The Effects of Genotype, Physiological Age, and Substrate on the Ca and Fe Content in the Leaves of Micropropagated Clones of *Amaranthus dubius*

Shakira Shaik* ⓘD, Phindile Nana Dladla, and Maria Paula Watt ⓘD

University of KwaZulu-Natal, School of Life Sciences, Westville Campus, Private Bag X54001, Durban 4000, South Africa

*Corresponding author: shaiksh@ukzn.ac.za

Compliance with Ethical Standards
The authors declare no conflict of interest.

Competing Interests
The authors have declared that no competing interests exist.

Abstract

This study aimed to screen and clonally propagate high and low Ca- and Fe-producing *Amaranthus dubius* genotypes and to test the resulting clonal progeny ex vitro for their Fe and Ca content. Seven genotypes (G) with varying levels of Ca and Fe were selected from 50 *A. dubius* seedlings and then micropropagated using nodal explants. They were acclimatised and grown in the greenhouse for 90 days on either soil or soil and vermiculite (1:1). Ca and Fe levels of the clones were determined at days 15, 30, 60, 80, and 90 following 21 days of acclimatisation and compared to those of 60-day-old parent genotypes. In general, the Ca levels of the clones of the tested genotypes were similar to those of their parents at day 15 and then increased up to 90 days, regardless of the substrate. In contrast, on both substrates, at day 15, the Fe levels were the same or higher in the clones compared with their parents and then decreased with time. Amongst the clonal genotypes, the mean plasticity indices (MPIs) were low and ranged from 0.12 to 0.43, 0.13 to 0.42, and 0.11 to 0.21 for shoot growth, Ca content, and Fe content, respectively. The clones of G41 were found to be the most plastic (MPI = 0.28), while the clones of G15 were the least plastic (MPI = 0.15). Overall, there is an indication that the number of leaves, their biomass, and their Ca and Fe contents are not negatively affected by low nutrient soils, which may be useful to communities who cannot afford to apply fertilisers.

Additional key words: biomass, direct organogenesis, fertiliser, micronutrients, phenotypic plasticity

Introduction

Amaranthus is a large taxonomic group with an estimated $60-70$ diverse species worldwide that have been extensively reviewed (Achigan-Dako et al., 2014; Venskutonis and Kraujalis, 2013 to name a few). They are often referred to as belonging to the African leafy vegetable group, which is a general term usually given to non-commercial vegetables with high micronutrient content; as such, they are purported to have the potential to alleviate problems related to malnutrition (Gerrano et al., 2015). Although some amaranths are cultivated, most are harvested in the wild for food and used for medical purposes (Achigan-Dako et al., 2014). They can be categorised according to their use, namely, grain amaranths, such as *A. caudatus*; vegetable amaranths, such as *A. tricolor*; and weed

amaranths, such as *A. dubius* (Das, 2012). Even though the latter is classified as a weed, this species is used as a vegetable to supplement the diets of many rural populations in South Africa (Gerrano et al., 2015). *A. dubius* is reported to contain high levels of Ca, Fe, K, Al, Mn, and Mg (Odhav et al., 2007; Yang and Keding, 2009; Molina et al., 2011; Muriuki et al., 2014). Studies in which micronutrient analyses of *A. dubius* were conducted in Africa document the ranges for Ca and Fe as $22.3 - 3014.7$ mg 100 g^{-1} dry mass (DM) and $3.4 - 96.2$ mg 100 g^{-1} DM, respectively (Odhav et al., 2007; Yang and Keding, 2009; Molina et al., 2011; Akinwunmi and Omotayo, 2016). This variation could be attributed to the tetraploid ($n = 32$) genome of *A. dubius* (Gerrano et al., 2015); such variability has also been reported in other polyploid amaranths (Ranade et al., 1997; Lymanskaya, 2012; Štefúnová et al., 2015). Therefore, there is evidence for a strategy that screens for high micronutrient-yielding genotypes and multiplying them in vitro to produce sexual breeding populations for community gardens. Towards this end, the objectives of this study were to screen a small population of *A. dubius* for the Ca and Fe content, to select and clonally propagate high and low Fe- and Ca-producing genotypes from the screened population, and to test the resulting clonal progeny ex vitro for their Fe and Ca content.

Materials and Methods

Experimental Design

The experimental design is illustrated in Fig. 1, and the details are provided in the sections that follow.

Plant Material and Growth Conditions

Seeds of *Amaranthus dubius* were randomly collected from a plant population at Randles Nursery, Durban, South Africa (29°49' S, 30°58' E), germinated and grown in potting soil for 60 days in a greenhouse (29°52' S, 30°59'E; 25°C day (d)/18°C night; $84 - 94\%$ relative humidity). An overhead irrigation system was used to water the plants for 3 min three times daily (20 mL·min^{-1}). The plants were treated with fungicides bi-weekly: 2 g·L^{-1} Dithane (Efeckto, South Africa) and 1 mL·L^{-1} Supremo (Grovida, South Africa) applied as a foliar spray and 1 mL·L^{-1} Chronos (Makhteshin-Agan, South Africa) and 1.25 mL·L^{-1} Orius (Makhteshin-Agan, South Africa) applied to the soil. Fertilisers (1 mL·L^{-1} Trelmix (Hubers, South Africa) and 2.5 g·L^{-1} Multifeed® (Nulandis®, South Africa) were also applied as foliar sprays every alternate week.

