Variations in the Chemical Composition of Cassava (*Manihot esculenta* Crantz) Leaves and Roots As Affected by Genotypic and Environmental Variation

Anna Elizabeth Burns,[†,§] Roslyn Margaret Gleadow,[†] Anabela M. Zacarias,[#]
Constantino Estevão Cuambe,[⊥] Rebecca Elizabeth Miller,[†,‡] and Timothy Richard Cavagnaro*,[†,‡]

[†]School of Biological Sciences, Faculty of Science, Monash University, 3800 Victoria, Australia
[#]Mozambique Agricultural Research Institute (IIAM), P.O. Box 3658, Mavalane, Maputo, Mozambique
[⊥]Mozambique Agricultural Research Institute (IIAM), Posto Agronomico de Nampula, Nampula, Mozambique
[‡]Australian Centre for Biodiversity, Monash University, 3800 Victoria, Australia

Ⓢ *Supporting Information*

ABSTRACT: The purpose of this study was to assess the quality of cassava cultivars, in terms of cyanogenic potential and composition of macro- and micronutrients, sampled from different locations in rural Mozambique. Total cyanide concentrations in fresh cassava tissues were measured using portable cyanide testing kits, and elemental nutrients were later analyzed from dried plant tissue. Variation in cyanogenic potential and nutrient composition occurred both among cultivars and across locations. The majority of cultivars contained >100 ppm total cyanide, fresh weight, and are therefore considered to be dangerously poisonous unless adequately processed before consumption. Leaf cyanogenic and nutrient content varied with plant water status, estimated using carbon isotope discrimination ($\delta^{13}C$). The colonization of roots of all cultivars by arbuscular mycorrhizal fungi was also quantified and found to be high, indicating that mycorrhizas could play a key role in plant nutrient acquisition in these low-input farming systems.

KEYWORDS: *manioc, cyanide, cyanogenic glycosides, nutrients/nutrient deficiency, arbuscular mycorrhizas, C-isotope discrimination, cassava roots and leaves*

INTRODUCTION

Cassava is one of the most important staple food crops in tropical parts of the world. Production of this crop plays a role in the maintenance of food security in much of the developing world, including Africa, the Asian Pacific, and South America.[1,2] More than 750 million people, including 45% of sub-Saharan Africans, currently rely on cassava as their primary food source.[3,4] The area under cultivation has almost doubled in the past 30 years[5] and is expected to expand further in the future.[6,7] The important role cassava plays in maintaining food security can be attributed to its ease of cultivation and tolerance of poor soils, low rainfall, and high temperatures.[8−11] Such characteristics are especially relevant in the face of projected climate change. Cassava roots and leaves are a good source of carbohydrates and some minerals and vitamins; however, the roots are a poor source of protein, and all parts of the plant are toxic.[2] Cassava is one of dozens of crops that contain cyanogenic glycosides (α-hydroxynitrile glucosides), which break down to release toxic cyanide (HCN) when plant tissue is crushed or chewed, disrupting the cells.[12] Cassava is alone among regularly consumed staple crops in that it can directly cause serious illness or death of consumers if it is not first processed adequately.[13−15]

Cassava breeding by farmers and scientists has led to the development of thousands of cultivars of this crop. All contain the cyanogenic glycosides linamarin (approximately 93% of total glucosides; 2-(β-ᴅ-glucopyranosyloxy)isobutyronitrile;

derived from valine) and lotaustralin (2-(β-ᴅ-glucopyranosyloxy)-2-methylbutyronitrile, derived from isoleucine).[16,17] The highest concentrations are in the cortex (or peel) of the tuberous root (hereafter termed "tuber" for ease of presentation) and leaves.[18] The tuber parenchyma (or flesh) is less cyanogenic, but a wide range of concentrations among cultivars has been reported,[19] from 1 to 2000 mg HCN equiv/kg fresh weight (or ppm). An extensive survey of 1750 cultivars[20] found the tuber parenchyma of the majority of cultivars contained <100 ppm cyanide, but many had >50 ppm, the International Codex Standard for the upper limit in "sweet cassava",[21] and many were significantly higher than the World Health Organisation's recommended safe limit of 10 ppm.[22] The potential for poisoning is further complicated by the fact that the cyanide content of cassava is known to vary with environmental conditions, such as drought (leading to an increase in cyanogenic potential) and soil nutrient supply as reviewed in Burns et al.[23] Another confounding variable, in regions and during periods of time when dependence on cassava is high, is the low concentration of other key nutrients. For example, cassava leaves and tubers contain very low concentrations of the sulfur-containing amino acids methionine

and cysteine,[2,24,25] which are important for detoxification of ingested cyanide.[26,27]

Most debate on the possible impact of climate change on food security has focused on yields, but not the nutritive value of crops.[28] It is this aspect of food security that we address here. There is a need for more detailed assessment of both the cyanogenic glycoside and nutrient content of cultivars of cassava being bred in regions of the world where cassava is a major part of the diet. Cassava is an important crop in Mozambique and is grown over a wide climatic range. Here we present the results of a survey of new cultivars growing in breeding trials at different latitudes in Mozambique. We examined the influences of genotypic variation, geographic location, water stress (using C isotope analysis), and mycorrhizal colonization on the micro- and macronutrient composition and cyanogenic glycoside concentration of tubers and leaves.

