. 4.4A). The plant height of the G39 clones grown in S was significantly longer at d 21 (12.33 ± 0.27 cm) than that grown in (v/v) 1S:1V (8.33 ± 0.27 cm) (Fig. 4.7B).

When grown in S, the plant height and root length of G8 (Fig. 4.4B,C) and G45 (Fig. 4.9B,C) clones remained the same from d 0 to 21 but the number of leaves of G39 (Fig. 4.7A) and G47 (Fig. 4.10A) significantly increased from 7 ± 0.94 at d 0 to 11 ± 0.54 at d 21 and 7 ± 0.81 at d 0 to 13 ± 0.72 at d 21, respectively.

In (v/v) 1S:1V, the leaf number of the G39 (Fig. 4.7A) and G47 (Fig. 4.10A) clones, the plant height of G11 (Fig. 4.5B) and the root length of G8 (Fig. 4.4C) and G47 (Fig. 4.10C) remained the same from d 0 to 21. However, the plant height of G8 (Fig. 4.4B) and G47 (Fig. 4.10B) significantly increased from 5 ± 0.47 cm at d 0 to 9.67 ± 0.27 cm at d 21 and 6 ± 0.27 cm at d 0 to 10.67 ± 0.72 cm at d 21, respectively. Also, the root length of G45 (Fig. 4.9C) significantly increased from 7 ± 0.81 cm at d 0 to 14.67 ± 1.65 cm at d 21.

When grown in S or in (v/v) 1S:1V, the number of leaves of G8 (Fig. 4.4A), G11 (Fig. 4.5A), G15 (Fig. 4.6A), G41 (Fig. 4.8A) and G45 (Fig. 4.9A), the plant height of G15 (Fig. 4.6A), G39 (Fig. 4.7B), G41 (Fig. 4.8) and G45 (Fig. 4.9B) and the root length of G11 (Fig. 4.5C), G15 (Fig. 4.6C), G39 (Fig. 4.7C) and G47 (Fig. 4.10C) did not change from d 0 to 21.

The shoot and root fresh and dry masses of the clones of the parent genotypes (G8, G11, G15, G39, G41, G45 and G47) were determined over a 90 d period after acclimatisation and transfer to the greenhouse on clones grown in S and (v/v) 1S:1V (Table 4.6). The shoot fresh masses amongst the genotypes were from 1.32 ± 0.56 g to 3.07 ± 0.80 g when grown in S and from

1.32 ± 0.66 g to 2.98 ± 0.31 g for clones grown in (v/v) 1S:1V (Table 4.6). Ranges of 0.30 ± 0.15 g to 0.63 ± 0.13 g in S and 0.28 ± 0.14 g to 0.75 ± 0.08 g in (v/v) 1S:1V were observed for the shoot dry masses (Table 4.6). Amongst the genotypes the fresh masses of the roots of the clones varied from 2.29 ± 1.54 g to 5.72 ± 0.77 g and from 0.63 ± 0.13 g to 3.09 ± 0.15 g when grown in S and in (v/v) 1S:1V, respectively (Table 4.6). The dry masses of the roots of the clones were between 0.33 ± 0.19 g and 0.78 ± 0.22 g in S and 0.42 ± 0.21 g and 0.84 ± 0.21 g in (v/v) 1S:1V (Table 4.6). No significant differences were observed in the shoot fresh and dry masses and in the root fresh and dry masses amongst the clones of the parent genotypes and between the substrates (Table 4.6). The root:shoot biomass amongst the genotype clones grown in S was from 0.64 ± 0.34 g to 2.08 ± 0.61 g and from 0.98 ± 0.22 g to 2.07 ± 0.56 g for those grown in (v/v) 1S:1V. No significant differences were observed in the root:shoot biomass between the two substrates (Table 4.6).

After 21 d of acclimatisation, the substrate did not affect biomass (both shoots and roots) (Figs. 4.4 – 4.10) with the exception of the G8 clones at d 21 where a significantly higher number of leaves were observed when G8 was grown in (v/v) 1S:1V than in S (Fig. 4.4A). This is significant because the leaves of *A. dubius* are harvested for consumption and therefore should one wish to increase biomass, other environmental parameters (light intensity, water regimes, salinity and heat stress) need to be investigated.

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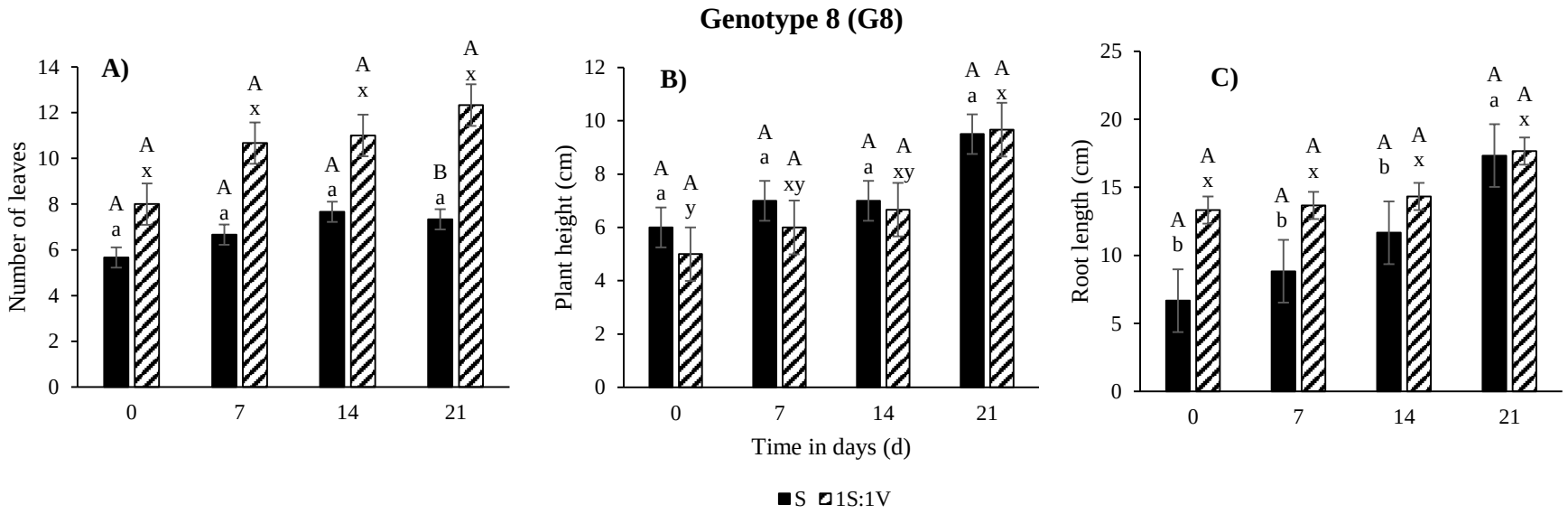

Figure 4.4: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length for G8 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) or (v/v) 1S:1V (x – y) over time; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 10, mean ± SE; t-test, comparison of each growth parameter at each time interval between substrates: A – B.

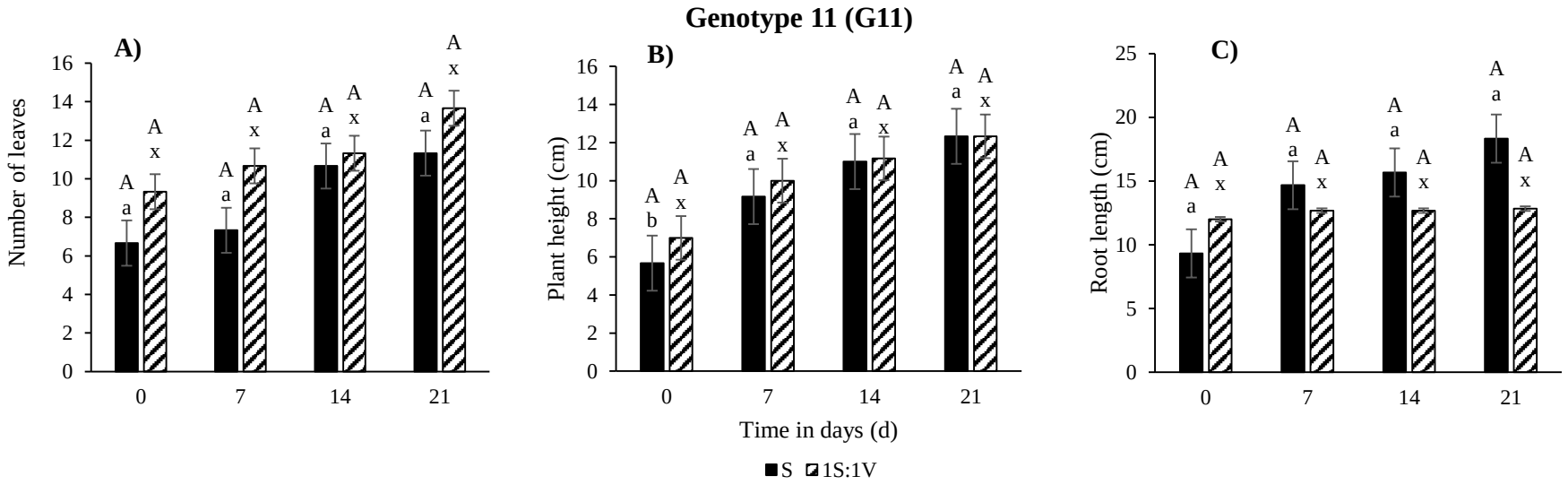

Figure 4.5: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length for G11 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) or (v/v) 1S:1V (x – y) over time; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; t-test, comparison of each growth parameters at each time interval between substrates: A – B.

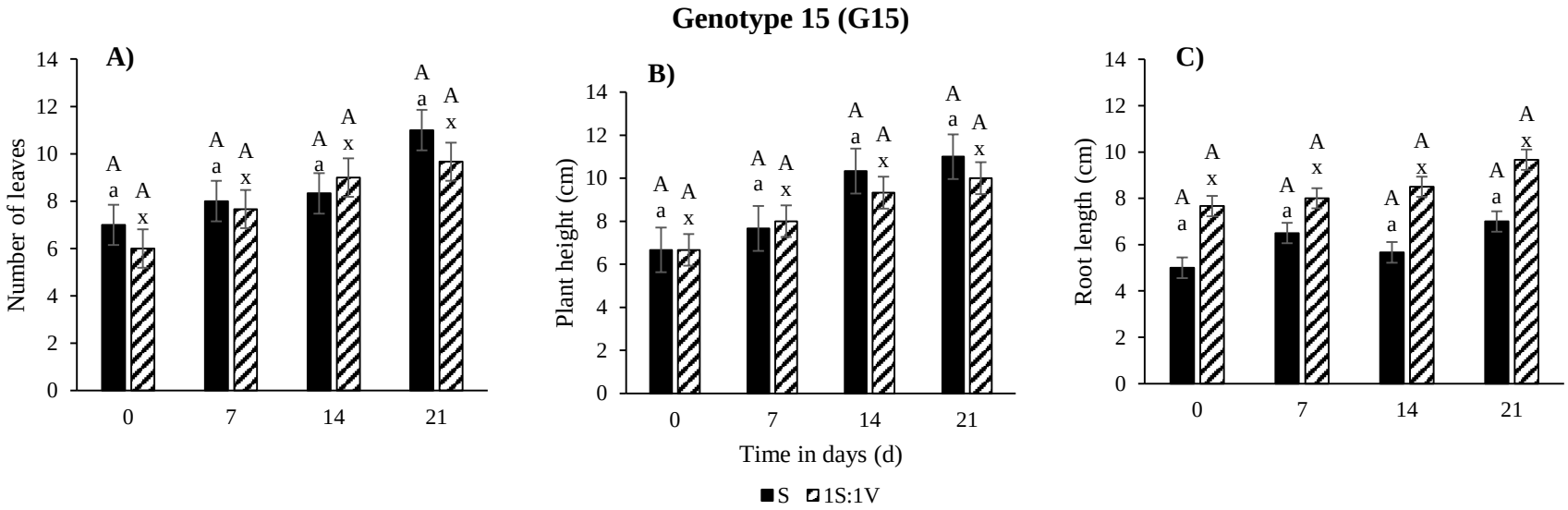

Figure 4.6: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length for G15 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) or (v/v) 1S:1V (x – y) over time; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; t-test, comparison of each growth parameters at each time interval between substrates: A – B.