Elemental Analysis

After 60 days of growth in a greenhouse, the top $5 - 6$ leaves of 50 seedling plants (genotypes) (30 cm height and $\pm 25 - 30$ leaves) were oven dried at 80°C for 48 h. Then, 0.15 g leaf material of each genotype was heat digested in 5 mL of nitric acid (HNO_3) for 3 min, increased to 25 mL with deionised water, and filtered (0.22 μm, Pall Corporation, USA). Ca and Fe contents were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Perkin Elmer 5300, Germany), in the ranges of $0.1 - 5$ and $1 - 150$ ppm for Fe and Ca, respectively. The extent of the Ca and Fe levels amongst the 50 tested genotypes was $246.3 \pm 1.1 - 765.3 \pm 6.1$ and $5.3 \pm 0.1 - 26.7 \pm 0.1$ mg 100 g^{-1} DM, respectively, in accordance with previous reports (Odhav et al., 2007; Yang and Keding, 2009; Molina et al., 2011; Akinwunmi and Omotayo, 2016). Seven genotypes, ranked as G47 > G45 > G11 > G41 = G8 > G39 > G15 for Ca levels
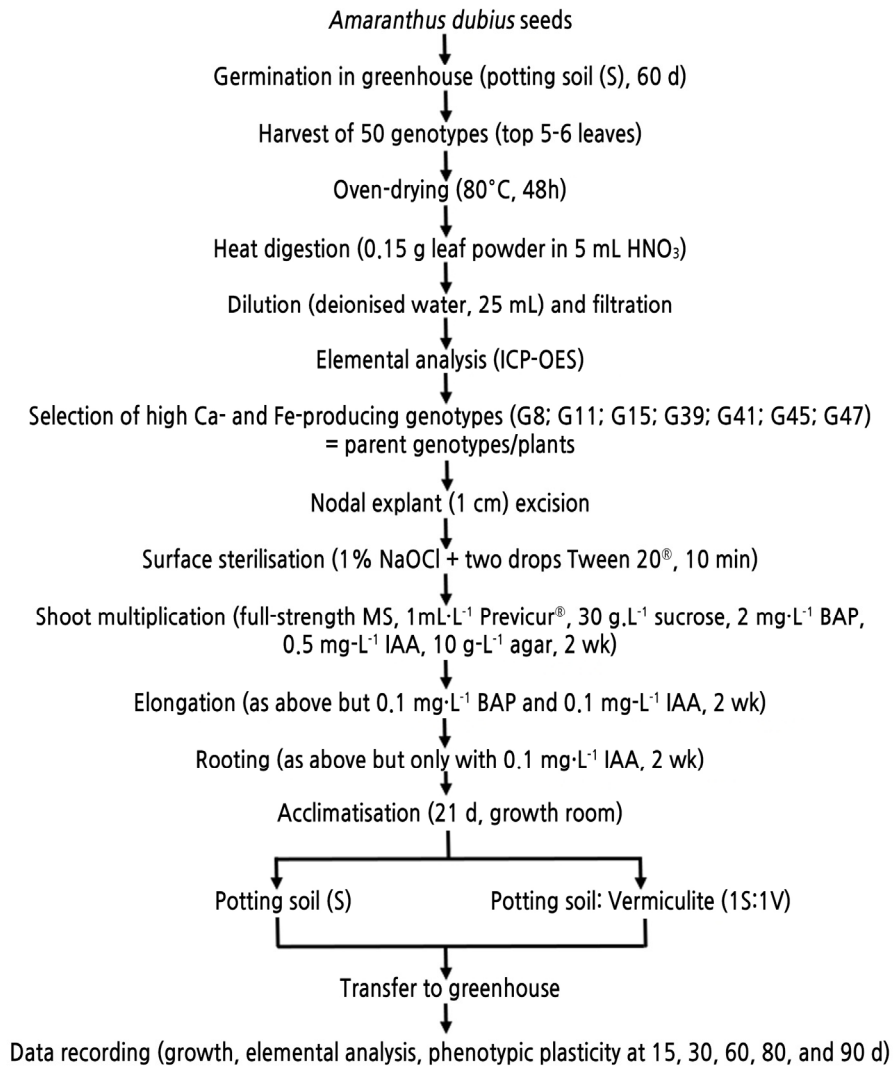
Amaranthus dubius seeds
↓
Germination in greenhouse (potting soil (S), 60 d)
↓
Harvest of 50 genotypes (top 5-6 leaves)
↓
Oven-drying (80°C, 48h)
↓
Heat digestion (0.15 g leaf powder in 5 mL HNO_3)
↓
Dilution (deionised water, 25 mL) and filtration
↓
Elemental analysis (ICP-OES)
↓
Selection of high Ca- and Fe-producing genotypes (G8; G11; G15; G39; G41; G45; G47)
= parent genotypes/plants
↓
Nodal explant (1 cm) excision
↓
Surface sterilisation (1% NaOCl + two drops Tween 20®, 10 min)
↓
Shoot multiplication (full-strength MS, 1mL·L⁻¹ Previcur®, 30 g.L⁻¹ sucrose, 2 mg·L⁻¹ BAP, 0.5 mg·L⁻¹ IAA, 10 g·L⁻¹ agar, 2 wk)
↓
Elongation (as above but 0.1 mg·L⁻¹ BAP and 0.1 mg·L⁻¹ IAA, 2 wk)
↓
Rooting (as above but only with 0.1 mg·L⁻¹ IAA, 2 wk)
↓
Acclimatisation (21 d, growth room)
↓
Potting soil (S)   Potting soil: Vermiculite (1S:1V)
↓
Transfer to greenhouse
↓
Data recording (growth, elemental analysis, phenotypic plasticity at 15, 30, 60, 80, and 90 d)

Fig. 1. Experimental design for the study on the effects of genotype, physiological age, and substrate on the leaf Ca and Fe content in clones of *Amaranthus dubius*. d = days, G = genotype, HNO_3 = nitric acid, ICP-OES = inductively coupled plasma-optical emission spectrometry, NaOCl = sodium hypochlorite, MS = Murashige & Skoog basal salt medium, BAP = 6-benzylaminopurine, IAA = indole-3-acetic acid, wk = weeks.

and G47 = G45 > G39 = G41 > G8 > G15 > G11 for Fe levels were used as parent plants (parent genotypes) in the cloning experiments. Although the seven selected genotypes differed in their Ca and Fe contents, they displayed similar morphological traits.