■ MATERIALS AND METHODS

Site Descriptions. A survey of cassava cultivars was undertaken at three field stations of the Agricultural Research Institute of Mozambique (i.e., Instituto de Investigação Agrária de Moçambique, IIAM), where ongoing cassava breeding trials are undertaken. Two of the stations are located in the north of the country in the province of Nampula (sites 1 and 2) and one in the south (site 3) in the province of Maputo, as follows: (site 1) Nampula station, 15° 08′ 53″ S, 39° 18′ 40″ E (WGS84, elevation 400 m asl, 7 km east of the center of Nampula city); (site 2) Liupo station, near the town of Liupo at 15° 36′ 11″ S, 39° 56′ 36″ E (WGS84, elevation 130 m asl, 100 km southeast of Nampula); and (site 3) Umbeluzi 26° 03′ 05″ S, 32° 22′ 22″ E (WGS84, elevation 13 m asl), 29 km southwest of the capital city, Maputo (25° 58′ 08″ S, 32° 34′ 10″ E, WGS84). Soil types at sites 1 and 2 are arenosols, that is, sandy soils with a predominance of laterite at site 1 (Nampula) and sandy soils to sandy-clay-loam at site 2 (Liupo).[29]

The climates at the field sites are tropical, with a wet season from November to April and a dry season from May to October. The average annual rainfall at Maputo (close to Umbeluzi, site 3) and Nampula, recorded from 1971 to 2000, was 860 and 1075 mm, respectively.[30] Average monthly air temperatures ranged from 14 to 22 °C minimum and from 25 to 30 °C maximum in Maputo and from 16 to 22 °C minimum and from 25 tp 33 °C maximum in Nampula (recorded from 1971 to 2000).[30] Thus, the sites in the north and south have different soil types and climates. Furthermore, sites 1 and 2 have slightly different climates because site 2 is closer to the coast (within 100 km), whereas site 1 is approximately 200 km inland (C. Cuambe, personal communication).

Plant Material. The cassava cultivars used in this study were developed in Mozambique by scientists at IIAM. The genetic diversity of the parents of each cultivar was tested by means of DNA fingerprinting and morphological methods, and the offspring were tested by morphological methods. Clonal propagules were produced from stem cuttings.

Field Sampling Protocol. *Survey 1: Northern Mozambique, Nampula (Site 1) and Liupo (Site 2).* Four cultivars of cassava were grown and sampled at both the Nampula and Liupo field stations (Table 1). These cultivars, which were clonally propagated in the field in January 2010, are part of IIAM's Time of Harvest Trial to test the best time to harvest each cultivar. We sampled tissues from three to six replicate plants of each cultivar on September 1, 2010 (from one plot of each cultivar per site). Leaves were collected from the first fully unfurled leaf to the 15th node (i.e., the equivalent leaf material that is used to produce food from cassava leaves, for example, "mathapa"[31]) and placed in a plastic bag. The tuberous roots were carefully exposed, and a tuber of diameter 30–80 mm was removed and stored in a plastic bag. Upon return to the laboratory, the samples were stored in a refrigerator (4 °C) until analysis the next day (see below). Fine roots were collected from plants at the time of excavation of tuberous roots

Table 1. Cultivar Codes Used in This Study and the Full Code Assigned by the Agricultural Research Institute of Mozambique (IIAM)[a]

cultivar code	IIAM cultivar code	site
008	MZMG07/008	1 and 2
041	MZMG07/041	1 and 2
170	MZNp05/170	1 and 2
578	MZMG08/578	1 and 2
A	MZUB04030	3
B	MZUB04012	3
C	Chinhembwe	3
D	MZUB04030	3
E	MZUB04596	3
F	MZUB04539	3
G	MZUB043698	3
H	MZUB04045	3
I	MZUB04107	3
J	MZUB04104	3

[a]See text for site descriptions. (Cultivars A and D, MZUB4030, were from different sources.)

and stored in 70% ethanol for later analysis of mycorrhizal colonization (see below).

Survey 2: Southern Region, Umbeluzi (Site 3). Ten cultivars were sampled at the Umbeluzi field station (Table 1). The cultivars were clonally propagated in September 2009 and sampled in August 2010. Samples were collected for analysis from five to nine replicate plants (depending on the number of plants available) of each cultivar. The stem of each plant was cut at the base, and all of the tuberous roots were up-rooted. The top of a stem of each replicate plant was removed, to about the 15th node, and stored in a bag until return to the laboratory. For collection of tuberous root samples, the entire plant was excavated, and a length of 40–80 mm was cut from the middle of the largest tuber (30–60 mm diameter). Leaf samples were stored in a refrigerator (4 °C), and the tuber samples were stored in a freezer (−20 °C), until analysis the next day (see below). Fine roots were collected from plants at the time of excavation and stored in 70% ethanol for later analysis of mycorrhizal colonization (see below).

Analytical Techniques. *Preparation of Samples.* The tuber samples were washed, the outermost "papery" layer of skin (the periderm) was removed, and a 1–2 mm thick transverse section was taken from the middle of each tuber sample. The next outermost layer of each tuber sample, the cortex (or peel), was then removed and cut into small sections (3–5 mm lengths), and a 100 ± 5 mg sample was used for quantification of total hydrogen cyanide (see below). A metal cork-borer (9 mm diameter) was then used to take a 100 ± 5 mg sample of tuber parenchyma (or flesh) adjacent to the center of the transverse section for later analysis of total hydrogen cyanide. Tuber sampling was therefore standardized to account for known radial and longitudinal variation in cyanogenic glycoside concentration.[32] Leaf samples of 100 ± 5 mg of fresh leaf tissue were sampled from each replicate plant, by taking leaf discs (using a 9 mm diameter punch) from the lamina of several leaves, from the youngest fully unfurled leaf to the leaf at the 15th node.