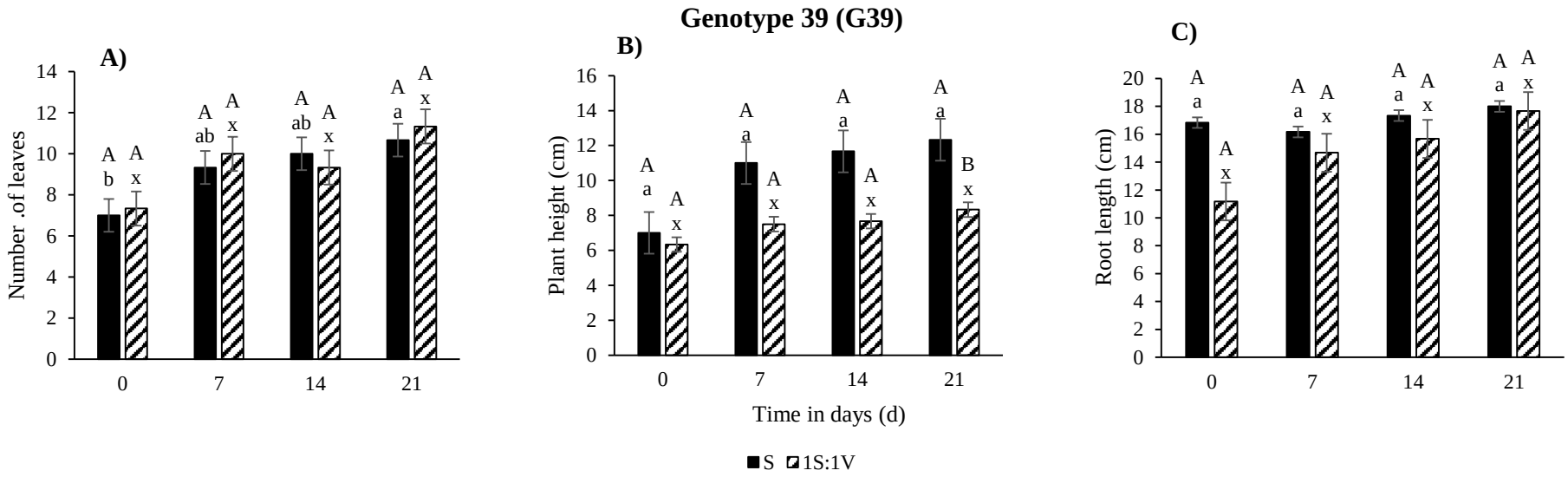

Figure 4.7: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length for G39 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) or (v/v) 1S:1V (x – y); One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; t-test, comparison of each growth parameters at each time interval between substrates: A – B.

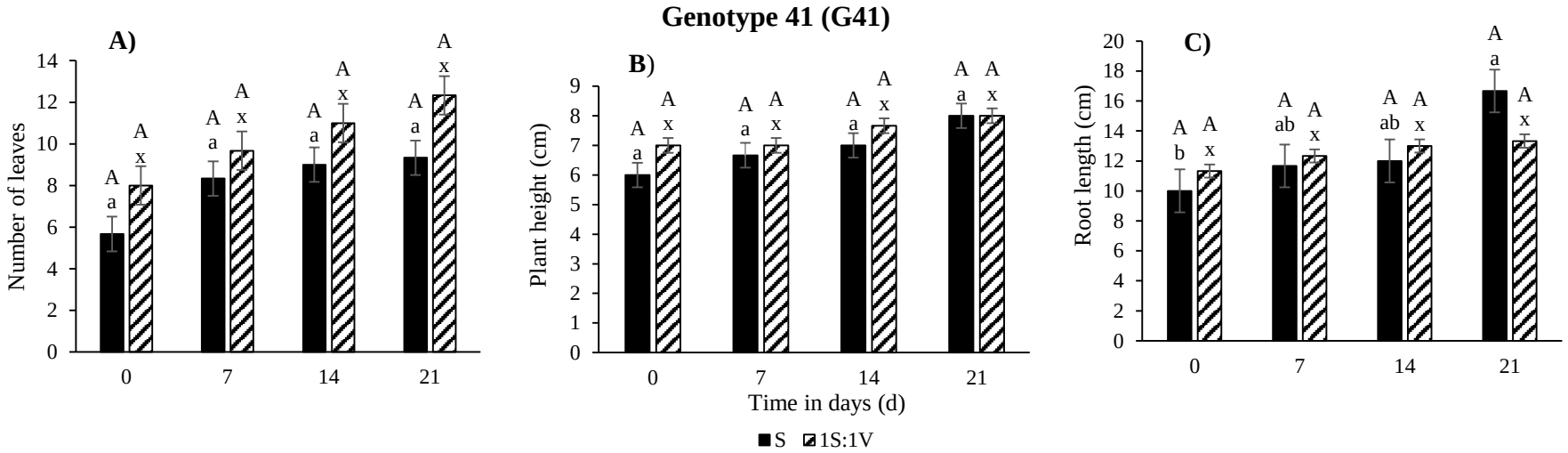

Figure 4.8: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length for G41 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) and (v/v) 1S:1V (x – y) over time; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; t-test, comparison of each growth parameters at each time interval between substrates: A – B.

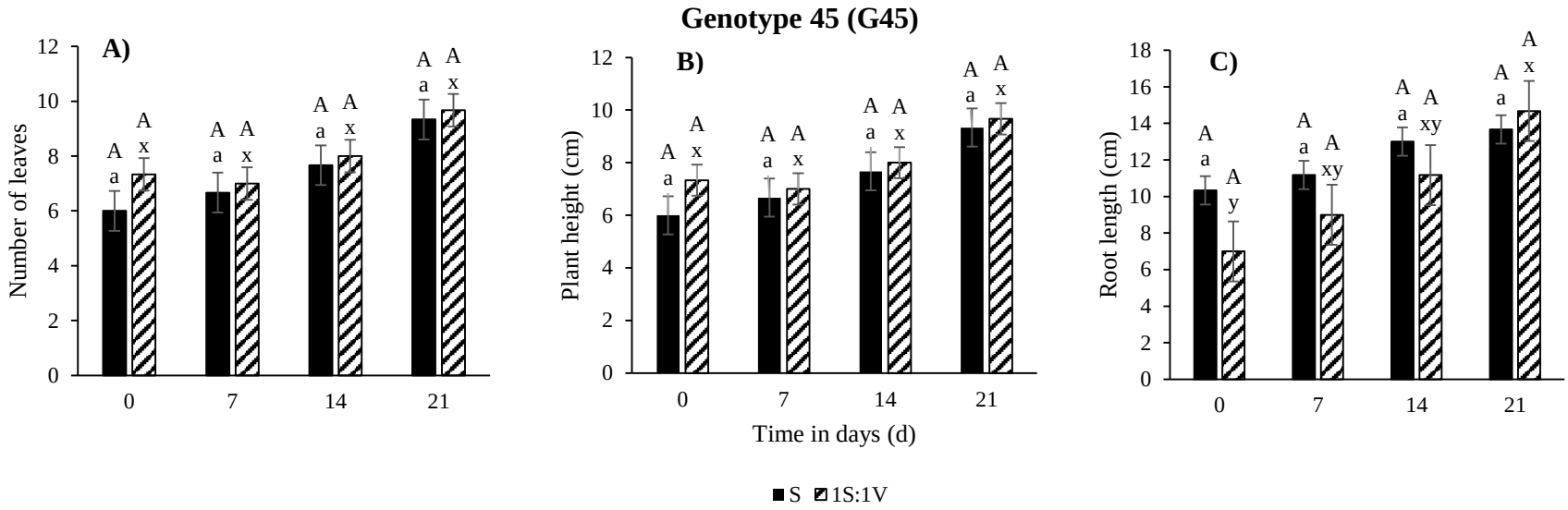

Figure 4.9: Growth parameters as indicated by A) number of leaves, B) plant height and C) root length for G45 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences for each growth parameter in S (a – b) and (v/v) 1S:1V (x – y) over time; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; t-test, comparison of each growth parameters at each time interval between substrates: A – B.

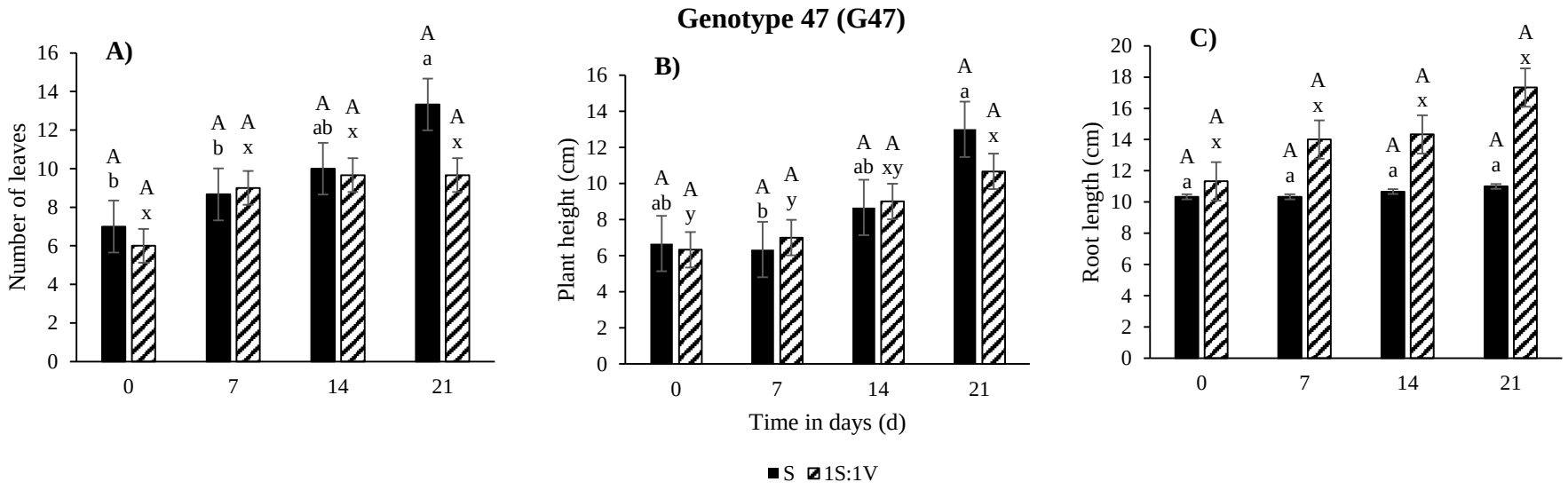

Figure 4.10: Growth parameter as indicated by A) number of leaves, B) plant height and C) root length for G47 clones over a 21 d period of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar lowercase alphabet characters denote statistical differences of each growth parameter in S (a – b) and (v/v) 1S:1V (x – y); One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE; t-test, comparison of each growth parameters at each time interval between substrates: A – B.

Table 4.6: The effect of soil (S) and soil:vermiculite (v/v) (1S:1V) on the fresh and dry masses of shoots, roots and root:shoot biomass of clones of the parent genotypes harvested at 90 d after transfer to the greenhouse, post 21 d of acclimatisation. Similar lowercase alphabets (a and b) denote no statistical differences for each growth parameter in S (a) and (v/v) 1S:1V (b) amongst the genotypes; One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE.