Micropropagation

Nodal explants (1 cm) of the parent genotypes were sterilised (1% (v/v) NaOCl with two drops of Tween 20® for 10 min), trimmed, and cultured on a medium common to all stages: Murashige and Skoog basal salts with vitamins (Murashige and Skoog, 1962; Highveld Biological, South Africa), 1 ml·L⁻¹ Previcur® (Propamocarb 600 g·L⁻¹) (Bayer, South Africa), 10 g·L⁻¹ agar (Sigma, South Africa), and 30 g·L⁻¹ sucrose, pH 5.6−5.8. After 2 weeks on shoot multiplication medium (2 mg·L⁻¹ 6-benzylaminopurine [BAP] and 0.5 mg·L⁻¹ indole-3-acetic acid [IAA]), shoots >2 cm

were elongated (0.1 mg·L^{-1} BAP and 0.1 mg·L^{-1} IAA) individually in culture tubes and, 2 weeks later, they were transferred onto rooting medium (0.1 mg·L^{-1} IAA) for 2 weeks. All the cultures were kept in a growth room (24°C day/18°C night and 16-h fluorescent light at 200 μmol·M^{-2}·S^{-1}/8 h dark photoperiod). Plantlets (>3 cm in shoot length) were potted (1 plant/pot; 10-cm diameter, 8-cm height) in potting soil (S) or 1 soil:1 vermiculite (v/v) (S:V) with decreasing humidity for 21 days. Each plant was then transplanted into a larger pot (14.5-cm height, 17.5-cm diameter) and transferred to a greenhouse (27 − 39°C, 85 − 90% RH and 500 − 1,100 μmol·m^{-2}·s^{-1}) for 90 days. The pH of both substrate types (5.92 and 6.26 for S and S:V, respectively) was determined not to be significantly different (mean of three values for pH of leachate, *t*-test). Leaves were counted before harvesting for shoot biomass and chemical analyses (at different physiological ages) as for the parent plants.

Phenotypic Plasticity

The phenotypic plasticity index was calculated for each of the shoot growth parameters and micronutrient contents measured. The index was calculated as the difference between the minimum and the maximum mean values between S- and S:V-grown clones divided by the maximum mean value (Valladares et al., 2000). The index ranged from zero (no plasticity) to one (maximum plasticity). Mean phenotypic plasticity was calculated for each measured parameter and genotype by averaging the indices of plasticity obtained.

Statistical Analyses

The experiments were conducted in a randomised block design. All values are presented as mean of three replicates per genotype. Data were analysed using the Genstat statistical package 17th edition (VSN International, Hernal Hempstead, UK). Prior to analysis, data were tested for normality using D'Agostino-Pearson test. One-way ANOVA was used to test for differences amongst and within genotypes for each measured parameter. Three-way ANOVA was used to test for interactions amongst the genotype, physiological age, and substrate. Means for analyses of variance were separated using a Tukey post-hoc test. A paired *t*-test was used to test for differences between substrates. All differences were considered significant at the 0.05 level.

Results and Discussion

To minimise somaclonal variation, and as no such protocol has been published, preliminary studies were undertaken to establish a protocol for direct organogenesis from nodal segments of *A. dubius.* That reported here produced an average of only two plants/explant which, although a low yield, was deemed adequate for this study. Although the parent genotypes were grown only in potting soil, the two substrates used for the ex vitro clones were chosen to investigate the effect of a relatively low nutrient supply [a common limiting factor in the production of traditional vegetables in rural communities (Matenge et al., 2011)] on the leaf biomass (the consumed part of the plant) and on their Ca and Fe content. Comparisons were also made between the parent genotypes and their clones to determine how many days of growth in S or S:V it took for the clones to accumulate similar Ca and Fe contents to those of their respective parents (which were determined 60 days after germination) (Tables 1 and 2).

The only observed significant difference in the effect of the substrates on the number of leaves was in the G8 clones, which produced significantly the most leaves in the low nutrient substrate (results not shown). There was no effect of the tested substrates on the shoot fresh ($1.3 \pm 0.6 - 3.1 \pm 0.8$ and $1.3 \pm 0.7 - 3.0 \pm 0.3$ g in S and S:V, respectively) and dry masses ($0.3 \pm 0.2 - 0.6 \pm 0.0$ g and $0.3 \pm 0.1 - 0.8 \pm 0.1$ g in S and S:V, respectively) of the clones of all seven genotypes (results not shown). However, the results demonstrate a low phenotypic plasticity as represented by the mean indices for overall shoot growth (MPI = 0.26) and for each shoot parameter (MPI = 0.19, 0.29, and 0.29 for the number of leaves, shoot fresh mass, and dry mass, respectively, Table 3) and for shoot growth of the clones of each genotype (MPI$_{SG}$ = 0.16 to 0.43, Table 3). This indicates that leaf biomass is not negatively affected by low nutrient soils, characteristic of those in rural communities who cannot afford to apply fertilisers. However, other environmental stresses that may affect leaf biomass in *A. dubius* as

Table 1. Comparison of Ca levels between 60-day-old parent plants of *A. dubius* and their clones, and the rankings of the cloned genotypes at 15, 30, 60, 80, and 90 days (d) of ex vitro growth on soil (S) and soil:vermiculite (S:V)