Total Cyanide Content. The total cyanide concentration of the cassava leaf and tuber samples was determined using a picrate paper method,[33] which measures the concentration of all cyanogens (i.e., linamarin, acetone cyanohydrin and free cyanide) in cassava tissue, volatilized as hydrogen cyanide. The only modification of the published method was the use of pH 6 buffer.[34] A freeze−thaw method[35] was used to rupture the plant tissue and bring linamarin in contact with linamarase, by placing the vials with plant tissue in a freezer (−20 °C) for 40–60 min; water was then water added to the plant tissue, and it was macerated using a pestle (for 20–30 s). It was assumed that the activity of the enzymes involved in cyanogenesis would be decreased by freezing, thus limiting the loss of cyanide during the maceration step. A picrate paper was added to each vial

immediately after tissue maceration, followed by incubation for at least 16 h at ambient room temperature (ca. 28−32 °C). The total cyanide concentration of the tissues is reported on a dry weight basis by conversion according to the percentage dry weight (DW) of leaf tissue measured from subsamples of the cassava leaves in this study (i.e., Umbeluzi samples (n = 19), 65% DW; Nampula samples (n = 12), 60% DW; Liupo samples (n = 12), 54% DW) and using the published standard for the average dry weight of cassava tuberous roots (whole root), 40%.[2] Specifically, the conversions are as follows: all tuber cyanide concentrations (parenchyma and peel tissues), 1 ppm HCN FW = 2.50 ppm HCN DW; Umbeluzi leaves, 1 ppm HCN FW = 1.53 ppm HCN DW; Nampula leaves, 1 ppm HCN FW = 1.73 ppm HCN DW 1 ppm HCN FW = 1.86 ppm HCN DW.

Nutrient and Isotope Analyses. All remaining leaf and tuber tissue was dried at 70−80 °C for 48−60 h and ground to a fine powder for nutrient analyses as follows. Total N content was determined by dry combustion using a CHNOS Elemental Analyzer (vario MICRO cube, Varian, Germany). Ground tissue samples were digested in nitric and perchloric acids to determine the concentrations of micro- and macronutrients by radial CIROS inductively coupled plasma atomic emission spectrometry (ICP-AES) (http://www.adelaide.edu.au/was/). The concentrations of Fe and Zn are reported here, and the concentrations of the other nutrients are reported in the Supporting Information.

The use of stable isotopes is becoming increasingly common in ecological research as a means of measuring water stress. The photosynthetic enzyme RuBisCO discriminates against the heavier $^{13}CO_2$, but when the stomata are closed, ^{13}C accumulates in the interstitial spaces and, therefore, more is assimilated. A strong correlation exists between ^{13}C abundance ($\delta^{13}C$) and water use efficiency (WUE), which is determined by the carbon assimilation to transpiration ratio.[36] A relatively high WUE is generally characteristic of water-stressed plants because stomatal conductance is low relative to photosynthetic capacity. Here, the stable isotope ratios of carbon were determined (i.e., $\delta^{13}C$) from leaf tissue of three replicates of selected cultivars at each site (Nampula, n = 3; Liupo, n = 3; Umbeluzi, n = 4). The isotopic composition of finely ground leaf tissue was determined using a Fisons Isochrom continuous-flow isotope ratio mass spectrometer, after combustion (at 1050 °C) in a Carlo Erba 1110 CHN-S elemental analyzer. Laboratory standards were analyzed at the same time to determine the carbon isotope ratios of the samples, that is, C_3 beet sucrose with a δ value of −24.62‰ VPDB (relative to the Vienna PeeDee Belemnite scale) and C_4 cane (ANU) sucrose (an international standard), which is accepted as −10.45‰ VPDB, as well as USGS Glutamic Acids 40 and 41, which provide very depleted and very enriched carbon. In interpreting the data, the carbon isotope ratio of source air was necessarily assumed to be similar at all sites and over the growing season of the plants.

Mycorrhizal Colonization. Colonization of fine roots (ca. <2 mm in diameter) by arbuscular mycorrhizal fungi (AMF) was assessed at 200× magnification using the gridline-intersect method,[37] following clearing of roots by immersion in 10% potassium hydroxide (w/v) and staining with Trypan Blue (omitting phenol from all reagents), using a modification of the method of Phillips and Hayman.[38]

Statistical Analysis. Differences in mean cyanide and nutrient concentrations, carbon isotope signatures, and mycorrhizal colonization were analyzed using general linear models (GLM) and analysis of variance (ANOVA) in PASW (formally SPSS) version 18.0 or JMP v.8 (SAS Institute). Data were tested for normality by the Shapiro−Wilk W statistic (used for sample sizes of <100) and for homogeneity of variance by Levene's method[39] and transformed when necessary to satisfy the assumptions of the statistical methods. Natural log transformations were performed on concentrations of total cyanide, Fe, and Zn in all tissues of samples from the Nampula and Liupo sites. For the data from the Umbeluzi site, cyanide concentrations of all tissues were square root transformed, leaf and tuber Fe and Zn concentrations were natural log transformed, and tuber N was square root transformed.