		Shoot		Root		
Genotype	**Substrate (%)**	**Fresh mass (g)**	**Dry mass (g)**	**Fresh mass (g)**	**Dry mass (g)**	**Root:Shoot (Dry mass)**
G8	S	2.18 ± 0.03 [a]	0.57 ± 0.04 [a]	4.26 ± 0.72 [a]	0.47 ± 0.06 [a]	0.82 ± 0.05 [a]
	1S:1V	1.57 ± 0.24 [b]	0.38 ± 0.06 [b]	5.74 ± 1.41 [b]	0.76 ± 0.19 [b]	2.07 ± 0.46 [b]
G11	S	2.04 ± 0.44 [a]	0.52 ± 0.13 [a]	3.41 ± 0.34 [a]	0.40 ± 0.06 [a]	0.85 ± 0.17 [a]
	1S:1V	2.98 ± 0.31 [b]	0.75 ± 0.08 [b]	4.65 ± 0.59 [b]	0.71 ± 0.07 [b]	0.98 ± 0.14 [b]
G15	S	1.43 ± 0.74 [a]	0.33 ± 0.17 [a]	2.29 ± 1.54 [a]	0.33 ± 0.19 [a]	0.96 ± 0.10 [a]
	1S:1V	1.32 ± 0.66 [b]	0.28 ± 0.14 [b]	3.09 ± 1.55 [b]	0.42 ± 0.21 [b]	1.49 ± 0.11 [b]
G39	S	3.07 ± 0.80 [a]	0.51 ± 0.05 [a]	5.37 ± 1.09 [a]	0.67 ± 0.12 [a]	1.33 ± 0.16 [a]
	1S:1V	2.27 ± 0.23 [b]	0.62 ± 0.07 [b]	5.59 ± 1.66 [b]	0.58 ± 0.09 [b]	0.98 ± 0.18 [b]
G41	S	1.32 ± 0.56 [a]	0.30 ± 0.15 [a]	3.40 ± 0.77 [a]	0.48 ± 0.10 [a]	2.08 ± 0.49 [a]
	1S:1V	2.65 ± 0.47 [b]	0.65 ± 0.16 [b]	4.66 ± 0.60 [b]	0.67 ± 0.11 [b]	1.13 ± 0.15 [b]
G45	S	1.92 ± 0.29 [a]	0.48 ± 0.04 [a]	5.72 ± 0.77 [a]	0.78 ± 0.22 [a]	1.59 ± 0.24 [a]
	1S:1V	2.43 ± 0.38 [b]	0.63 ± 0.16 [b]	4.83 ± 0.98 [b]	0.84 ± 0.21 [b]	1.38 ± 0.20 [b]
G47	S	2.59 ± 0.52 [a]	0.63 ± 0.13 [a]	3.51 ± 1.70 [a]	0.66 ± 0.15 [a]	1.05 ± 0.10 [a]
	1S:1V	1.61 ± 0.24 [b]	0.43 ± 0.06 [b]	4.65 ± 0.59 [b]	0.65 ± 0.09 [b]	1.54 ± 0.02 [b]

4.3.2 The effect of physiological age (time) on the Ca and Fe content of the clones of the parent genotypes grown in soil and soil:vermiculite (1:1) over 90 d after transfer to the greenhouse

Comparisons were made between the parent genotypes and each of their clones to determine how many days of growth in soil (S) or soil:vermiculite (1:1) (v/v) (1S:1V) it took for the clones to accumulate similar Ca and Fe contents to those of their respective parents (Figs. 4.11, 4.12). Although the parents were grown only in S and harvested at 60 d, comparisons of the Ca and Fe content of the parent plants and clones grown in (v/v) 1S:1V were also performed to determine if the clones would exhibit similar Ca and Fe levels or if they would outperform their respective parents.

4.3.2.1 Ca content over time

When grown in S, the Ca levels of the G11, G39, G41 and G47 clones remained the same from d 15 to 90 (Fig. 4.11) while that of the G8 clones significantly increased from 302.73 ± 157.89 mg 100 g^{-1} DM at d 15 to 999.57 ± 34.04 mg 100 g^{-1} DM at d 90 (Fig. 4.11A). The Ca content of G15 (Fig. 4.11C) and G45 (Fig. 4.11F) significantly increased from d 80 to 90. The lowest and highest content of Ca were found in genotypes G8 and G45 with values of 302.73 ± 157.89 mg 100 g^{-1} DM at d 15 and 1127.75 ± 94.84 mg 100 g^{-1} DM at d 90, respectively (Fig. 4.11).

As observed in the (v/v) 1S:1V substrate, the Ca contents of the G11, G15, G41, G45 and G47 clones remained the same from d 15 to 90 (Fig. 4.11). The Ca content of the G8 clones (Fig. 4.11A) in (v/v) 1S:1V significantly increased from 525.79 ± 7.25 mg 100 g^{-1} DM at d 15 to 1148.11 ± 26.07 mg 100 g^{-1} DM at d 80. The Ca content of the G39 clones (Fig. 4.11D) remained the same from d 30 to 80 but significantly increased from d 15 (423.70 ± 5.68 mg 100 g^{-1} DM) to d 30 (631.14 ± 53.97 mg 100 g^{-1} DM). In contrast to the clones grown in S, the lowest and highest Ca producing cloned genotypes were G47 (106.93 ± 34.58 mg 100 g^{-1} DM) at d 15 and G8 (1204.01 ± 94.10 mg 100 g^{-1} DM) at d 90 in (v/v) 1S:1V, respectively (Fig. 4.11).

The Ca levels of the G8, G11, G15, G39, G41 and G45 clones were similar to their respective parents at d 15 in both S and (v/v) 1S:1V (Fig. 4.11). The Ca content of G47 was similar to its respective parent at d 15 when grown in S and at d 30 when grown in (v/v) 1S:1V (Fig. 4.11G).

The clones of the selected parent genotypes responded differently with regards to their Ca content in the two different substrates and over time (Fig. 4.11). Significant increases in the Ca

contents of G8 when grown in S and in (v/v) 1S:1V, G15 and G45 when grown in S, and G39 and G41 when grown in (v/v) 1S:1V were observed from d 15 to 90 (Fig. 4.11). On the other hand, when grown in S or in (v/v) 1S:1V, no significant increases in the Ca contents of the G11 and G47 clones were observed over time (Fig. 4.11).

4.3.2.2 Fe content over time

As observed in the S substrate, the Fe content of the clones of the parent genotypes (G8, G11, G15, G39, G45 and G47) significantly decreased from d 15 to 90 but that of the G41 clones remained the same (Fig. 4.12). The Fe level of G11 at d 15 (16.01 ± 0.00 mg 100 g^{-1} DM) was significantly higher than that at d 30 (8.99 ± 0.11 mg 100 g^{-1} DM). Also, for G15 the Fe content at d 15 was higher than that at d 30 (9.78 ± 0.20 mg 100 g^{-1} DM, 7.56 ± 0.30 mg 100 g^{-1} DM, respectively. The Fe content of 15.77 ± 0.51 mg 100 g^{-1} DM at d 15 and 11.91 ± 0.54 mg 100 g^{-1} DM at d 30 for G45 clones where significantly higher at d 15 than at d 30 (Fig. 4.12). However, the Fe content of the G8, G39 and G47 clones remained constant from d 15 to 30 (Fig. 4.12). The Fe content of the G15 and G47 clones were the same at d 60 to 80 but significantly decreased from d 80 to 90 (Figs. 4.12). The Fe levels of the G39 and G45 clones stayed constant from d 60 to 90 and that of G8 from d 80 to 90 (Fig. 4.12). The lowest and highest Ca producing genotypes were G15 (2.91 ± 0.05 mg 100 g^{-1} DM) at d 90 and G11 (16.01 ± 0.00 mg 100 g^{-1} DM) at d 15, respectively (Fig. 4.12).

When grown in (v/v) 1S:1V (Fig. 4.12), the Fe contents of the G11, G15 and G45 clones significantly decreased from d 15 to 60, after which they stayed constant. The Fe level of G8 clones significantly decreased from 13.42 ± 0.43 mg 100 g^{-1} DM at d 15 to 9.38 ± 0.12 mg 100 g^{-1} DM at d 30 and did not change from d 60 to 80 and from d 80 to 90 (Fig. 4.12A). In contrast, the Fe content of the G39 clones significantly increased from 12.38 ± 1.74 mg 100 g^{-1} DM at d 15 to 12.94 ± 1.02 mg 100 g^{-1} DM at d 30 (Fig. 4.12D). Also, the Fe content of the G41 clones significantly decreased from d 15 (11.70 ± 1.60 mg 100 g^{-1} DM), to d 60 (7.39 ± 0.57 mg 100 g^{-1} DM) to d 90 (5.53 ± 0.61 mg 100 g^{-1} DM) but remained the same from d 15 to 30 and d 60 to 90 (Fig. 4.12E). However, for G47, the Fe content significantly decreased from d 15 (10.20 ± 0.55 mg 100 g^{-1} DM) to d 90 (7.21 ± 0.19 mg 100 g^{-1} DM) (Fig. 4.12G). The lowest Fe content observed from the clones grown in (v/v) 1S:1V was G45 (2.45 ± 0.14 mg 100 g^{-1} DM) at d 90 and the highest was G8 (13.42 ± 0.43 mg 100 g^{-1} DM) at d 15 (Fig. 4.12).

The Fe contents of the G15 and G45 clones were similar to their respective parents at d 30 in S and in (v/v) 1S:1V (Fig. 4.12). The clones of G41 and G47 accumulated similar Fe levels to

their respective parents after 15 d when grown in S and in (v/v) 1S:1V (Fig. 4.12). When grown in S, the Fe levels of G8 at d 30, that of G11 at d 80 and that of G39 at d 30 were similar to their respective parents (Fig. 4.12). In 1(v/v) S:1V, G8 at d 30, G11 at d 60 and G39 at d 15 exhibited similar Fe levels to their respective parents (Fig. 4.12).

The Fe levels of G8, G11, G15, G39, G45 and G47 significantly decreased from d 15 to 90 when grown in S and in (v/v) 1S:1V but that of G41 significantly decreased from d 15 to 90 only when grown in (v/v) 1S:1V (Fig. 4.12).

4.3.3 The effect of substrate on the Ca and Fe content of the clones of the parent genotypes over 90 d after transfer to the greenhouse

4.3.3.1 Ca content

When grown in S, there were no significant differences in the Ca content amongst the clones of the parent genotypes at d 15, 30 and 90 (Table 4.7). However, at d 60 and 80 there were significant differences in the Ca contents of some of the clones, *viz.* at d 60, G39 (800.89 ± 118.58 mg 100 g^{-1} DM) clones exhibited significantly higher Ca levels than G15 (435.06 ± 13.42 mg 100 g^{-1} DM) and G47 (412.65 ± 65.07 mg 100 g^{-1} DM). At d 80, that of G39 (961.09 ± 157.19 mg 100 g^{-1} DM) was significantly higher than those of G11 (512.43 ± 0.88 mg 100 g^{-1} DM), G15 (486.26 ± 12.36 mg 100 g^{-1} DM) and G47 (533.66 ± 2.03 mg 100 g^{-1} DM) clones (Table 4.7).

When grown in (v/v) 1S:1V, the Ca levels exhibited by the clones of all parent genotypes were similar at d 60, 80 and 90 (Table 4.7). However, significant differences in the Ca contents were observed at d 15 and 30, *viz.* G8 (525.79 ± 7.25 mg 100 g^{-1} DM), G11 (689.42 ± 89.22 mg 100 g^{-1} DM), G15 (598.39 ± 100.1 mg 100 g^{-1} DM) and G45 (552.57 ± 140.0 mg 100 g^{-1} DM) clones' Ca contents were significantly higher than that of the G47 (106.93 ± 34.58 mg 100 g^{-1} DM) clones (Table 4.7). Also, the Ca content of the G11 (838.30 ± 67.99 mg 100 g^{-1} DM) clones at d 30 was significantly higher than those of G41 (475.35 ± 63.95 mg 100 g^{-1} DM) and G47 (432.4 ± 97.44 mg 100 g^{-1} DM) (Table 4.7).

A paired t-test was performed to determine on which substrate and at which time interval did the clones of the parent genotypes produce the most Ca. It was found that the Ca contents of G11 at d 30 and G8 and G11 at d 80 were significantly higher than those of their respective clones when grown in (v/v) 1S:1V than in S. Further, a three-way ANOVA revealed that only the genotype and substrate interaction significantly affected the Ca content of the clones.

In conclusion, the substrate had an effect on the Ca content of the clones G8 and G11 at d 30 and 80, they accumulated higher Ca levels when grown in (v/v) 1S:1V than in S, thereby confirming that there was a significant interaction between genotype and substrate.

4.3.3.2 Fe content

In S, the Fe content of the clones of G45 was 15.77 ± 0.51 mg 100 g^{-1} DM at d 15 and 11.91 ± 0.54 mg 100 g^{-1} DM at d 30 which were significantly higher than those of G15 (9.78 ± 0.20 mg 100 g^{-1} DM and 7.56 ± 0.30 mg 100 g^{-1} DM) at d 15 and 30, respectively, and G41 (9.62 ± 0.95 mg 100 g^{-1} DM and 8.53 ± 0.89 mg 100 g^{-1} DM) at d 15 and 30, respectively (Table 4.8). The Fe levels of G47 at d 60 (10.23 ± 0.79 mg 100 g^{-1} DM) and 80 (9.66 ± 0.77 mg 100 g^{-1} DM) were significantly higher than those of G11 (6.07 ± 0.03 mg 100 g^{-1} DM and 4.04 ± 0.01 mg 100 g^{-1} DM) at d 60 and 80, respectively, G15 (6.53 ± 0.22 mg 100 g^{-1} DM and 5.60 ± 0.40 mg 100 g^{-1} DM) at d 60 and 80, respectively, and G45 (7.97 ± 0.07 mg 100 g^{-1} DM and 6.12 ± 0.46 mg 100 g^{-1} DM at d 60 and 80, respectively. At d 90, the Fe content of G39 (8.16 ± 0.29 mg 100 g^{-1} DM) clones was significantly higher than those of G8, G11, G41 and G45 clones (Table 4.8).