Genotype (G)	Substrate	Parent plant (mg 100 g^{-1})	Clones (mg 100 g^{-1})				
		60 d	15 d	30 d	60 d	80 d	90 d
8	S	534.9 ± 1.0 BC,c	302.7 ± 157.9 C	562.3 ± 153.1B C	649.9 ± 139.8 B	780.2 ± 15.0 AB	999.6 ± 34.0 A
	S:V	-	525.8 ± 7.3 c	562.4 ± 24.8 bc	796.5 ± 69.3 b	1148.1 ± 113.5 a*	1204.0 ± 94.1 a
11	S	604.1 ± 1.5 A,a	383.5 ± 91.1 A	430.0 ± 5.3 A	486.1 ± 123.5 A	512.4 ± 0.9 A	651.7 ± 155.6 A
	S:V	-	689.4 ± 89.2 a	838.3 ± 68.0 a,*	876.2 ± 163.5 a*	888.2 ± 58.3 a*	912.2 ± 154.3 a
15	S	402.0 ± 1.4 B,b	369.7 ± 14.0 B	427.3 ± 88.8 B	435.1 ± 13.4 B	486.3 ± 12.4 B	723.0 ± 62.1 A
	S:V	-	598.4 ± 100.1 ab	658.2 ± 25.5 ab	812.1 ± 146.7 ab	993.4 ± 161.7 a	1017.7 ± 154.5 a
39	S	453.5 ± 1.5 B,c	484.3 ± 227.7 AB	752.7 ± 52.0 AB	800.9 ± 118.6 AB	961.1 ± 157.2 AB	1077.2 ± 34.8 A
	S:V	-	423.7 ± 5.7 c	631.1 ± 54.0 b	740.3 ± 12.2 b	765.5 ± 57.4 b	1152.9 ± 35.4 a
41	S	535.8 ± 3.8 A,b	467.6 ± 126.5 A	571.6 ± 160.2 A	626.4 ± 36.0 A	628.4 ± 156.6 A	896.5 ± 145.4 A
	S:V	-	410.5 ± 54.8 b	475.4 ± 64.0 b	769.4 ± 153.9 ab	826.1 ± 19.0 ab	1176.5 ± 130.9 a
45	S	641.6 ± 1.0 B,a	641.6 ± 43.3 B	516.4 ± 115.5 B	793.7 ± 3.8 B	812.9 ± 10.4 B	1127.8 ± 94.8 A
	S:V	-	552.5 ± 140.0 a	570.9 ± 73.1 a	600.2 ± 46.8 a	723.1 ± 386.6 a	943.3 ± 129.3 a
47	S	765.3 ± 6.1 A,a	342.4 ± 154.2 A	370.6 ± 59.2 A	412.7 ± 65.1 A	533.7 ± 2.0 A	656.6 ± 229.8 A
	S:V	-	106.9 ± 34.6 b	432.4 ± 97.4 ab	554.0 ± 284.9 ab	590.4 ± 38.7 ab	679.5 ± 145.0 ab

Interaction			Rankings		
Parameters	F-value	Age (days)	S		S:V
Genotype × Substrate	6.4**	15	8 = 11 = 15 = 39 = 41 = 45 = 47		8 = 11 = 15 = 45 ≥ 39 = 41 ≥ 47
Genotype × Physiological Age	0.9 NS	30	8 = 11 = 15 = 39 = 41 = 45 = 47		11 ≥ 8 = 15 = 39 = 45 ≥ 41 = 47
Substrate × Physiological Age	0.7 NS	60	39 = 45 ≥ 8 = 11 = 41 ≥ 15 = 47		8 = 11 = 15 = 39 = 41 = 45 = 47
Genotype × Substrate × Physiological Age	0.6 NS	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
		Parents (60 d on soil)	47 > 45 > 11 > 41 = 8 > 39 > 15		

Parent plants were only grown in S. Dissimilar letters denote statistical differences between parent plants and their clones grown on S (A-C) and their clones grown on 1S:1V (a-b) with increasing physiological age (one-way ANOVA, Tukey's post hoc test, $p < 0.05$, $n = 3$, mean $\pm$ SE). * = significant differences between substrates for each clone at each physiological age (paired *t*-test, $p < 0.05$, $n = 3$, mean $\pm$ SE). NS = No significance or ** = significance for each interaction amongst genotype, physiological age, and substrate (three-way ANOVA, $p < 0.05$, $n = 3$).

reported for *A. tricolor*, *A. cruentus*, and *A. blitum* under water stress (Liu and Stützel, 2004) and for *A. tricolor* under varying light intensities (Singh et al., 2009) still need to be investigated for *A. dubius*.

In this study, the genotype, physiological age, and substrate type had varying effects on the leaf Ca and Fe levels (determined using ICP-OES) of the clones of *A. dubius* obtained by micropropagation. Clonal variability (reflected as rankings, Table 1) was observed as significant differences in leaf Ca amongst the clones of each of the parent genotypes at days 60 and 80 on S and days 15 and 30 on S:V. Translocation of Ca occurs mainly in the xylem with water and is therefore dependent on transpiration (Clarkson 1984). High humidity, which causes reduced transpiration, in the greenhouse could have affected Ca mobility in certain genotypes leading to variability in their Ca concentrations. By day 90, the clones of all parents had similar Ca levels to each other regardless of the substrate (Table 1). They also produced similar Ca levels to their parents by day 15 on both substrates, except for G47. As physiological age increased up to day 90, Ca levels increased significantly in G8, G15, and G45 on S and in G8, G39, and G41on S:V (Table 1). Due to the scarcity of studies on *A. dubius* and on micropropagated clones, comparisons can only be made with related species and common vegetables. Generally, Ca accumulates with advancing maturity of plant organs because of its immobile nature (Chapin, 1980; Noodén, 1988; Khader and Rama, 2003); for example, the leaf Ca content increased from day 33 to 61 in two accessions of *Cicer arietinum* (chickpea) leaves (Ibrikci et al., 2003) grown in a greenhouse. However, in field-grown *A. cruentus* (a close relative of *A. dubius*), Moussa et al. (2020) reported a decrease in leaf Ca content with increasing harvest time (up to 8 weeks after transplanting). In terms of consumption, leaf age has an effect on palatability and digestibility, which are reportedly lower in older leaves than young ones; more mature leaves may also contain anti-nutrients (e.g. oxalate and phytate), which decrease mineral nutrient bio-availability to humans and animals (Ross and Graham, 1999).