For the Nampula−Liupo data set we analyzed data for each tissue type separately using two-way ANOVA (i.e., with location and cultivar

as factors in the model), with Tukey's HSD posthoc tests (p = 0.05) for pairwise differences among cultivar means. For the Umbeluzi data set, one-way ANOVAs were performed to compare cultivars, for each tissue type separately, with Tukey's HSD posthoc tests (p = 0.05) to determine the pairwise differences among cultivar means. In addition, targeted t tests were used to assess differences between sites for some parameters. Mycorrhizal colonization data were arcsine transformed before analysis; untransformed data are presented for ease of interpretation.

Bivariate correlations in total cyanide concentration between the tissue types were analyzed with log transformed data from the Nampula and Liupo sites. Only samples that included young leaves were included for consistency (i.e., samples that contained mature leaves only were excluded). Similar analyses could not be performed with the Umbeluzi data because leaf and root samples from the same plant could not be matched. Simple linear regressions were also performed between leaves and tuber parenchyma, and between leaves and tuber peel, with leaf total cyanide concentration as the independent variable because the majority of linamarin (the main cyanogenic glycoside in cassava and a precursor to cyanide) is produced in the leaves and transported to the roots.[40,41] A simple linear regression was also performed between leaf cyanide concentration and leaf $\delta^{13}C$ signature of selected cultivars. Additional analyses to assess Pearson's correlation coefficients between total N and HCN and $\delta^{13}C$ on pooled data were performed using Minitab16 .

■ RESULTS

Differences in Total Cyanide Concentrations. *Survey 1: Nampula and Liupo.* The concentration of cyanide in the tissues of cassava plants grown at the Nampula and Liupo field sites differed among cultivars and locations for each tissue type (Figure 1). For tuber parenchyma (or flesh; Figure 1a), the concentration of cyanide was significantly higher (ca. 3−4 times) in cultivars 170 and 578 than in the other cultivars, when pooled over sites. Further analysis (targeted t tests) revealed that the concentrations of cyanide in the flesh of cultivars 170 (p = 0.0015) and 578 (p = 0.0493) were significantly higher in plants grown at the Nampula site than at the Liupo site. In the case of tuber peel (or cortex; Figure 1b), the concentration of cyanide varied between both cultivars and locations, as indicated by a significant two-way interaction (p = 0.002). As with the tuber parenchyma, the concentration of cyanide was highest in the tuber peel of cultivar 578 when plants were grown at the Nampula field site. On average, the concentration of cyanide was 5.6 times higher in the peel than the flesh across all cultivars and both sites (compare panels a and b of Figure 1). The concentration of cyanide in the leaves (Figure 1c) differed significantly among genotypes and sites, as indicated by a significant two-way interaction (p = 0.0013). The only significant difference between sites in foliar cyanide content was in cultivar 170, which had significantly higher concentrations in plants grown at Liupo than at Nampula. Leaf cyanide concentrations were relatively low for cultivars 8 (91−159 CN ppm) and 41 (107−140 CN ppm) at both sites.

The concentration of cyanide in the tuber peel was much higher (an order of magnitude for some cultivars) than that of the tuber parenchyma and leaves. Further analysis revealed a significant positive correlation between the cyanide concentrations of leaves and tuber peel across all cultivars and sites (r = 0.58, r^2 = 0.33, p < 0.01, n = 35; data not shown); there was no correlation between tuber parenchyma and tuber peel cyanide concentrations. Interestingly, a significant positive correlation between tuber parenchyma and leaf cyanide concentrations was detected across the four cultivars at the Nampula site (r = 0.66, r^2 = 0.43, p = 0.0034), whereas no
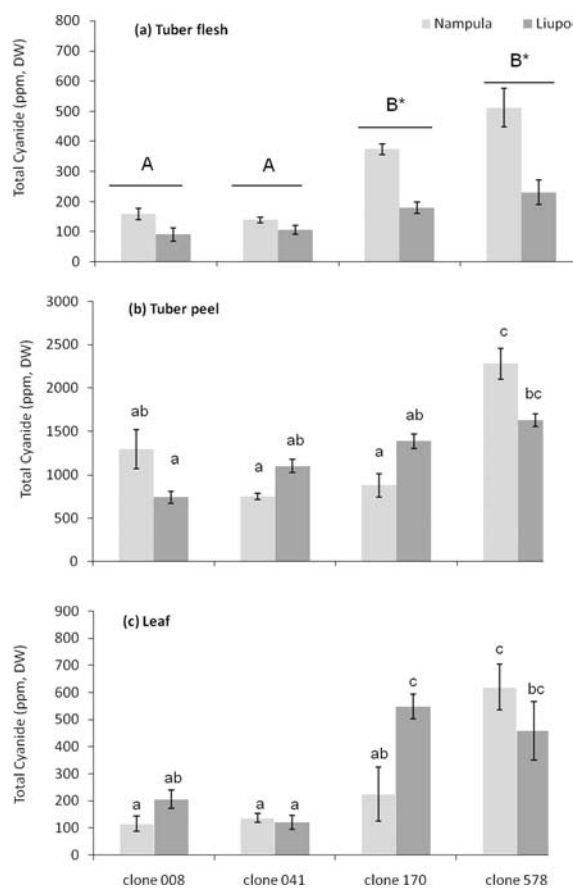
Figure 1. Total cyanide concentrations of four cassava cultivars sampled at two locations, Nampula and Liupo, in three tissue types: (a) tuber parenchyma (flesh); (b) tuber cortex (peel); (c) leaf tissues. Values are the mean ± 1SE. Cultivar (clone) means followed by the same letter were not significantly different at the $p < 0.05$ level; capital letters were used in (a) because there was no significant interaction between cultivar and location; * indicates significant differences between sites, determined using targeted t tests (see text for full details of data analysis).