When grown in (v/v) 1S:1V, the Fe content of G45 (15.24 ± 0.62 mg 100 g^{-1} DM) clones was significantly higher than that of G47 (10.20 ± 0.55 mg 100 g^{-1} DM) (Table 4.8) at d 15. At d 30, the Fe levels of G8, G11, G15, G41 and G47 remained the same but were significantly lower than that of G39 (12.94 ± 1.02 mg 100 g^{-1} DM). The Fe contents of G47 (8.63 ± 0.03 mg 100 g^{-1} DM) clones at d 80 and at d 90 (7.21 ± 0.19 mg 100 g^{-1} DM) were significantly higher than the Fe levels of G8, G11, G15, G41 and G45 on the same days (Table 4.8). The Fe levels of G39 were significantly higher than those of G11 and G15 at d 30 and 60 (Table 4.8).

A paired t-test was performed to determine on which substrate type and at which time interval did the clones of the parent genotypes produce the most Fe. It was found that G11 at d 60, G15 at d 60, G39 at d 90, G45 at d 80 and 90 and G47 at d 15 accumulated more Fe when grown in S than in (v/v) 1S:1V. In contrast, the clones of G11 at d 80, G15 at d 15 and 90 and G47 at d 90 exhibited higher Fe levels when grown in (v/v) 1S:1V than in S. Additionally, a three-way ANOVA showed that the genotype and time and genotype and substrate interactions significantly affected the Fe content of the clones.

In conclusion, the substrate had an effect on the Fe levels of the clones of G11 at d 60, G39 at d 90, G45 at d 80 and 90 and G47 at d 15, all of which accumulated higher levels of Fe when

grown in S than in (v/v) 1S:1V. Additionally, when grown in (v/v) 1S:1V, the clones of G11 at d 60, G15 at d 60 and G47 at d 15 accumulated higher Fe levels than those in S, confirming that there was significant interaction between the genotype and time and between genotype and substrate (Table 4.8).

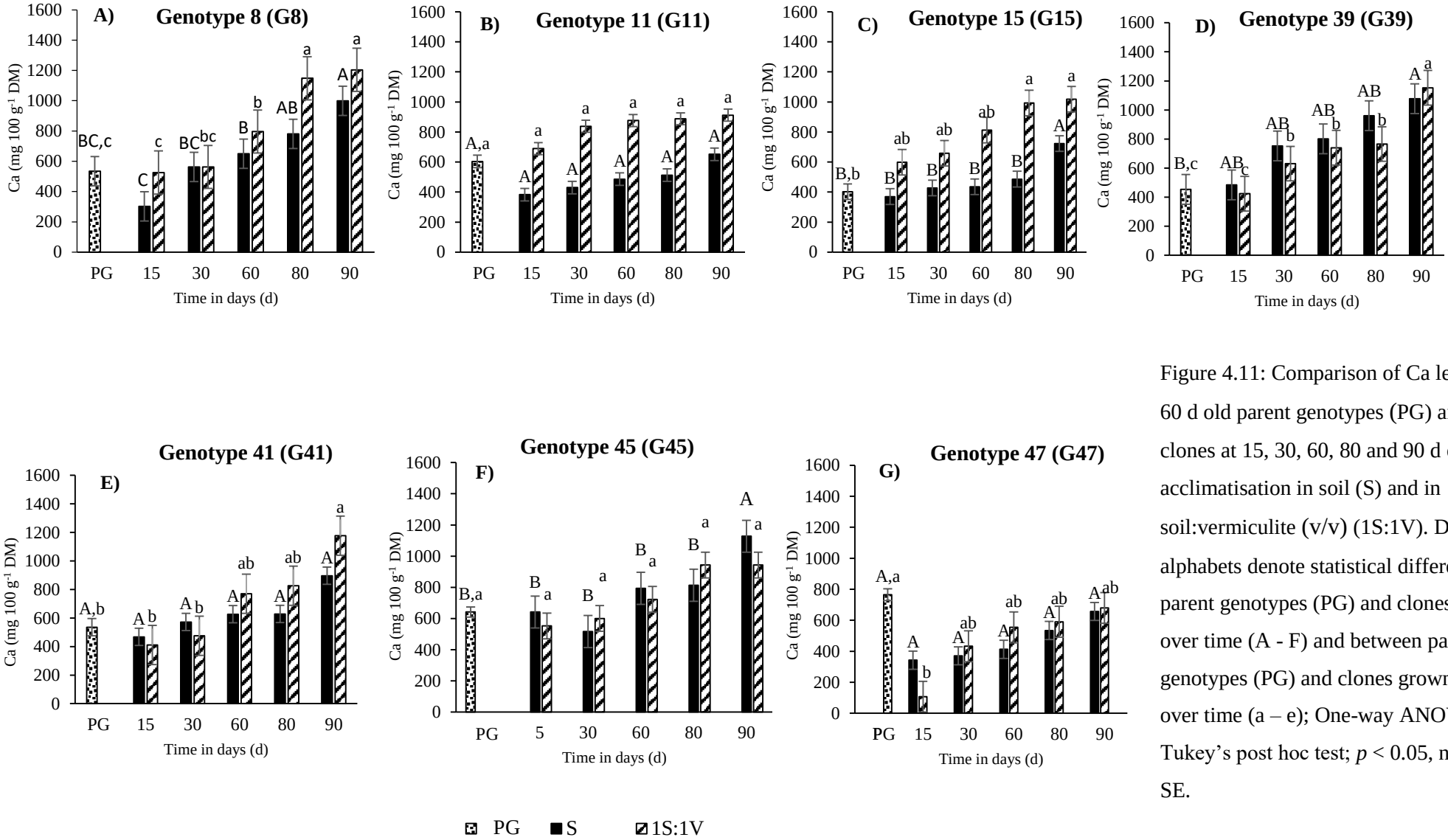

Figure 4.11: Comparison of Ca levels between 60 d old parent genotypes (PG) and their clones at 15, 30, 60, 80 and 90 d of acclimatisation in soil (S) and in soil:vermiculite (v/v) (1S:1V). Dissimilar alphabets denote statistical differences between parent genotypes (PG) and clones grown in S over time (A - F) and between parent genotypes (PG) and clones grown in 1S:1V over time (a – e); One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE.

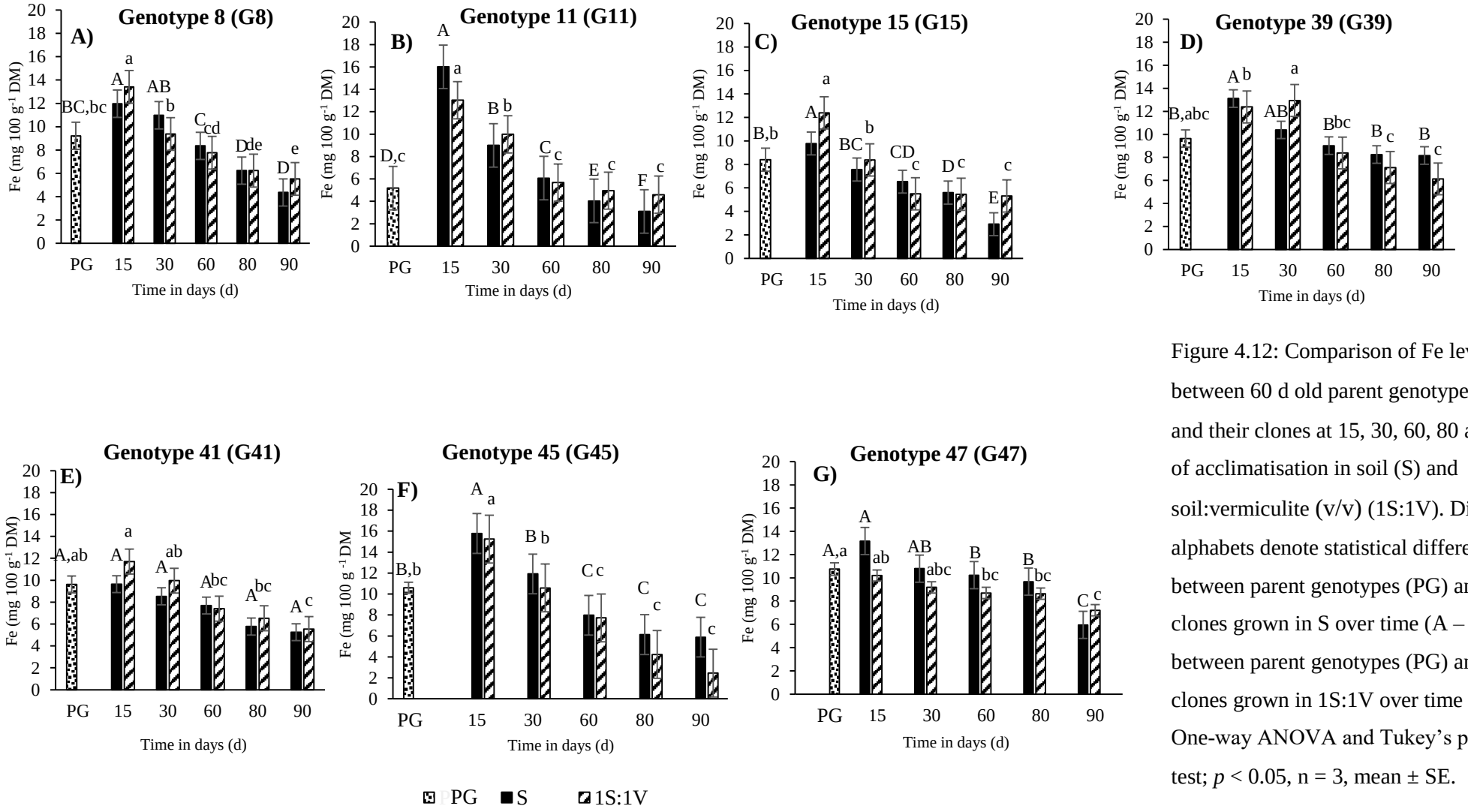

Figure 4.12: Comparison of Fe levels between 60 d old parent genotypes (PG) and their clones at 15, 30, 60, 80 and 90 d of acclimatisation in soil (S) and soil:vermiculite (v/v) (1S:1V). Dissimilar alphabets denote statistical differences between parent genotypes (PG) and clones grown in S over time (A – F) and between parent genotypes (PG) and clones grown in 1S:1V over time (a – e); One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE.

Table 4.7: Calcium content amongst selected *A. dubius* clones grown in the greenhouse over a 90 d period in soil (S) or soil:vermiculite (v/v) (1S:1V) after 21 d of acclimatisation. Dissimilar alphabet characters denote statistical differences across each row, comparison of the Ca content amongst the selected parent genotypes grown in S (a – b), comparison of the Ca content amongst the clones of the parent genotypes grown in 1S:1V (x – y); One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE. A paired t-test was used to determine the statistical differences in the Ca content between substrates at each time interval, and are represented by an asterisk (*). Three-way ANOVA is represented by F-values. Data from Fig. 4.11.