The leaf Ca content over time was influenced by the substrate type in the clones of only two parent genotypes, for example, at day 80, G8 clones exhibited significantly more leaf Ca on S:V (1148.1 ± 113.5 mg 100 g^{-1} DM) than on S (780.2 ± 14.0 mg 100 g^{-1} DM), while the levels of Ca in the G11 clones were significantly greater on days 30, 60, and 80 (838.3 ± 68.0, 876.2 ± 163.5, and 888.2 ± 58.3, respectively) on S:V than those in the plants growing on S (430.0 ± 5.3, 486.1 ± 123.5, and 512.4 ± 0.9, respectively). Variability in genotype Ca content between substrates could be attributed to root cation exchange capacity in that certain cations, e.g. K, Mg, Na, NH^{4+}, and H^+, and even trace elements may directly or indirectly depress Ca uptake and distribution (Wallace and Mueller, 1980). In *A. hybridus*, Mziray et al. (2001) found that leaf Ca content did not differ amongst plants from populations grown in different field locations fed with the same concentration of chicken manure (4 kg·M^{-2}). In three cultivars of *Cichorium endivia* (endive) grown under hydroponic conditions, the leaf Ca content was higher when on an organic (coconut fibre, nutrient-rich) rather than an inorganic (rockwool) substrate (Kowalczyk et al., 2015). Similarly, in this study, two genotypes (G8 and G11) produced more Ca on S:V (nutrient-poor) than on S (nutrient-rich) but only at certain ages. However, all clonal genotypes demonstrated low levels of phenotypic plasticity (MPI$_{PAC}$ = 0.13 − 0.40, Table 3) for Ca content in the tested (albeit small) sample. The MPIs for Ca content were the highest and lowest at days 15 (0.33) and 90 (0.18), respectively. As for shoot growth, there is an indication that the Ca content is not negatively affected by low nutrient soils more so at specific physiological ages. In *Lactuca sativa* var. *capitata* (lettuce) and *Apium graveolens* var. *dulce* (celery), increased nitrogen supply increased Ca content but decreased it in *Brassica oleracea* var. *acephala* (curly kale) (Pitura and Michalojć, 2015).

Variability amongst the clones of the selected parent genotypes was more pronounced for Fe than for Ca levels, at all

tested days and for both tested substrates (reflected as rankings, Table 2). On S, all clones produced more Fe than their respective parents by day 15. On S:V, the same was true for G8, G11, G15, and G45, whereas the clones of G39, G41, and G47 produced similar Fe levels to those of their parents by day 15 (Table 2). As physiological age increased from days 15 to 90, the Fe levels decreased significantly in the clones of all genotypes on both substrates with the exception of G41 on S (Table 2). Similar results were reported for chickpea (Ibrikci et al., 2003). In contrast, leaf Fe content of many different field- and glasshouse-gown *Amaranthus* species has been found to increase with age (*A. blitum*, Khader and Rama, 1998; *A. blitum*, *A. hybridus*, *A. hybridus* var. *cruentus*, *A. hypochondriacus*, *A. tricolor*, and *A. thunbergia*, Modi, 2007; *A. cruentus*, Moussa et al., 2020). Khader and Rama (1998) proposed that a decrease in Fe content with age may indicate redistribution for other organ development (e.g. flowers). In this study, five to six apical leaves were collected at each harvest, and as the plants grew bigger over time, it is possible that *A. dubius* is similar to chickpea in that a decreased

Table 2. Comparison of Fe levels between 60-day-old parent plants of *A. dubius* and their clones, and the rankings of the cloned genotypes at 15, 30, 60, 80, and 90 days (d) of ex vitro growth on soil (S) and soil:vermiculite (S:V)

Genotype (G)	Substrate	Parent plant (mg 100 g^{-1})	Clones (mg 100 g^{-1})				
		60 d	15 d	30 d	60 d	80 d	90 d
8	S	9.22 ± 0.10 BC,bc	12.0 ± 0.6 A	10.98 ± 0.6 AB	8.4 ± 0.2 C	6.2 ± 0.2 D	4.4 ± 0.5 D
	S:V	-	13.4 ± 0.4 a	9.4 ± 0.1 b	7.8 ± 0.6 cd	6.2 ± 0.3 de	5.5 ± 0.2 e
11	S	5.18 ± 0.06 D,c	16.0 ± 0.0 A	9.0 ± 0.1 B	6.1 ± 0.0 C*	4.0 ± 0.0 E	3.1 ± 0.0 F
	S:V	-	13.0 ± 0.0 a	10.0 ± 0.6 b	5.7 ± 0.1 c	5.0 ± 0.0 c*	4.6 ± 0.1 c*
15	S	8.40 ± 0.06 B,b	9.8 ± 0.2 A	7.6 ± 0.3 BC	6.5 ± 0.2 CD*	5.6 ± 0.4 D	2.9 ± 0.1 E
	S:V	-	12.4 ± 0.6 a*	8.4 ± 0.3 b	5.5 ± 0.3 c	5.5 ± 0.2 c	5.3 ± 0.2 c*
39	S	9.64 ± 0.04 B,abc	13.1 ± 1.5 A	10.4 ± 0.2 AB	9.0 ± 0.3 B	8.3 ± 0.2 B	8.2 ± 0.3 B*
	S:V	-	12.4 ± 1.7 a	12.9 ± 1.0 a	8.4 ± 0.4 bc	7.1 ± 0.6 c	6.1 ± 0.2 c
41	S	9.62 ± 0.06 A,ab	9.6 ± 1.0 A	8.5 ± 0.9 A	7.7 ± 1.8 A	5.8 ± 1.4 A	5.3 ± 0.4 A
	S:V	-	11.7 ± 1.6 a	10.0 ± 0.5 ab	7.4 ± 0.6 bc	6.5 ± 0.6 bc	5.5 ± 0.6 c
45	S	10.60 ± 0.07 B,b	15.8 ± 0.5 A	11.91 ± 0.54 B	8.0 ± 0.1 C	6.1 ± 0.5 C*	5.9 ± 0.6 C*
	S:V	-	15.2 ± 0.6 a	10.6 ± 0.3 b	7.7 ± 0.6 c	4.2 ± 0.4 d	2.5 ± 0.1 d
47	S	10.76 ± 0.11 AB,a	13.2 ± 0.3 A*	10.8 ± 0.6 AB	10.2 ± 0.8 B	9.7 ± 0.8 B	6.0 ± 0.4 C
	S:V	-	10.2 ± 0.6 ab	9.2 ± 0.6 abc	8.7 ± 0.6 bc	8.6 ± 0.1 bc	7.2 ± 0.2 c*