Figure 2. Total cyanide concentrations of 10 cassava cultivars sampled at the Umbeluzi Agricultural Research Station in three tissue types: tuber parenchyma (flesh); tuber cortex (peel); leaf tissue ($n = 4-9$ per cultivar and tissue type except cultivar I, $n = 1$ young leaf sample, see text for details). Values are the mean ± 1SE. See Table 1 for full cultivar codes. Cultivar means followed by the same letter were not significantly different at the $p < 0.05$ level; see text for full details of data analysis.

significant correlations were detected across both sites or at the Liupo site alone.

Survey 2: Umbeluzi. The concentrations of cyanide in plant tissues sampled from the Umbeluzi field site varied considerably among cultivars and tissue types (Figure 2). When tissue types were compared, the concentration of cyanide was 3.3 times greater (when pooled across cultivars) in the tuber peel than the tuber parenchyma. Similarly, the concentration of cyanide was on average 1.8 times higher in leaves than in tuber parenchyma. Within each tissue type, significant differences in total cyanide concentrations among cultivars were detected. For example, cyanide concentration in tuber parenchyma ranged from 162 to 706 ppm cyanide (on a dry weight basis) across the 10 cultivars (Figure 2). There was a 2-fold difference between the highest and lowest ranked cultivars for tuber peel cyanide concentrations; a similar pattern was also seen in leaf cyanide (Figure 2).

Plant Fe, Zn, and N Concentrations. *Survey 1: Nampula and Liupo.* The mean concentration of iron in the leaves of cassava plants grown at Nampula (284 ± 33 mg/kg, across cultivars) was significantly higher than in those grown at Liupo (93 ± 3.6 mg/kg) and differed among cultivars (Figure 3a; significant two-way interaction, $p < 0.001$). Interestingly,
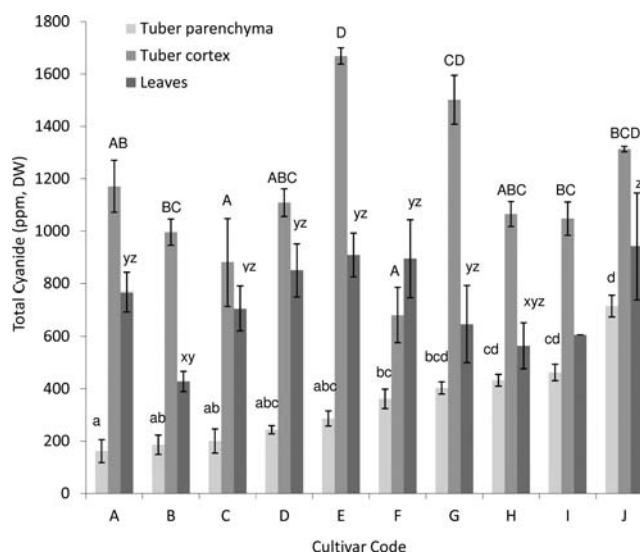
although there were no differences in leaf iron concentrations among cultivars when grown at Liupo, there was considerable variation in leaf iron among cultivars grown at Nampula (from 178 to 497 mg/kg DW). In contrast to the leaves, no differences in iron concentrations of tuber parenchyma were detected between sites or cultivars (Figure 4a). When pooled over sites and cultivars, the concentration of iron in the tuber parenchyma was 10.75 ± 1.03 mg/kg DW (mean ± SE), 21 times lower than in the leaves.

For foliar zinc, there were some differences among cultivars, as indicated by a significant main effect of cultivar ($p < 0.001$), with cultivar 578 having the lowest concentration (Figure 3b). Interestingly, for cultivar 578, plants grown at the Liupo field site had significantly higher tissue zinc concentrations in the leaves (47.8 mg/kg) than when grown at the Nampula field site (27.0 mg/kg; t test, $p < 0.05$). In contrast, no differences in tuber parenchyma zinc concentrations were detected among cultivars grown at the Nampula and Liupo field sites (Figure 4b). The mean tuber parenchyma zinc concentration across all sites and cultivars was 6 times lower than that of leaves (compare Figures 3b and 4b).

Leaf nitrogen concentrations (Figure 3c) differed among cultivars, irrespective of locations, as indicated by a significant main effect of cultivar ($p = 0.0028$). Specifically, the concentration of nitrogen was lower in cultivar 578 (mean = 4.6%) than the other cultivars (mean = 5.5%). Leaf nitrogen was also lower when plants were grown at the Nampula field site, for three of the four cultivars (Figure 3c), as indicated by a significant main effect of location ($p = 0.0002$). Tuber parenchyma nitrogen, although low across all samples (Figure 4c), was higher in plants grown at the Liupo field site ($p < 0.0001$) and differed significantly among cultivars ($p < 0.0001$).