		Genotypes						
Substrate	**Time in days (d)**	**8**	**11**	**15**	**39**	**41**	**45**	**47**
S	15	302.73 ± 157.89^{a}	383.46 ± 91.05^{a}	369.70 ± 13.99^{a}	484.18 ± 227.65^{a}	467.58 ± 126.54^{a}	641.50 ± 43.34^{a}	342.42 ± 154.23^{a}
	30	562.30 ± 153.19^{a}	430.07 ± 5.29^{a}	427.29 ± 88.78^{a}	752.66 ± 52.02^{a}	571.62 ± 160.23^{a}	516.37 ± 115.46^{a}	370.58 ± 59.19^{a}
	60	649.90 ± 161.41^{ab}	486.12 ± 123.51^{ab}	435.06 ± 13.42^{b}	800.89 ± 118.58^{a}	626.43 ± 36.04^{ab}	793.73 ± 3.80^{a}	412.65 ± 65.07^{b}
	80	780.24 ± 14.99^{ab}	512.43 ± 0.88^{b}	486.26 ± 12.36^{b}	961.09 ± 157.19^{a}	628.42 ± 156.55^{ab}	812.90 ± 10.40^{ab}	533.66 ± 2.03^{b}
	90	999.57 ± 34.04^{a}	651.73 ± 220.10^{a}	723.01 ± 62.14^{a}	1077.16 ± 34.81^{a}	896.53 ± 145.39^{a}	1127.75 ± 94.84^{a}	656.65 ± 229.82^{a}
1S:1V	15	525.79 ± 7.25^{x}	689.42 ± 89.22^{x}	598.39 ± 100.10^{x}	423.70 ± 5.68^{xy}	410.36 ± 54.83^{xy}	552.57 ± 140.00^{x}	106.93 ± 34.58^{y}
	30	562.44 ± 24.75^{xy}	838.30 ± 67.99^{x*}	658.22 ± 25.46^{xy}	631.14 ± 53.97^{xy}	475.35 ± 63.95^{y}	570.94 ± 73.06^{xy}	432.40 ± 97.44^{y}
	60	796.51 ± 69.30^{x}	876.19 ± 163.53^{x}	812.45 ± 146.69^{x}	740.34 ± 12.19^{x}	796.35 ± 153.91^{x}	600.24 ± 46.84^{x}	553.80 ± 284.85^{x}
	80	1148.11 ± 26.07^{x*}	888.23 ± 58.29^{x*}	993.38 ± 161.73^{x}	765.48 ± 57.42^{x}	826.14 ± 18.99^{x}	723.12 ± 386.55^{x}	590.37 ± 38.66^{x}
	90	1204.01 ± 94.10^{x}	912.15 ± 154.31^{x}	1017.70 ± 154.45^{x}	1152.29 ± 35.40^{x}	1176.48 ± 130.91^{x}	943.29 ± 129.29^{x}	679.53 ± 144.97^{x}

Results of a three-way ANOVA test	**F-value**
G×S	6.38*
G×T	0.92^{NS}
S×T	0.71^{NS}
G×S×T	0.56^{NS}

G, genotype, T, time S, substrate, ns, Not significant, *$p < 0.05$

Table 4.8: Iron content amongst selected *A. dubius* clones grown in the greenhouse over a 90 d period in soil (S) or soil:vermiculite (v/v) (1S:1V) after 21 d of acclimatisation. Dissimilar alphabet characters denote statistical differences across each row, comparison of the Fe content amongst the clones of the parent genotypes grown in S (a – b), comparison of the Fe content amongst the clones of the parent genotypes grown in 1S:1V (w – z); One-way ANOVA and Tukey's post hoc test; $p < 0.05$, n = 3, mean ± SE). A paired t-test was used to determine the statistical differences in Fe content between substrates at each time interval, and are represented by an asterisk (*). Three-way ANOVA is represented by F-values. Data from Fig. 4.12.

		Genotypes						
Substrate	**Time in days (d)**	**8**	**11**	**15**	**39**	**41**	**45**	**47**
S	15	11.97 ± 0.62^{bc}	16.01 ± 0.00^{a}	9.78 ± 0.20^{c}	13.11 ± 1.53^{ab}	9.62 ± 0.95^{c}	15.77 ± 0.51^{a}	$13.16 \pm 0.31^{ab*}$
	30	10.98 ± 0.58^{ab}	8.99 ± 0.11^{bcd}	7.56 ± 0.30^{d}	10.39 ± 0.24^{abc}	8.53 ± 0.89^{cd}	11.91 ± 0.54^{a}	10.78 ± 0.58^{ab}
	60	8.36 ± 0.21^{abc}	$6.07 \pm 0.03^{d*}$	$6.53 \pm 0.22^{cd*}$	9.02 ± 0.31^{ab}	7.70 ± 1.80^{ab}	7.97 ± 0.07^{bcd}	10.23 ± 0.79^{a}
	80	6.24 ± 0.17^{b}	4.04 ± 0.01^{c}	5.60 ± 0.40^{bc}	8.25 ± 0.20^{a}	5.77 ± 1.41^{bc}	$6.12 \pm 0.46^{b*}$	9.66 ± 0.77^{a}
	90	4.37 ± 0.47^{bc}	3.10 ± 0.00^{c}	2.91 ± 0.05^{c}	$8.16 \pm 0.29^{a*}$	5.25 ± 0.41^{b}	$5.88 \pm 0.64^{b*}$	5.95 ± 0.35^{b}
1S:1V	15	13.42 ± 0.43^{wx}	13.03 ± 0.00^{wx}	$12.39 \pm 0.64^{wx*}$	12.38 ± 1.74^{wx}	11.70 ± 1.60^{wx}	15.24 ± 0.62^{w}	10.20 ± 0.55^{x}
	30	9.38 ± 0.12^{x}	9.98 ± 0.58^{x}	8.38 ± 0.30^{x}	12.94 ± 1.02^{w}	9.96 ± 0.50^{x}	10.57 ± 0.30^{wx}	9.18 ± 0.57^{x}
	60	7.77 ± 0.56^{wx}	5.67 ± 0.05^{x}	5.49 ± 0.29^{x}	8.38 ± 0.37^{w}	7.39 ± 0.57^{wx}	7.73 ± 0.59^{wx}	8.70 ± 0.61^{w}
	80	6.24 ± 0.33^{xy}	$4.95 \pm 0.03^{yz*}$	5.45 ± 0.24^{xyz}	7.12 ± 0.55^{wx}	6.53 ± 0.59^{xy}	4.23 ± 0.37^{z}	8.63 ± 0.05^{w}
	90	5.53 ± 0.21^{xy}	4.59 ± 0.10^{y}	$5.31 \pm 0.20^{xy*}$	6.14 ± 0.16^{wx}	5.53 ± 0.61^{xy}	2.45 ± 0.14^{z}	$7.21 \pm 0.19^{w*}$

Results of a three-way ANOVA test	**F-value**
G×S	5.69*
G×T	8.01*
S×T	1.23^{NS}
G×S×T	3.18^{NS}

G, genotype, T, time, S, substrate, ns, Not significant, *$p < 0.05$

4.3.4 Comparison of Ca and Fe content 'rankings' of the selected parent genotypes to their clones

4.3.4.1 Ca 'rankings' for parent genotypes and their clones

The significant differences in the Ca content of the parent plants in relation to each other ('rankings') when grown in S were established at d 60 (Fig. 4.2). It can be presented as G47 > G45 > G11 > G41 = G8 > G39 > G15. A similar illustration is shown in Table 4.9 for all the tested clones of the parent genotypes grown in S and in (v/v) 1S:1V. It was observed that at no tested time or tested substrate did the clones exhibit the same 'rankings' as the parents. When grown in S, the 'rankings' of the selected clones were similar at d 15, 30 and 90 but at d 60 and 80, the 'rankings' changed and G39 had the highest Ca content. In (v/v) 1S:1V, the Ca 'rankings' of the clones differed only at d 15 and 30.

Table 4.9: Ca 'rankings' of the clones of the parent genotypes at each time interval in S and in (v/v) 1S:1V. Parents were 'ranked' as G47 > G45 > G11 > G41= G8 > G39 > G15 at d 60. Data from Table 4.7.

Time in days (d)	Ca in S	Ca in 1S:1V
15	8 = 11 = 15 = 39 = 41 = 45 = 47	8 = 11 = 15 = 45 ≥ 39 = 41 ≥ 47
30	8 = 11 = 15 = 39 = 41 = 45 = 47	11 ≥ 8 = 15 = 39 = 45 ≥ 41 = 47
60	39 = 45 ≥ 8 = 11 = 41 ≥ 15 = 47	8 = 11 = 15 = 39 = 41 = 45 = 47
80	39 ≥ 8 = 41 = 45 ≥11 = 15 = 47	8 = 11 = 15 = 39 = 41 = 45 = 47
90	8 = 11 = 15 = 39 = 41 = 45 = 47	8 = 11 = 15 = 39 = 41 = 45 = 47

It can be concluded that neither the time intervals nor the substrate type resulted in the clones exhibiting similar 'rankings' to their respective parents with regards to their Ca content.

4.3.4.2 Fe 'rankings' for parent genotypes and their clones

At d 60, the Fe 'ranking' of the parent genotypes was established and illustrated as G47 = G45 > G39 = G41 > G8 > G15 > G11 (Fig. 4.3). Of the tested clones grown in S and in (v/v) 1S:1V, the Fe 'rankings' were established and are shown in Table 4.10. The Fe content of the clones

of G47 and G11 at d 60 and of G11 at d 80 in S exhibited similar ‘rankings’ to their respective parents. In (v/v) 1S:1V, the clones of G47 were ‘ranked’ similar to its respective parent at d 80 and 90.

Table 4.10: Fe ‘rankings’ of the clones of the parent genotypes at each time interval in S and in (v/v) 1S:1V. Parents were ‘ranked’ as G47 = G45 > G39 = G41 > G8 > G15 > G11 at d 60. Data from Table 4.8.

Time in days (d)	**Fe in S**	**Fe in 1S:1V**
15	11 = 45 ≥ 39 = 47 ≥ 8 = 15 = 41	45 ≥ 8 = 11 = 15 = 39 = 41 ≥ 47
30	45 ≥ 47 = 8 ≥ 39 = 11 ≥ 41 = 15	39 ≥ 45 ≥ 8 = 11 = 15 = 41 = 45 = 47
60	47 ≥ 41 = 39 ≥ 8 ≥ 45 ≥ 15 ≥ 11	39 = 47 ≥ 8 = 41 = 45 ≥ 11 = 15
80	39 = 47 ≥ 8 = 45 ≥ 15 = 41 ≥ 11	47 ≥ 39 ≥ 8 = 15 = 41 ≥ 11> 45
90	39 ≥ 41 = 45 = 47 ≥ 8 ≥ 11 = 15	47 ≥ 39 ≥ 8 = 41 ≥ 15 ≥ 11 ≥ 45

It was concluded that time intervals and substrate type had no effect on the Fe ‘rankings’ of G47 and G11 clones at d 60 when grown in S and of G47 clones at d 80 and 90 when grown in (v/v) 1S:1V as the ‘rankings’ of these clones were similar to their respective parents.

Chapter 5. Discussion

5.1 Establishment of a micropropagation protocol for *A. dubius* field-derived nodal explants and the application of that protocol to the nodal explants of the selected parent genotypes

As emphasised previously, ALVs are a rich source of micronutrients, in particular calcium (Ca) and iron (Fe), and thus have the potential to supplement the diets of impoverished communities (Gockowski et al., 2003; Modi et al., 2006; Odhav et al., 2007; Faber et al., 2010; Achigan-Dako et al., 2014; Mavengahama et al., 2014; Cardi et al., 2017). However, variations amongst plant genotypes have been reported for several traits including micronutrient content, protein levels and various morphological characteristics (Ono et al., 1993; Doorne et al., 1995; Kintzios and Taravira, 1997; Sairam et al., 2003; Schween and Schwenkel, 2003; Bhatia et al., 2005; Jabeen et al., 2005; Sutan et al., 2010; Borjian and Arak, 2013; Mwase et al., 2014; Shumilina et al., 2015; Srivastava, 2015; Naranjo et al., 2016). Therefore, the strategy proposed in this study was to select genotypes of *Amaranthus dubius* with desirable Ca and Fe levels to be mass propagated and kept in the greenhouse for the purpose of distribution to communities suffering from micronutrient malnutrition. Biotechnological strategies have been reported as tools to micropropagate genotypes selected for specific traits of interest (Dubois, 2009; Butt et al., 2015; Opabode, 2017). With regards to *Amaranthus* species, *in vitro* propagation via indirect (Flores et al., 1982; Bennici et al., 1992; Bennici et al., 1997; van Le et al., 1998) and direct (Pannu et al., 2013) organogenesis has been reported. Based on those published protocols, the present study set out to establish a micropropagation protocol for field grown *A. dubius* plants, found locally, using nodal explants. Additionally, a seedling population of *A. dubius* was used to screen and select genotypes with varying Ca and Fe levels (hereafter referred to as parent genotypes). Thereafter, the established micropropagation protocol was applied using the nodal explants of the parent genotypes and the effects of micropropagation, physiological age (time) and substrate type on the growth and the Ca and Fe content of their clones were determined.

Ideally, *in vitro* culture techniques should produce plants free from contaminants but due to the presence of undetectable endogenous bacteria and fungi in plants, this is unlikely (George, 1993; Orlikowska et al., 2017). Several methods of limiting contaminants such as the application of fungicides (de Oliveira et al., 2010; Orlikowska et al., 2017), the use of biocides (ethanol, calcium [CaOCl] or sodium hypoclorite [NaOCl]), and of mercuric chloride ($HgCl_2$) amongst others (Liefert and Waites, 1991) and the use of 'inner' sterilisation such as soaking

explants in antibiotics (Falkiner 1997) or in 8-hydroxyqinoline-citrate (Orlikowska et al., 2017) have been used prior to, or during culture, to eradicate contaminants. The plant species, type and source of explant and the nature of the contaminants influence the effectiveness of these methods (Niedz and Bausher, 2002). Pannu et al. (2013) successfully surface sterilised leaves of *A. spinosus* with 0.5% (v/v) NaOCl for 15 – 20 min. Flores et al. (1982) used 70% (v/v) ethanol for 2 min followed by immersion in 10% (v/v) Clorox® (bleach) for leaf explants of *A. cruentus*, *A. hypochondriacus* and *A. tricolor* and reported 0 – 5% contamination.