Interaction			Rankings		
Parameters	F-value	Age (days)	S		S:V
Genotype × Substrate	5.7**	15	11 = 45 ≥ 39 = 47 ≥ 8 = 15 = 41		45 ≥ 8 = 11 = 15 = 39 = 41 ≥ 47
Genotype × Physiological Age	8.0**	30	45 ≥ 47 = 8 ≥ 39 = 11 ≥ 41 = 15		39 ≥ 45 ≥ 8 = 11 = 15 = 41 = 47
Substrate × Physiological Age	1.2 NS	60	47 ≥ 41 = 39 ≥ 8 ≥ 45 ≥ 15 ≥ 11		39 = 47 ≥ 8 = 41 = 45 ≥ 11 = 15
Genotype × Substrate × Physiological Age	3.2 NS	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
		Parents (60 d on S)	47 = 45 > 39 = 41 > 8 > 15 > 11		

Parent plants were only grown in S. Dissimilar letters denote statistical differences between parent plants and their clones grown on S (A-C) and their clones grown on S:V (a-b) with increasing physiological age (one-way ANOVA, Tukey's post hoc test, $p < 0.05$, $n = 3$, mean ± SE). * = significant differences between substrates for each clone at each physiological age (paired *t*-test, $p < 0.05$, $n = 3$, mean ± SE). NS = No significance or ** = significance for each interaction amongst genotype, physiological age, and substrate (Three-way ANOVA, $p < 0.05$, $n = 3$).

delivery of Fe to the apical leaves may be caused by the apportioning of root-absorbed nutrients to the more mature leaves (Ibrikci et al., 2003).

In this study, only the clones of G8 and G41 were not influenced by the substrate in the production of leaf Fe. All other clonal genotypes exhibited some phenotypic variability in leaf Fe levels as affected by the substrate (S or S:V), although the mean plasticity indices were higher for Ca (0.25) than for Fe (0.16) (Table 3). By day 90, G11, G15, and G47 produced significantly more Fe on the nutrient-poor substrate (4.6 ± 0.1, 5.3 ± 0.2, and 7.2 ± 0.2, respectively) than on the nutrient-rich one (3.1 ± 0.0, 2.9 ± 0.1, and 6.0 ± 0.4, respectively), while the opposite was true for G39 and G45 (8.2 ± 0.3 and 5.9 ± 0.6 on S and 6.1 ± 0.2 and 2.5 ± 0.1 on S:V, respectively). 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

Table 3. Phenotypic plasticity index (PPI) for shoot growth and Ca and Fe content at different physiological ages for the cloned genotypes of *A. dubius* parent plants

Parameter		PPI							MPI
		G8	G11	G15	G39	G41	G45	G47	
Shoot growth									
no. of leaves		**0.41**	0.17	0.12	0.06	0.24	0.04	0.27	*0.19*
shoot fresh weight		0.28	0.32	0.08	0.26	0.50	0.21	0.38	*0.29*
shoot dry weight		0.33	0.31	0.15	0.18	0.54	0.24	0.32	*0.29*
MPI$_{SG}$		*0.34*	*0.26*	*0.12*	*0.17*	*0.43*	*0.16*	*0.32*	
Physiological Age									
Ca	15 d	0.13	0.12	0.42	0.38	0.69	0.44	0.14	*0.33*
	30 d	0.16	**0.17**	0.00	0.35	0.14	0.49	0.10	*0.20*
	60 d	0.08	**0.19**	0.18	0.46	0.26	0.45	0.24	*0.26*
	80 d	**0.20**	**0.24**	0.32	0.51	0.10	0.42	0.11	*0.27*
	90 d	0.07	0.24	0.17	0.29	0.03	0.29	0.16	*0.18*
	MPI$_{PAC}$	*0.13*	*0.19*	*0.22*	*0.40*	*0.24*	*0.42*	*0.15*	
Fe	15 d	0.06	0.18	**0.11**	0.21	0.22	0.19	**0.03**	*0.14*
	30 d	0.20	0.14	0.15	0.10	0.15	0.10	0.11	*0.14*
	60 d	0.07	**0.04**	**0.07**	0.16	0.15	0.07	0.03	*0.08*
	80 d	0.14	**0.12**	0.00	0.03	0.11	**0.18**	0.31	*0.13*
	90 d	0.25	**0.05**	0.21	**0.45**	0.17	**0.32**	**0.58**	*0.29*
	MPI$_{PAF}$	*0.14*	*0.11*	*0.11*	*0.19*	*0.16*	*0.17*	*0.21*	
	MPI$_{G}$	*0.20*	*0.20*	*0.15*	*0.25*	*0.28*	*0.25*	*0.23*	
Summary of plasticity									
Shoot growth (*MPI$_{SG}$*)		G41 > G8 > G47 > G11 > G39 > G45 > G15							*0.26*
Ca content (*MPI$_{PAC}$*)		G45 > G39 > G41 > G15 > G11 > G47 > G8							*0.25*
Fe content (*MPI$_{PAF}$*)		G47 > G39 > G45 > G41 > G8 > G11 = G15							*0.16*
Overall (*MPI$_{G}$*)		G41 > G39 = G45 > G47 > G8 = G11 > G15							*0.22*

G = genotype, MPI = mean plasticity index across genotypes for each shoot growth parameter or physiological age, MPI$_{SG}$ = mean plasticity index for shoot growth for each genotype, MPI$_{PAC}$ = mean plasticity index for Ca content at each physiological for each genotype, MPI$_{PAF}$ = mean plasticity index for Fe content at each physiological for each genotype, MPI$_{G}$ = mean plasticity index for each genotype. Bold font indicates parameters that significantly differed ($p < 0.05$) between soil and soil:vermiculite substrates within each genotype.