Survey 2: Umbeluzi. The mean concentrations of iron and zinc in cassava leaves differed significantly among the cultivars ($p < 0.0001$) (Table 2), whereas nitrogen concentration did
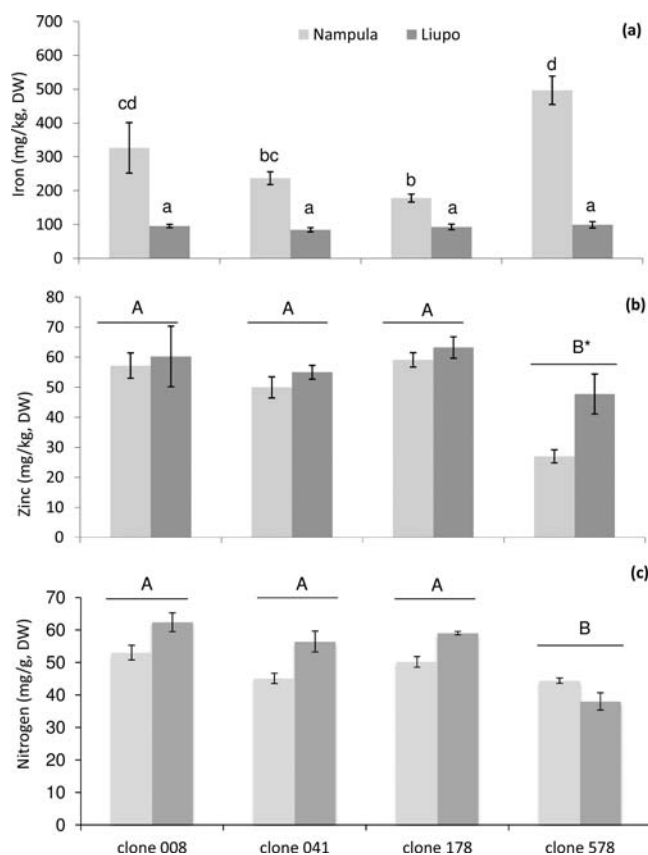
Figure 3. Total (a) iron, (b) zinc, and (c) nitrogen concentrations in leaves of four cassava cultivars sampled at two locations, Nampula and Liupo. Values are the mean ± 1SE. Cultivar (clone) means followed by the same letter were not significantly different at the $p < 0.05$ level; capital letters were used to show significant differences among cultivars ($p < 0.05$) when the interaction between cultivar and location was not significant. ∗ indicates a significant difference between sites, determined using a targeted t test (see text for full details of data analysis).
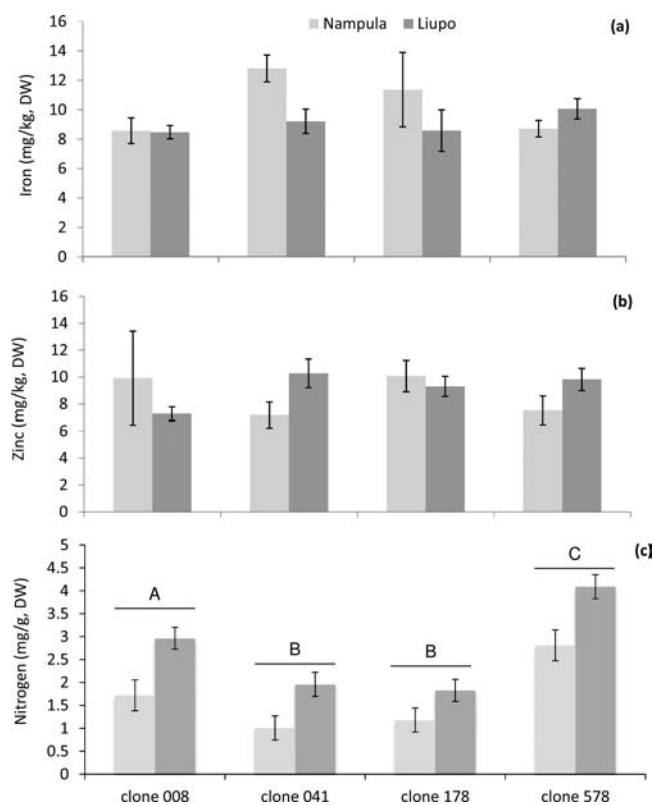

Figure 4. Total (a) iron, (b) zinc, and (c) nitrogen concentrations in tuber parenchyma of four cassava cultivars sampled at two locations, Nampula and Liupo. Values are the mean ± 1SE. Capital letters were used to show significant differences among cultivars ($p < 0.05$) when the interaction between cultivar and location was not significant (see text for full details of data analysis).

not. Foliar iron concentrations varied 5-fold among cultivars, and foliar zinc varied 2-fold among cultivars. As with leaves, iron and zinc concentrations of tuber parenchyma differed significantly among cultivars (iron, $p < 0.001$; zinc, $p = 0.0162$), ranging from 8 to 24 mg/kg and from 8 to 19 mg/kg, respectively, whereas tuber nitrogen concentrations were similar (Table 2).

C-Isotope Discrimination: Surveys 1 and 2. Carbon isotope signatures ($\delta^{13}C$ values) can be used to indicate the water status of plants,[36] with relatively high (less negative) values typical in more water-stressed (or water use efficient) plants. We measured foliar $\delta^{13}C$ on a selection of plants from each of the three field sites, using common cultivars when possible. Overall, pooling all data, there was no significant difference in $\delta^{13}C$ among sites (Table 3). However, when comparisons were made among leaves of the same cultivars across sites, $\delta^{13}C$ values were significantly higher at Nampula than at Liupo (cultivars 41 and 170, $p < 0.05$), but lower at Nampula compared with Umbeluzi (cultivar MZUB04539, $p < 0.05$; Table 3). When sites were examined separately, significant differences among cultivars were detected at each site. In each case, one cultivar had a significantly lower mean $\delta^{13}C$ value than other cultivars growing at the same site ($p < 0.05$; Table 3). At Umbeluzi, for example, the mean $\delta^{13}C$ of cultivar I was