In the present study, 89.3 ± 2.0 % fungal and bacterial contamination were observed in field-derived nodal explants after 3 d in culture when they were surface sterilised using 1% (v/v) NaOCl and 70% ethanol (protocol A) (Table 4.1). To reduce the contamination, the field plants maintained in the greenhouse were sprayed with fungicides twice a week (Table 3.1) and Previcur® was added to the multiplication medium during the nodal explant culture (protocol B). Although this approach reduced the fungal contamination, 49.3 ± 2.57% explants with bacterial contamination were still evident after 14 d (Table 4.1). Methylene blue was then added to the multiplication medium to reduce the bacterial contamination; although this effectively reduced the contamination to 18.7 ± 2.01% after 14 d (Table 4.1), the bacterial contamination increased to 50%, after another two weeks in culture. Hence, various antibiotic treatments were tested for their effectiveness in eradicating the two bacterial strains that were visually identified, and subsequently, streptomycin/penicillin (100 µg l^{-1}) and rifampicin (50 µg l^{-1}) were selected (Table 4.2) for the decontamination protocol. Therefore, surface sterilisation of field-derived nodal explants in 1% (v/v) NaOCl, 1 ml l^{-1} (v/v) Previcur® and 2 drops of Tween 20® for 10 min followed by three rinses in sterile distilled water, shaking (1500 rpm) for 5h in an antibiotic solution (¼ strength Murashige and Skoog (MS) basal salt medium, 100 µg l^{-1} streptomycin/penicillin, 50 µg l^{-1} rifampicin) prior to culture in semi-solid and liquid multiplication media resulted in 30% contamination.

Shoots were induced from field-derived nodal explants in semi-solid and liquid media containing various concentrations and combinations of benzylaminopurine (BAP) and indole-3-acetic acid (IAA) resulting in 60 ± 0.3 – 70 ± 0.1% and 10 ± 0.3 – 11 ± 0.3% explants producing shoots, respectively (Table 4.3). These were lower than that (93.3 ± 0.27%) reported by Pannu et al. (2013) when leaves of *A. spinosus* were cultured on 0.5 mg l^{-1} BAP and 0.1 mg l^{-1} naphthalene acetic acid (NAA). In that study, using the same PGR combination, the shoot length was higher (6.78 ± 0.21 cm) than those reported in the current study (1.1 ± 0.1 – 2.7 ± 0.1 and 0.5 ± 0.0 – 1.2 ± 0.2 cm for semi-solid and liquid media, respectively) using various

BAP and IAA combinations and concentrations. Due to the lack of literature on the number of shoots/nodal explant derived from direct organogenesis of *Amaranthus* species, comparisons were made between number of shoots/nodal explant of *A. dubius* obtained in the current study and those of the number of shoots/leaf explant of *A. spinosus* obtained via indirect organogenesis (Pannu et al. 2013). In the current study, 4 shoots/nodal explant were obtained in the media with 1 mg l^{-1} BAP + 1 mg l^{-1} IAA and 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA (Table 4.3). These were lower than that reported by Pannu et al. (2013) (8.66 ± 0.32) using leaf explants of *A. spinosus*.

In the present study, as the field nodal explants that were cultured in semi-solid media had the best *in vitro* shoot growth responses those from 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA were chosen for the rooting investigations. Three different IAA concentrations were tested for rooting (Table 4.4). All the shoots cultured in the medium with 0.1 mg l^{-1} IAA rooted within 7 – 14 d but only 40% of the shoots rooted in the lower tested concentrations. In other studies, on *Amaranthus* (*A. caudatus, A. cruentus, A. hybridus, A. hypochondriacus, A. edulis*, *A. spinosus*), 100% rooting was obtained with 1 – 2 mg l^{-1} indole-3-butyric acid (IBA) (Bennici et al., 1997; van Le et al., 1998; Gajdošová et al., 2013; Pannu et al., 2013). All of these studies and the current study demonstrate that various *Amaranthus* species root well with different auxins. Following rooting, plantlets were acclimatised in two substrates *viz.* S (soil, i.e. nutrient-rich) and (v/v) 1S:1V (1soil:1vermiculite, i.e. nutrient-poor). Although it was found that 80% of the plants survived acclimatisation in both substrates, the yield (plant/nodal explant) averaged at only 2 ± 0.2 (Table 4.4) for the tested rooting media. In contrast, the yield obtained by Pannu et al. (2013) from *A. spinosus* was 7 plants/leaf explant following indirect organogenesis. These differences are likely due to the explant type and the pathway of morphogenesis (Nicole 1998; Gubiš et al., 2003; Jabeen et al., 2005; Khar et al., 2005; Ishag et al., 2009; Shojaei et al., 2010; Sutan et al., 2010; Yan et al., 2009; Yang et al., 2010; Grozeva and Velkov, 2014; Amin et al., 2015; Kaur et al., 2015).

Altering environmental conditions can affect the development and physiology of plants and those changes express themselves in the phenotype of the plant (Brenner et al., 2010; Khanam and Oba, 2014). This is referred to as phenotypic plasticity and has been tested on clones and on inbred plants (Sultan, 2000; Sultan, 2003; Nicotra et al., 2010). In this study, the effects of physiological age (time) and substrate type on three growth parameters (number of leaves, plant height and root length) were recorded for *A. dubius* clones acclimatised in S and (v/v) 1S:1V (Fig. 4.1). These parameters were recorded during 21 d of acclimatisation. When the clones

were grown in (v/v) 1S:1V, there were no effects of time on plant height and root length but the number of leaves changed over time. They increased significantly from d 0 (6 ± 0.35) to 7 (16 ± 0.54), decreased from d 7 to 14 (10 ± 0.71) and increased from d 14 to 21 (13 ± 0.54). When grown in S, there were significant increases in the number of leaves from d 0 (8 ± 0.3) to d 14 (14 ± 0.52), in the plant height from d 0 (10 ± 0.34 cm) to d 14 (17.67 ± 0.5 cm) and for the root length from d 0 (12.33 ± 0.43 cm) to d 14 (22.67 ± 0.6 cm). Oyedeji et al. (2014) investigated the effects of NPK (nitrogen:phosphorus:potassium) and poultry manure on some growth parameters (number of leaves, plant height, stem girth, leaf area and number of branches) of *A. hybridus*, *A. cruentus* and *A. deflexus* plants grown in a greenhouse. Generally, because poultry manure releases micronutrients into the soil at a slower rate than NPK fertilisers it is therefore considered as nutrient-poor (Oyedeji et al., 2014). Those authors, found that all the growth parameters increased on both substrates, while in this current study such increases occurred only in S only (Fig. 4.1). In the present study, at d 7 there were more leaves on the plants grown in (v/v) 1S:1V than in S, and at d 14 and 21, longer plant heights and root lengths were observed in plants grown in S than those grown in (v/v) 1S:1V (Fig. 4.1). However, the substrate only influenced the number of leaves in the study by Oyedeji et al. (2014), which were higher in poultry manure (7 and 22) than in the NPK (4 and 12) substrate for *A. hybridus* and *A. cruentus*, at 1 and 2 weeks of growth, respectively, for each species. In the current study, the number of leaves increased on both substrates (S and (v/v) 1S:1V) with time an indication that both substrates can be practically used for producing plants with many leaves (Fig. 4.1). The results by Oyedeji et al. (2014) discussed above are similar to those of the current study, as they also showed increases in the tested growth parameters with increases in physiological age on substrates of different fertility.

Following the tested multiplication and rooting stages, *A. dubius* nodal explants were best propagated in semi-solid medium with 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA, elongated in 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} IAA and rooted in 0.1 mg l^{-1} IAA. However, during the 21 d of acclimatisation of field-derived clones, both substrates resulted in a similar increase in the tested growth parameters. Since the leaves which are harvested from the plant increased on both substrates during acclimatisation, both substrates were used in the subsequent study when the selected parent genotypes were micropropagated using this protocol.

Fifty seeds of *A. dubius* were germinated and the seedlings screened for their Ca and Fe contents following which seven genotypes (parent genotypes) were selected for further study. These were G8, G41, G45 and G47 for high Ca and Fe content, G15 for low Ca and Fe levels

and G39 for high Fe and low Ca content and G11 for high Ca and low Fe (Figs. 4.2 and 4.3). The established micropropagation protocol was then applied to the nodal explants of the selected parent genotypes (Table 4.5) and the *in vitro* growth responses of these explants were compared to those of the field-derived nodal explants (Table 4.3). It was observed that explants of the parent genotypes had less bacterial contaminants in culture (5.0 ± 08 – 12.5 ± 1.9%) and resulted in 26% more explants producing shoots than those of the field-derived nodal explants (Table 4.5). Due to the harsh environmental (exposure to microbial organisms) conditions, field-derived explants often have a higher contamination percent in culture (George et al., 2008). Hence, the bacterial contamination observed in field-derived explants in the current study. In contrast, the shoot lengths of the clones of the parent genotypes (0.6 ± 0.2 to 1.9 ± 0.1 cm) were shorter than those of the field-derived clones (1.1 ± 0.1 to 2.7 ± 0.1 cm). The number of shoots/nodal explant were similar for the parent genotypes (2.0 ± 0.6 to 5 ± 1.4) to those that were field-derived (2.0 ± 0.1 to 4.0 ± 0.4). The yield (plants/nodal explant) of the field-derived clones was 2.0 ± 0.2 (Table 4.4) but ranged from 2.0 ± 0.3 to 4.0 ± 0.3 (Table 4.5) for the clones of the parent genotypes. The latter yield was still lower than that of 7 plants/leaf explant reported by Pannu et al. (2013). It is possible that the differences in the growth responses were the result of the age of both the explant sources, *viz.* field-derived (age of the field-derived explants was unknown) and selected parent genotypes (age was 60 d), and also the environmental conditions of the field-derived explants prior to housing them in the greenhouse. The environmental conditions, i.e. soil microbial contaminants of the field plants, could have also contributed to the high bacterial contamination in culture. Explant age has been found to have significant effects on shoot regeneration in that the older plants have less ability to regenerate shoots than the younger explants (Niedz et al., 1989; Dong and Jia., 1991; Ono et al., 1993; Bhatia et al., 2005; Mohebodini et al., 2011; Yildiz, 2012; Ambajo and Matheka, 2016). When the *in vitro* responses of the clones of the parent genotypes were compared amongst each other, only the shoot length and yield showed significant differences amongst them (Tables 4.5). Significantly longer shoot length was observed for G47 than those of G11, G39 and G45 and the plant yield of G45 was higher than those of G8, G39 and G41 (Table 4.5). Genotypic effects have also been reported to influence the growth of plants (Mohebodini et al., 2011; Yildiz, 2012).

Finding the most suitable substrate and optimal age to produce more leaves and bigger plants is important for the nutritional (Ca and Fe) benefits of *A. dubius* leaf consumers. In this study, the effects of physiological age (time) and substrate type on selected growth parameters of the

clones of the parent genotypes were investigated over 21 d of acclimatisation in S or (v/v) 1S:1V (Figs. 4.4 – 4.10). Significant increases were observed from d 0 to 21 in the number of leaves of G39 (d 0: 7 ± 0.5; d 21: 10.67 ± 0.53, Fig. 4.7A) and G47 (d 0: 7 ± 0.54; d 21: 13.33 ± 0.55, Fig. 4.10A), the plant heights of G11 (d 0: 5.67 ± 0.33; d 21: 12.33 ± 0.53, Fig. 4.5B) and the root lengths of G8 (d 0: 6.67 ± 0.42; d 21: 17.33 ± 0.53, Fig. 4.4C) and G41 (d 0: 10 ± 0.49; d 21: 16.67 ± 0.51, Fig. 4.8C) grown in S. The plant height of the genotypes G8 (d 0: 5 ± 0.41; d 21: 9.67 ± 0.5, Fig. 4.4B) and G47 (d 0: 6.33 ± 0.43; d 21: 10.67 ± 0.53, Fig. 4.10B) significantly increased from d 0 to 21 when grown (v/v) 1S:1V. Similar reports were made by Horak and Loughin (2000) where the leaf number, plant height and root length of *A. palmeri, A. rudis, A. retroflexus* and *A. albus* plants increased with an increase in time.