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 that sequester Fe in the cellular structures of the plant, also influences the available Fe in each plant (Morrissey and Guerinot, 2009). Mean phenotypic plasticity indices for Fe content were low and ranged from 0.11 to 0.21 for the clonal genotypes, and the MPIs for Fe content were the highest and lowest at days 90 (0.29) and 60 (0.08), respectively (Table 3). This study has indicated that the Fe levels are not negatively affected by low nutrient soils. Genotypes and their interactions with fertiliser are known to influence plant responsiveness to nutrient availability (Sossa-Vihotogbé et al., 2013; Fonge et al., 2016); other substrate minerals may have an impact on Fe uptake as reported for *Vigna radiata* (mung bean) (Liu et al. 2000) and *Brassica napus* (rapeseed) (Mendoza-Cózatl et al., 2008). In *Amaranthus*, the evidence to date is that the process of Fe acquisition in leafy vegetables differs amongst species. For example, Mziray et al. (2001) found that leaf Fe content did not differ amongst *A. hybridus* plants from different field populations and fed with the same concentration of chicken manure (4 kg·M^{-2}), and Moussa et al. (2020) showed that leaf Fe content in *A. cruentus* was not affected by different urea micro-doses in the field. In lettuce and celery, increasing N doses in a greenhouse caused leaf Fe content to decrease, while it increased and then decreased in curly kale (Pitura and Michalojć, 2015).

It must be stressed that, in this study and all the cited ones, the Ca and Fe contents were determined on raw leaves, but *A. dubius* is ordinarily consumed after cooking. However, there are some reports that leaf Ca content is not affected by microwaving (*A. dubius*, *A. blitum*, *A. polygonoides*, *A. spinosus*, and other leafy vegetables, Amalraj and Pius, 2015). In contrast, leaf Ca and Fe content in *A. hybridus* reportedly decreased by boiling (Mziray et al., 2001).

In this study, there may have been unknown effects caused by the micropropagation stages, which may have affected the growth and physiology of the clones. Nonetheless, this study has shown that even within a small population of *A. dubius* seedlings, genotypic variability was evident for leaf number and Ca and Fe contents, and low levels of phenotypic plasticity were obtained for all tested parameters, e.g. shoot growth (0.26) > Ca content (0.25) > Fe content (0.16) (Table 3). The overall levels of plasticity for the clones of the tested genotypes were as follows: G41 (0.28) > G39 = G45 (0.25) > G47 (0.23) > G8 = G11 (0.20) > G15 (0.15), but these levels differed for each measured parameter (Table 3) with a final MPI of 0.22 for all tested parameters and genotypes. Although the study had its limitations and the detected overall levels of plasticity were low, this strategy may be potentially useful in the selection of specific genotypes, not only high yielding Ca and Fe, but also other traits of interest for subsequent 'fixing' of such genotypes or their incorporation into breeding programmes. Such approaches are being pursued as they may prove critical in exploring the use of *A. dubius* (and other African leafy vegetables) as a potential crop species for food security in parts of Africa; such approaches may prove invaluable under a deteriorating climate and environmental degradation especially in instances where access to nutritious soil is lacking.

Literature Cited

Achigan-Dako EG, Sogbohossou OED, Maundu P (2014) Current knowledge on *Amaranthus* spp.: Research avenues for improved nutritional value and yield in leafy amaranths in sub-Saharan Africa. Euphytica 197:303-317. doi:10.1007/s10681-014-1081-9

Akinwunmi OA, Omotayo FO (2016) Proximate analysis and nutritive values of ten common vegetables in South-West (Yoruba Land) Nigeria. Comm Appl Sci 4:79-91

Amalraj A, Pius A (2015) Bioavailability of calcium and its absorption inhibitors in raw and cooked green leafy vegetables commonly

consumed in India - An in vitro study. Food Chem 170:430-436. doi:10.1016/j.foodchem.2014.08.031

Chapin FS III (1980) The mineral nutrition of wild plants. Annu Rev Ecol Systemat 11:233-260. doi:10.1146/annurev.es.11.110180.001313

Clarkson DT (1984) Calcium transport between tissues and its distribution in the plant. Plant Cell Environ 7:449-456. doi:10.1111/j.1365-3040.1984.tb01435.x

Das S (2012) Systematics and taxonomic delimitation of vegetable, grain and weed amaranths: a morphological and biochemical approach. Genet Resour Crop Evol 59:289-303. doi:10.1007/s10722-011-9684-7

Fonge BA, Bechem EE, Awo EM (2016) Fertiliser rate on growth, yield, and nutrient concentration of leafy vegetables. Int J Veg Sci 22:274-288. doi:10.1080/19315260.2015.1005726

Frossard E, Bucher M, Mächler F, Mozafar A, Hurell R (2000) Potential for increasing the content and bioavailability of Fe, Zn and Ca in plants for human nutrition. J Sci Food Agric 80:861-879. doi:10.1002/(SICI)1097-0010(20000515)80:7<861::AID-JSFA601>3.0.CO;2-P

Gerrano AS, Jansen van Rensburg WS, Adebola PO (2015) Genetic diversity of *Amaranthus* species in South Africa. S Afr J Plant Soil 32:39-46. doi:10.1080/02571862.2014.973069

Ibrikci H, Knewtson SJB, Grusak MA (2003) Chickpea leaves as a vegetable green for humans: evaluation of mineral composition. J Sci Food Agric 83:945-950. doi:10.1002/jsfa.1427