3.3‰ lower than the mean $\delta^{13}C$ of cultivars A, D, and F, which did not differ from each other ($p < 0.0001$). A highly significant positive correlation was detected between leaf cyanide and leaf $\delta^{13}C$ when data from all sites were pooled (leaf CN = 10936 + 426$\delta^{13}C$, $p < 0.001$; Pearson's coefficient (r) = 0.72). This positive correlation between foliar CN and $\delta^{13}C$ was stronger when only cultivars growing at a single site, Umbeluzi, were examined (leaf CN = 7278 + 272$\delta^{13}C$, $p < 0.0001$; $r = 0.94$). Also at Umbeluzi, highly significant correlations between plant water status and foliar nutrient concentrations were detected among cultivars. Specifically, $\delta^{13}C$ was strongly positively correlated with foliar N ($r = 0.97$; $p < 0.0001$; N = 19.9 + 0.66$\delta^{13}C$) and S ($r = 0.97$; $p < 0.0001$; S = 12085 + 368$\delta^{13}C$) and, to a lesser extent, with Na ($r = 0.61$, $p = 0.037$) (data not shown). Conversely, negative correlations were found between $\delta^{13}C$ and the macronutrients Mg ($r = 0.68$, $p = 0.014$), K ($r = 0.59$, $p = 0.043$), and Ca ($r = 0.74$, $p = 0.006$) and the micronutrients Fe ($r = 0.64$, $p = 0.024$), Mn ($r = 0.69$, $p = 0.014$), Al ($r = 0.74$, $p = 0.0006$), and Ti ($r = 0.70$, $p = 0.011$) (data not shown).

Mycorrhizal Colonization: Surveys 1 and 2. The roots of cassava plants of all cultivars, grown at all sites, had high levels of mycorrhizal colonization ranging from 51 to 93% (Figure 5). Whereas the percentage of root length colonized by AMF was significantly higher ($p < 0.001$) at the Liupo field site than at the Nampula field site (91 versus 64%), there were no differences in colonization among cultivars (Figure 5a). Similarly, there was no difference in the level of mycorrhizal colonization among any of the cultivars grown at the Umbeluzi

Table 2. Mean ($\pm$1SE) Concentrations of Fe, Zn, and N in Cassava Leaves and Tuber Parenchyma of Different Cultivars Grown at the Umbeluzi Agricultural Research Station in Southern Mozambique[a]

cultivar	tissue type	N	Fe (mg/kg)	Zn (mg/kg)	N (mg/kg)
A	leaf	7	453 (23) c	95 (6) ab	47865 (1060)
	tuber	6	8 (1) A	12 (1) AB	2187 (205)
B	leaf	7	141 (7) a	87 (5) a	46862 (1432)
	tuber	7	20 (2) CD	16 (2) AB	1658 (520)
C	leaf	9	207 (20) ab	132 (12) abcd	43762 (2078)
	tuber	8	12 (1) ABC	8 (1) A	1835 (255)
D	leaf	8	235 (19) b	158 (17) d	48422 (1481)
	tuber	8	9 (1) A	9 (1) AB	1731 (193)
E	leaf	8	679 (56) c	137 (5) bcd	45552 (6082)
	tuber	9	9 (1) A	12 (3) AB	1562 (432)
F	leaf	6	206 (27) ab	124 (25) abcd	43079 (2232)
	tuber	5	24 (4) D	19 (3) B	2151 (128)
G	leaf	4	195 (24) ab	141 (7) bcd	45457 (2800)
	tuber	9	16 (2) BCD	12 (3) AB	1861 (457)
H	leaf	9	206 (19) ab	98 (4) abc	46647 (5458)
	tuber	8	10 (1) AB	8 (1) A	1453 (188)
I	leaf	6	608 (54) c	137 (8) bcd	40949[b]
	tuber	7	11 (1) AB	10 (1) AB	2577 (4558)
J	leaf	5	458 (54) c	150 (8) cd	38527 (2319)
	tuber	9	13 (3) ABC	11 (2) AB	3128 (356)

[a]For each nutrient and organ (leaf, tuber), means with different letters were significantly different at $p < 0.05$. Values are per dry mass of plant tissue. [b]Only one foliage sample was obtained for this cultivar for N determination due to leaf senescence.

Table 3. Carbon Isotope Discrimination ($\delta^{13}C$) of Leaf Tissue of Cassava Cultivars at Three Different Sites in Mozambique

location	cultivar code[a]	$\delta^{13}C$[b]
Nampula	041[#]	−24.12 (0.49) a
Nampula	170[#]	−24.36 (0.05) a
Nampula	MZUB04539*	−25.78 (0.26) b
Liupo	041[#]	−24.82 (0.03) A
Liupo	170[#]	−24.85 (0.15) A
Liupo	578	−26.18 (0.31) B
Umbeluzi	A (MZUB04030)	−23.39 (0.35) x
Umbeluzi	D (MZUB04030)	−23.04 (0.16) x
Umbeluzi	F (MZUB04539)*	−23.99 (0.14) x
Umbeluzi	I (MZUB04107)	−26.82 (0.12) y

[a]* indicates significant difference between sites using a targeted t test; [#] indicates significant main effect of site when comparing cultivars common to both sites; letters indicate significant differences among cultivars at each site ($p < 0.05$). [b]Values are the mean ($\pm$ 1SE) of $n = 3$ plants per cultivar.

field site (Figure 5b), although targeted t tests indicated a higher level of colonization in cultivar E than in cultivar J ($p = 0.026$).