With regards to the substrate effect, it was found that at d 21 the number of leaves of the G8 (12.33 ± 1.52 in (v/v) 1S:1V; 7.33 ± 0.6 in S; Fig. 4.4A) clones and the plant height of the G39 (12.33 ± 0.27 cm in S; 8.33 ± 0.9 cm in (v/v) 1S:1V; Fig. 4.7A) clones, were significantly higher and longer in (v/v) 1S:1V and S, respectively. This means that only one out of the seven selected parent genotypes preferred nutrient-poor soil to produce more leaves. However, there were no significant differences in the shoot dry masses of the G8 and G39 clones between the two substrates (Table 4.6). As there were no significant differences between the substrates in increasing leaf number in the other genotypes, or in increasing the plant height and root length, it can be concluded that nutrient-poor soil, typical of the targeted communities, can be used for the *ex vitro* growth of the clones of the parent genotypes. Peyvast et al. (2005), investigated the effects of four substrate types, i.e. peat, perlite, rice-hull and perlite:rice-hull (1:1) on the growth of greenhouse-grown *Cucumis sativus* plants. Those authors found that the best substrate was peat, in which the plants exhibited the longest plant length, largest fruit size and weight, and the best plant yield. They suggested that the positive effect of peat might be due to its high cation exchange capacity (CEC). A high CEC influences the ability of the substrate to hold on to more micronutrients, making them sufficient in Ca, Fe, magnesium (Mg) and other cation content (Sonon et al., 2014). In a similar study, Alan et al. (1994) investigated the effects of soil (nutrient-rich) and combinations of inorganic medium (perlite, peat, pumice–nutrient-poor) on the growth parameters of greenhouse-grown tomatoes (*Lycopersicon esculentum*). They reported that the tomato plants produced higher yield (2.87 kg/plant) in a mixture of 80% pumice + 10% perlite + 10% peat substrate than in 100% soil (2.05 kg/plant). Also, Agyare et al. (2017) reported that different mulches (black plastic, grass and no mulch) significantly influenced growth parameters, i.e. plant height (46.4 to 82.6) and diameter (14.9 to 16.0), fruit

length (10.9 to 16.1), number (65.0 to 118.0) and yield (2.5 to 7.1) of ten field-grown okra (*Abelmoschus esculentus* L.) genotypes. These findings, similar to the results of the current study, show that in some species nutrient-poor soils can produce plants with similar growth parameters to those grown in nutrient-rich soils.

The root:shoot biomass of the clones of the parent genotypes was recorded at 90 d, following transfer to the greenhouse (Table 4.6) after 21 d of acclimatisation. The root:shoot biomass indicates the allocation of resources in the soil for uptake into the plants via the roots (Horak and Loughin, 2000; Poorter et al., 2012). In the present study, the root:shoot ranged from 0.64 ± 0.34 to 2.08 ± 0.61 g in S and 0.98 ± 0.22 to 2.07 ± 0.56 g in (v/v) 1S:1V with no significant differences amongst the genotypes on each substrate or between substrates for each genotype, further indicating that *A. dubius* can be grown on nutrient-poor substrates. Future studies should investigate the effect of physiological age on the root:shoot of *A. dubius* clones and determine their photosynthetic ability in relation to light intensity in various regions of the country since the photosynthetic ability has been reported to influence the biomass in different environmental conditions e.g. legume genotypes (*Cyamopsis tetragonoloba*) (Singla et al., 2016). Additionally, other environmental conditions, such as water stress should be investigated for their impact on root:shoot since genotypic differences in biomass have been reported for *Amaranthus* (Liu and Stützel, 2004), light intensity since variation in leaf area, biomass or *A. tricolor* (Singh et al., 2009b) and other vegetables under these stresses e.g. *Brassica rapa* (Edwards et al., 2016).

Following micropropagation of the selected *A. dubius* genotypes using the established protocol, genotypic variations were observed for the *in vitro* shoot length and post acclimatisation yield. In addition, the number of leaves increased with time for the clones of all the genotypes on both substrates but only, G8 and G39 preferred nutrient-poor substrate to produce more leaves. In addition, the shoot and the root:shoot dry masses were not affected by the substrate type or the genotypes. Therefore, a nutrient-poor substrate can be used to grow clones of *A. dubius* of the selected parent genotypes to produce similar growth responses as those in a nutrient-rich substrate.

5.2 Leaf Ca and Fe content in the parent genotypes, and the effects of micropropagation and physiological age (time) on these contents in their clones grown in S

Knowledge of the genetic variation within or amongst different genotypes aids in the selection of progenies with desired traits (Shukla et al., 2010; Akaneme and Ani, 2013; Gerrano et al., 2014; Oduwaye, 2014). Besides being influenced by genotype, differences in traits for example micronutrient levels, can be dependent on several factors such as geographical location (source of material), physiological age, substrate type, secondary compounds (tannins, ascorbic acid, oxalates), substrate pH, substrate micronutrients and environmental conditions (Rangarajan and Kelly, 1998; Frossard et al., 2000; Gupta et al., 2005; Modi, 2007; van der Walt et al., 2009). The effects of environmental conditions on the Ca and Fe levels in the leaves of *Amaranthus* species were evident in studies by Modi (2007), Molina et al. (2011) and Ribeiro et al. (2017) on different *Amaranthus* species, and by Odhav et al. (2007), Yang and Keding (2009) and Molina et al. (2011) in leaves of *A. dubius*. Of particular interest in the current study were the differences in the leaf Ca and Fe levels of selected *A. dubius* (G8, G11, G15, G39, G41, G45 and G47) parent genotypes and their micropropagated clones. When the leaf micronutrient levels of fifty *A. dubius* genotypes from a seedling population were determined, it was found that the Ca content was 246.3 to 765.3 mg 100 g^{-1} DM (Fig. 4.2) which is within the lower levels cited in the literature (Table 1.2). In contrast, the Fe levels recorded in this study were 5.25 to 26.68 mg 100 g^{-1} DM (Fig. 4.3) and within the range of 3.4 to 95.15 mg 100 g^{-1} DM reported in the literature (Table 1.2).

The current study also investigated the effects of micropropagation and physiological age (time) on the Ca and Fe levels of the clones of the parent genotypes. The effects of micropropagation were determined by comparing the leaf Ca and Fe levels of the parent genotypes grown in S and harvested at 60 d to those of their respective clones grown in S (nutrient-rich substrate) harvested at 15, 30, 60, 80 and 90 d after transfer to the greenhouse following 21 d of acclimatisation (Figs. 4.11 and 4.12).

It was found that all the clones exhibited similar Ca levels to their respective parent genotypes at d 15 (Fig. 4.11). The ‘rankings’, i.e. significant differences in the Ca content of the parent genotypes in relation to each other, were established at d 60 (G47 > G45 > G11 > G41= G8 > G39 > G15) and at d 15, 30, 60, 80 and 90 for their clones (Table 4.9). It was observed that at no time interval did the clones in S show similar Ca ‘rankings’ to their parents (Table 4.9). This indicates that micropropagation influenced the ‘rankings’ of the clones at each time interval.

The *in vitro* culture conditions (light intensity, medium type etc) can induce variations in *in vitro* and *ex vitro* responses either temporarily as a result of the genotype and environment, or permanently, in that the cells undergo persistent genetic changes that are heritable from the source plant (Bairu et al., 2010; Krishna et al., 2016). Although it appears that the variations in the Ca levels of the leaves of the clones of the parent genotypes were due to micropropagation, it is unclear if this variation can be attributed to permanent or temporary factors.

Increases in the leaf Ca levels of the clones were observed with time (i.e. physiological age) for the clones of some parent genotypes but for G8 significant increases were observed from d 15 to 90 and for G15 and G45 from d 80 to 90. There were no changes in the leaf Ca levels of G11, G39, G41 and G47 from d 15 to 90. Similarly, studies by Modi (2007) on glasshouse-grown *A. hybridus var. cruentus, A. hypochondriacus, A. tricolor*, *A. thunbergii* and *A. hybridus* and by Khader and Rama (2003) on field-grown *A. blitum* and *A. gongeticus,* showed that the leaf Ca content of those plants increased with time (15 to 30 d old plants). According to Khader and Rama (2003), the observed effect of the accumulation of Ca in older leaves is due to the immobile nature of Ca and its inability to translocate from the older to the juvenile leaves. The immobility of Ca may also be attributed to genotypic effects, the plant's rooting system or the amount of inhibitory factors (oxalates, phenolic, phytic acid, tannins etc.) within the plants (Frossard et al., 2000; Khader and Rama 2003). In a study by Amalraj and Pius (2015), the authors found that some tested leafy vegetables (*Chenopodium album, Alternanthera philoxeroides* and *Centella asiatica*) with higher levels of inhibitory factors had less bioavailable Ca in both raw and cooked material. Nutrient absorption by the plant may also be influenced by the substrate type, the genotype or the type of root system (Nicole, 1998; Gupta et al., 2005; Ohshiro et al., 2016). The spatial distribution in the substrate influences the potential of the plant roots to exploit the soil for regions with higher micronutrients and also affects the roots' ability to increase in surface area by the production of root hairs for greater nutrient uptake (Nicole, 1998; Horn et al., 2009). It is possible that in the current study, these factors also affected the clones of the parent genotypes in accumulating Ca. However, in the case of this study, it was found that although there were significant increases over time in the Ca levels of some genotypes in S, the root:shoot biomass of G8 (0.82 ± 0.05), G15 (0.96 ± 0.10) and G45(1.59 ± 0.24) (Fig. 4.11 and Table 4.6) did not influence their ability to accumulate Ca, an indication that the accumulation of Ca was independent of the masses of the roots and root:shoot of the genotypes.

After micropropagation, the G15, G39, G41, G45 and G47 clones accumulated similar Fe levels to those of their respective parent genotypes prior to 60 d (Fig. 4.12). For the G41 and G47 clones, similar Fe levels to their respective parents were achieved at d 15 whilst this occurred for G15, G39 and G45 clones at d 30. Only the clones of G8 reached a similar Fe level to its parent genotype at d 60. On the other hand, at d 80, G11 was similar in Fe content to its respective parent (Fig. 4.12). As for Ca, the Fe 'rankings', i.e. the significant differences in the Fe content of the parent genotypes in relation to each other, were established at d 60 (G47 = G45 > G39 = G41 > G8 > G15 > G11) and at d 15, 30, 60, 80 and 90 for their clones (Table 4.10). It was observed that only the 'rankings' of G47 and G11 at d 60 ($47 \geq 41 = 39 \geq 8 \geq 45 \geq 15 \geq 11$) and of G11 at d 80 ($39 = 47 \geq 8 = 45 \geq 15 = 41 \geq 11$) were the same as their respective parents (Table 4.10). These results indicate that micropropagation affected the 'rankings' of five of the seven genotype clones at each time interval. Ferdausi et al. (2009) also reported higher Fe content (0.5531 ppm and 0.5007 ppm) in *in vitro* regenerated plants of two *Solanum melongena* L. genotypes (Jhumky and Islampuri) than in the seedlings of both the genotypes (0.2015 ppm and 0.2509 ppm), (unknown d age) in the greenhouse. Although, that study and the current one show that micropropagation significantly influences the Fe content of *in vitro* regenerated genotypes, it is unclear if this occurrence is due to a temporary effect or a permanent one. As a result, it is important for future studies to determine if the variations amongst the clones of the *A. dubius* parents are the result of a temporary environmental condition (epigenetic) or if they are heritable (permanent change).

A decline in the Fe levels was observed in most of the clones of the parent genotypes over time (i.e. physiological age) (Fig. 4.12). In contrast to what was determined with Ca, significant decreases in the Fe levels over time were observed for G8, G11, G15, G39, G45 and G47 from d 15 to d 90. However, only G11 exhibited significant decreases in Fe at all the tested times. A significant interaction (f-value = 8.01) was observed for the physiological age and genotype in support of these results (Table 4.8). In studies by Ibrikci et al. (2003) and Khader and Rama (1998), similar to the current study, decreases in Fe content were observed in 19 accessions of greenhouse-grown *Cicer arietinum* (chickpea) and on field-grown *A. blitum* and *A. gongeticus,* respectively. This may be an indication that Fe is redistributed to other plant parts for example into flower initiation and is therefore unavailable in the leaves (Khader and Rama, 1998). However, results by Cornforth et al. (1978), Khader and Rama (1998) and Modi (2007) differed from those of the current study, as they found that the leaf Fe content significantly increased with time in kale, *A. blitum,* and *A. hybridus var. cruentus, A. hypochondriacus, A. tricolor, A.*

thunbergii and *A. hybridus*, respectively. This is a disadvantage because older leaves have been suggested to have more anti-nutritional compounds making them less palatable than the juvenile leaves (Modi, 2007; Agbaire, 2012). Despite the fact that Fe is one of the most abundant micronutrients in the earth's crust, its accessibility to plant roots is very low as it relies on the soil redox potential and pH to convert insoluble Fe into a soluble form (Morrissey and Guerinot, 2009). According to Frossard et al. (2000), the development of a rooting system able to excrete protons along the root surfaces is essential in converting insoluble Fe^{3+} into soluble Fe^{2+}, for its bioavailability in plants. In addition, the presence of ferritin and frataxin, proteins which sequester Fe in the cellular structures of the plant also influence the available Fe in each plant (Morrissey and Guerinot, 2009). In the current study, although genotypes differed in their Fe contents with time and the Fe contents of some genotypes were influenced by the substrate (Fig. 4.12), it was clear that these contents were not influenced by their root nor their root:shoot dry masses, which was not affected by the substrate.There were no significant differences in Fe in S and (v/v) 1S:1V amongst the genotypes (0.64 ± 0.34 g to 2.08 ± 0.61 g in S and 0.98 ± 0.22 g to 2.07 ± 0.56 g in (v/v) 1S:1V (Table 4.6).