Khader V, Rama S (1998) Selected mineral content of common leafy vegetables consumed in India at different stages of maturity. Plant Foods Hum Nutr 53:71-78. doi:10.1023/A:1008061525579

Khader V, Rama S (2003) Effect of maturity on macromineral content of selected leafy vegetables. Asia Pac J Clin Nutr 12:45-49

Kowalczyk K, Gajc-Wolska J, Marcinkowska M, Jabrucka-Pióro E (2015) Assessment of quality attributes of endive (*Cichorium endivia* L.) depending on a cultivar and growing conditions. Acta Sci Pol-Hortoru 14:13-26

Liu F, Stützel H (2004) Biomass partitioning, specific leaf area, and water use efficiency of vegetable amaranth (*Amaranthus* spp.) in response to drought stress. Sci Hortic 102:15-27. doi:10.1016/j.scienta.2003.11.014

Liu J, Reid RJ, Smith FA (2000) The mechanism of cobalt toxicity in mung beans. Physiol Plant 110:104-110. doi:10.1034/j.1399-3054.2000.110114.x

Lymanskaya SV (2012) Estimation of the genetic variability of an amaranth collection (*Amaranthus* L.) by RAPD analysis. Cytol Genet 46:210-216. doi:10.3103/S0095452712040093

Matenge ST, Van der Merwe D, Kruger A, De Beer H (2011) Utilisation of indigenous plant foods in the urban and rural communities. Indilinga: Afr J Indig Syst 10:17-37

Mendoza-Cózatl DG, Butko E, Springer F, Torpey JW, Komives EA, Kehr J, Schroeder JI (2008) Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. Plant J 54:249-259. doi:10.1111/j.1365-313X.2008.03410.x

Modi AT (2007) Growth temperature and plant age influence on nutritional quality of *Amaranthus* leaves and seed germination capacity. Acta Hortic 33:369-376. doi:10.4314/wsa.v33i3.180598

Molina E, González-Redondo P, Montero K, Ferrer R, Moreno-Rojas R, Sánchez-Urdaneta A (2011) Effect of harvest time and organ of the plant on the metal content of *Amaranthus dubius* Mart. Ex Thell. Interciencia 35:386-391

Morrissey J, Guerinot ML (2009) Iron uptake and transport in plants: The good, the bad, and the ionome. Chem Rev 109:4553-4567. doi:10.1021/cr900112r

Moussa MID, Alashi AM, Sossa-Vihotogbé CNA, Akponikpè PBI, Baco MN, Djènontin AJ, Aluko RE, Akissoé NH (2020) Proximate composition, mineral profile and trypsin-inhibitory activity of West African leafy vegetables: influence of urea micro-dosing and harvest time. Polish J Food Nutr Sci 70:179-188. doi:10.31883/pjfns/119674

Murashige M, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497. doi:10.1111/j.1399-3054.1962.tb08052.x

Muriuki EN, Sila DN, Onyango A (2014) Nutritional diversity of leafy amaranth species grown in Kenya. J Appl Biosci 79:6818-6825. doi:10.4314/jab.v79i1.9

Mziray RS, Imungi JK, Karuri EG (2001) Nutrient and antinutrient contents of raw and cooked *Amaranthus hybridus*. Ecol Food Nutr 40:53-65. doi:10.1080/03670244.2001.9991637

Noodén LD (1988) Whole plant senescence. In: Noodén LD, Leopold AC (eds) Senescence and aging in plants, Academic Press Inc, San Diego, pp 391-439. doi:10.1016/B978-0-12-520920-5.50018-3

Odhav B, Beekrumb S, Akula US, Baijnath H (2007) Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. J Food Compos Anal 20:430-435. doi:10.1016/j.jfca.2006.04.015

Pitura K, Michalojć Z (2015) Influence of nitrogen doses on the chemical composition and proportions of nutrients in selected vegetable species. J Elem 20:667-676. doi:10.5601/jelem.2015.20.1.760

Ranade S, Kumar A, Goswami M, Farooqui N, Sane P (1997) Genome analysis of amaranths: determination of inter- and intra-species variations. J Biosci 22:457-464. doi:10.1007/BF02703191

Ross M, Graham RD (1999) A new paradigm for world agriculture: meeting human needs, productive, sustainable, and nutritious. Field Crops Res 60:1-10. doi:10.1016/S0378-4290(98)00129-4

Singh A, Kandasamy T, Odhav B (2009) *In vitro* propagation of *Alternanthera sessilis* (sessile joyweed), a famine food plant. Afr J Biotechnol 8:5691-5695. doi:10.5897/AJB09.307

Sossa-Vihotogbé CNA, Anihouvi BV, Akissoe HN, Amadji LG, Hounhouigan DJ (2013) Effect of organic and mineral fertilization on proximate composition of three leafy vegetables harvested at different periods. Int J Chem Biol Sci 7:271-286. doi:10.4314/ijbcs.v7i1.23

Štefúnová V, Bežo M, Žiarovská J, Ražná K (2015) Detection of the genetic variability of *Amaranthus* by RAPD and ISSR markers. Pak J Bot 47:1293-1301

Valladares F, Wright SJ, Lasso E, Kitajima K, Pearcy R (2000) Plastic phenotypic response to light of 16 congeneric shrubs from a Panamanian rainforest. Ecology 81:1925-1936. doi:10.1890/0012-9658(2000)081[1925:PPRTLO]2.0.CO;2

Venskutonis PR, Kraujalis P (2013) Nutritional components of amaranth seeds and vegetables: A review on composition, properties, and uses. Compr Rev Food Sci Food Saf 2:381-412. doi:10.1111/1541-4337.12021

Wallace A, Mueller RT (1980) Calcium uptake and distribution in plants. J Plant Nutr 2:247-256. doi:10.1080/01904168009362774

Yang YR, Keding GB (2009) Nutritional contributions of important African indigenous vegetables. In: Shackleton CM, Pasquini MW, Drescher AW (eds) African indigenous vegetables in urban agriculture, Earthscan, London, pp 105-135