The present study showed that the effects of micropropagation on the content of Ca in the leaves were twofold. Firstly, the clones of all the parent genotypes grown in S accumulated similar Ca levels to their respective parents at d 60, and secondly, the Ca 'rankings' of all these clones in S did not match their respective parent genotypes at any time interval. In the case of Fe, the contents of five of the seven genotype clones matched their respective parents prior to 60 d and the 'ranking' of five of the seven genotype clones was different to those of their respective parent genotypes at different time intervals. When the effects of physiological age on the leaf Ca and Fe levels were investigated, significant interactions between the physiological age and genotype were observed only in the case of the Fe levels, which decreased over time. In constrast, the Ca levels of the clones increased with time.

5.3 The effect of substrate type on the leaf Ca and Fe content of the clones of the parent genotypes

The type of substrate, its moisture content, cation exchange capacity (CEC) and pH are known to influence the availability of micronutrients within the substrate and subsequent uptake by plants (Cornforth et al., 1978; Rangarajan and Kelly, 1998; Frossard et al., 2000; Gupta et al., 2005; Yetsir et al., 2006; Modi, 2007; van der Walt et al., 2009; Olle et al., 2012; Ohshiro et al., 2016). Priya and Santhi (2014) investigated the effects of soil enriched with vermicompost

(enriched separately with the worms *Eudrilus eugeniae* and *Lampito mauritii*) on the growth and nutrient content of leaves of *A. gangeticus* and *A. blitum* and found significant differences in micro and macronutrient concentrations in the leaves of both species when compared to the control substrate without vermicompost. The Fe contents of *A. gangeticus* and *A. blitum* were higher when grown in vermicompost enriched with *Eudrilus eugeniae* (26.12 ppm and 25.66 ppm) than in that enriched with *Lampito mauritii* (24.76 ppm and 13.53 ppm). The Fe results in *A. gangeticus* and *A. blitum*, from soils enriched with vermicompost were higher than those from the control treatments (14.45 ppm and 20.45 ppm), respectively. In this regard, an enriched substrate (more fertile) favoured higher Fe content in the leaves of the *Amaranthus* plants (Priya and Santhi, 2014).

The current study investigated the effects of two substrates *viz.* nutrient-rich soil (S) and nutrient-poor soil (v/v) **(**1S:1V) on the accumulation of leaf Ca and Fe content in the clones of the parent genotypes. Since most of the genotypes accumulated similar Ca levels in S and (v/v) 1S:1V, this may be an indication that both substrates have a high CEC ability. This could also indicate that the genotypes have low levels of anti-nutrients (oxalates, tannins, dietary fibre and phytate), which have been reported to influence the availability of Ca in plants (Amalraj and Pius, 2015; Essack et al., 2017). Statistical analyses confirmed a significant interaction (f-value = 6.38 and 5.69 for Ca and Fe, respectively) between the genotype and the substrate (Table 4.7).

The effects of substrate were seen in the Ca levels of G8 at d 80 and of G11 at d 30 and 80 which were significantly greater when the clones were grown in the nutrient-poor substrate which had a pH of 6.26 ((v/v) 1S:1V) than in S with a pH of 5.92 (Table 4.7). The pH of the substrates could not have influenced the leaf Ca and Fe contents of the clones as the pH value of each substrate was not significantly different to each other. Furthermore, the ability of the G11 clones to accumulate more Ca in (v/v) 1S:1V than S, cannot be attributed to the clones' root system as there were no significant increases or decreases in the root length over 21 d of acclimatisation nor of root dry mass after 90 d transfer to the greenhouse when these clones were grown in (v/v) 1S:1V than in S (Fig. 4.5C and Table: 4.6). On the other hand, the roots of the clones of G8 could have influenced its ability to take up Ca because of the significant increase from d 0 to d 21 observed in its root length (Fig. 4.4C). However, at d 90, in both G8 and G11 clones as with all the other clones, the root:shoot DM was not significantly different on either substrate (Table 4.6).

When Fe levels were determined in the clones of the parent genotypes after transferring into the greenhouse for 90 d, there was a significant interaction between genotype and substrate in terms of Fe content. Clones of five genotypes, G11 and G15 at d 60, G39 at d 90, G45 at d 80 and 90 and G47 at d 15 exhibited higher Fe levels in S than in (v/v) 1S:1V (Table 4.8). In contrast, the Fe levels of three genotypes, G11 at d 80, G15 at d 15 and 90 and G47 at day 90 were higher in (v/v) 1S:1V than in S. According to Morrissey and Guerinot (2009), Fe is available in its soluble (Fe^{2+}) and insoluble (Fe^{3+}) forms and its ability to be absorbed is dependent on the pH and soil redox potential. Since the pH of the substrates in the current study were not significantly different to each other, the Fe content appears to be have been influenced by the genotype and other soil characteristics (e.g. porosity, CEC, etc.) that require further investigation. As with Ca, the clones' root lengths over time (21 d of acclimatisation) and root dry masses after 90 d transfer to the greenhouse, were not affected by the substrate type nor their pH. There were also no significant differences in the root:shoot between substrates for each genotype.

In addition to the investigations discussed above, the Ca and Fe contents of the parents, which were grown only in S (60 d), were compared to those of the clones of the parent genotypes grown in (v/v) 1S:1V to determine the effects of substrate on the accumulation of Ca and Fe. After micropropagation, all the genotypes matched the same Ca and Fe contents as their parents prior to 60 d. The clones of G8, G11, G15, G39, G41 and G45 accumulated similar Ca levels as their respective parents at d 15 and that of G47 at d 30. Similarly, the Fe levels of G39, G41, G47 and G8, G15, G45 clones were the same as their respective parents at d 15 and 30, respectively. The 'rankings' of the clones were also compared to that of their parents, and it was observed that in 1S:1V, the Fe 'rankings' of G47 at d 80 ($47 \geq 39 \geq 8 = 15 = 41 \geq 11 > 45$) and 90 ($47 \geq 39 \geq 8 = 41 \geq 15 \geq 11 \geq 45$) were similar to that of its parent (G47 = G45 > G39 = G41 > G8 > G15 > G11).

The effect of substrate on Ca and Fe content was evident in these investigations. In general, G11, G15 and G47 preferred nutrient-poor substrates at certain time intervals to accumulate higher Ca and Fe levels. On the other hand, G11, G15, G39, G45 and G47 preferred nutrient-rich substrate at certain time intervals. All the genotypes accumulated their Ca and Fe content independent of their root and their root:shoot DM, which had no significant differences on either substrate, at d 90.

5.4 Concluding remarks

1a) Established protocol

- The established protocol for *Amaranthus dubius* was to surface sterilse the field-derived nodal explants in a 10 min soak of 1% (v/v) NaOCl and 2 drops of Tween 20® followed by three rinses in sterile distilled water prior to immersion in an antibiotic solution (¼ strength Murashige and Skoog basal salt medium, 50 µg l^{-1} rifampicin, 100 µg l^{-1} streptomycin/penicillin) on a shaker at 1500 rpm for 5 h which resulted in 20% contamination. These explants were then cultured onto media containing 2 mg l^{-1} BAP + 0.5 mg l^{-1} IAA (shoot multiplication), 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} IAA (elongation) and 0.1 mg l^{-1} IAA (rooting). Thereafter, plantlets were acclimatised over 21 days (d) in nutrient-rich (S – soil) and nutrient-poor ((v/v) 1S:1V – 1soil:1vermiculite) substrates which resulted in increases in some of the growth parameters *viz.* number of leaves, plant height and root length in S and only number of leaves in (v/v) 1S:1V. The post-acclimatisation yield was 2 plants/nodal explants amongst the field-derived plants.

b) Genotypic variations in the *in vitro* and *ex vitro* growth amongst the clones of the selected parent genotypes

- After the established protocol was applied, genotypic variations were observed in the *in vitro* shoot lengths amongst the clones of the parent genotypes (G8, G11, G15, G39, G41, G45 and G47). A longer shoot length was observed for the G47 (1.9 cm) clones than in those of G11, G39 and G45 (0.6, 0.9 and 1.0 cm, respectively) and but was similar in length to those of G8, G15 and G41 (1.7, 1.1 and 1.2 cm, respectively).
- Genotypic variations were also observed in the *ex vitro* yield after 21 d of acclimatisation amongst the cloned genotypes. The clones of G45 (4 plants/nodal explant) had a significantly higher yield than those of G8, G39 and G45 (2 explants/nodal plant).
- At 90 d after transfer to the greenhouse, there were no genotypic variations in the root, shoot and root:shoot dry masses amongst the cloned genotypes in both of the tested substrates (S and (v/v) 1S:1V).

2. Differences in the Ca and Fe content in a seedling population

- Amongst the fifty seedlings grown in S, genotypic variations in their leaf Ca and Fe levels were observed. The Ca content ranged from 246.3 ± 1.14 to 765.3 ± 6.07 mg 100 g^{-1} DM and the Fe levels were 5.25 ± 0.06 to 26.68 ± 0.10 mg 100 g^{-1} DM amongst the parent genotypes, evidence that within a population variation exists in the micronutrient levels.

3. Variations in the Ca and Fe levels between the parents and the clones of the selected parent genotypes in S and the effects of physiological age (time) on these clones

- At 60 d in the greenhouse following 21 d of acclimatisation, the clones of G8, G11, G15, G39, G41, G45 and G47 exhibited similar Ca and Fe levels to those of their respective parents. However, G11 exhibited less Fe at d 60 than its parent plant, an indication of the negative effects of micropropagation.
- The 'rankings' of the Ca and Fe levels of the clones of the parent genotypes differed to that of the parents at all time points (15, 30, 60, 80 and 90 d) with the exception of G11 and G47, which 'ranked' the same in their Fe levels as their parents at 60 d.
- Ca content of the clones grown in S increased with the physiological age (time). The opposite was observed for the Fe levels.

4. Effects of substrate (S vs (v/v) 1S:1V) on the *ex vitro* growth and the Ca and Fe levels of the clones of the selected parent genotypes

- Significant differences were observed in the *ex vitro* growth parameters of some clones. The number of leaves of G39 and G47 clones in S, the plant height of G8 and G47 clones in (v/v) 1S:1V and of G11 clones in S and the root length of G8 and G41 in S, G45 in (v/v) 1S:1V which all increased from d 0 to d 21. However, no significant differences were observed in the root:shoot dry masses of the clones grown in S and (v/v) 1S:1V.
- G11, G15, G39, G45 and G47 exhibited higher Fe content in S than in (v/v) 1S:1V, indicating that these genotypes preferred a nutrient-rich substrate to accumulate high Fe levels.
- G8, G11, G15 and G47 accumulated more Ca and Fe in (v/v) 1S:1V than in S, an indication that these genotypes preferred a nutrient-poor substrate to accumulate high Ca levels.

5. Comparison of the Ca and Fe levels of the parent genotypes grown in S to their clones grown in (v/v) 1S:1V

- Only two clones (G11and G41) exhibited similar Ca and Fe levels to their respective parents at d 60 when grown in (v/v) 1S:1V. On the other hand, G8 and G39 exhibited more Ca than the parents at 60 d. In contrast, G15, G45 and G47 showed less Fe than their respective parents at 60 d.

Overall, the Ca and Fe levels of the clones of all the selected parent genotypes were influenced by micropropagation, physiological age and substrate type. However, significant interactions were observed between the physiological age and genotype for the Fe content and between genotype and substrate for both Ca and Fe. These responses were independent of the root, shoot and root:shoot DM for all the clones. Since the clones of some of the selected genotypes accumulated high Ca and Fe levels in nutrient-poor soils, these clones can be grown in a nutrient-poor substrate.

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