■ DISCUSSION

The results presented here show that the concentrations of cyanide in the edible portions of cassava cultivars currently being bred in Mozambique are, overall, relatively high, although they vary with both genotype and environmental factors. These data highlight the need for adequate processing of cassava-based foods prior to consumption and education of new
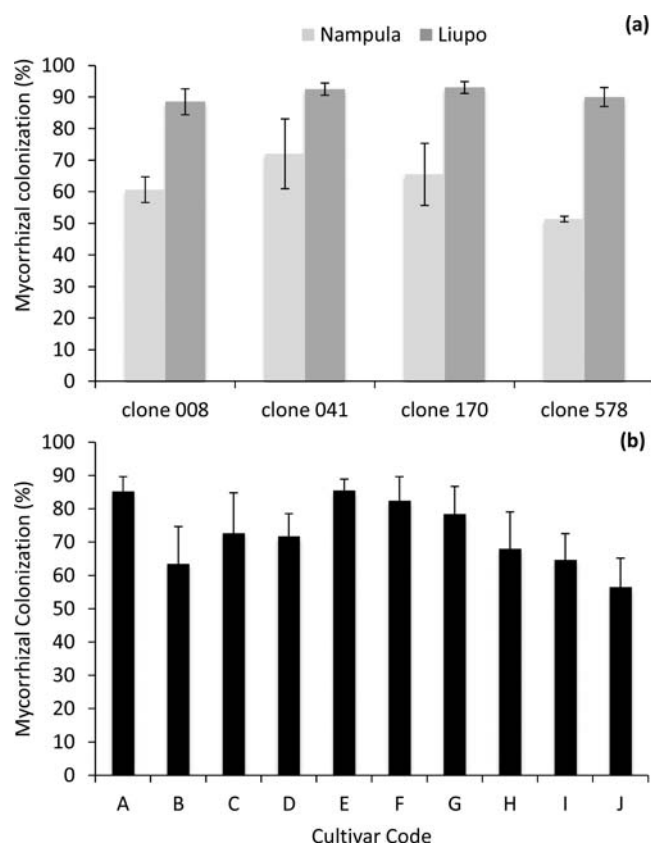
Figure 5. Mycorrhizal colonization of roots (%) of (a) 4 cultivars grown at the Nampula and Luipo field sites and (b) 10 cultivars grown at the Umbeluzi field site. Values are the mean $\pm$ 1SE; see text for full details of data analysis.

growers of cassava about the risks associated with, and appropriate processing procedures of, this important staple crop. Considerable genotypic and environmental variation in the concentrations of iron and zinc in the edible parts of cassava was also seen, further highlighting the value of conducting breeding trials at multiple locations, as occurs in Mozambique.

Cassava Toxicity. The mean cyanide concentrations of cultivars sampled in this study were 14−70 times higher (on a dry weight basis) than the World Health Organization's recommended safe limit for cassava flour 10 ppm,[22] consistent with earlier studies (see ref 19). Using a classification system for fresh, peeled tubers developed by Bourdoux et al. and others,[42−44] only two cultivars in this study (008 and 041) would be classified as "innocuous" (<50 ppm total cyanide, fresh weight). Eight cultivars would be considered to be dangerously poisonous (>100 ppm total cyanide, fresh weight). Even higher concentrations were observed in the leaves (120−940 ppm, dry weight), which are also sometimes eaten by humans,[2,30] and in the tuber peel (680−2280 ppm, dry weight), which is often fed to livestock.[45] These results emphasize the importance of adequate processing of cassava prior to consumption[46,47] and the potential danger to livestock. To this end, education programs on the risks associated with cassava consumption and appropriate methods for detoxification should accompany the expansion of this crop into new areas.

The cyanide concentration in plants is known to vary with environmental and edaphic conditions.[23,48] For example, higher cyanide concentrations have been reported in plants, including

cassava, subject to water stress.[49−52] Consistent with this was the significant positive correlation between leaf cyanide concentration and $\delta^{13}C$ found here; that is, leaves from plants that were experiencing greater water stress also had higher concentrations of leaf cyanide. Also, consistent with climatic differences within Mozambique, phenotypic variation in $\delta^{13}C$ among sites suggests greater water limitation at the southern site (Umbeluzi) and the inland northern site (Nampula). Significant differences in $\delta^{13}C$ among cultivars at each site ($\delta^{13}C$ varying by up to 3.8‰ at Umbeluzi) indicate potential differences in water use efficiency among cultivars, which were all sampled toward the end of the dry season.

Carbon isotope discrimination has been used as a tool in breeding programs to indirectly assess the relative water use efficiency (WUE) of crop genotypes (e.g., wheat, rice, peanuts, beet) grown under identical conditions (see, e.g., refs 53−55). Relationships between nutrient accumulation and WUE ($\delta^{13}C$), however, have been less explored.[56,57] The negative correlations between $\delta^{13}C$ and several nutrients here are consistent with a reduction in passive mineral transport in more WUE (higher $\delta^{13}C$) cultivars.[58,59] A positive correlation between leaf N and $\delta^{13}C$ has also previously been reported (see, e.g., ref 60). Given our results, more investigation of the relationships among WUE, yield, toxicity, and nutrient accumulation is warranted to determine the utility of $\delta^{13}C$ in cassava breeding.

Whereas plant water stress ($\delta^{13}C$) was associated with variation in foliar cyanide concentrations, foliar and tuber cyanide concentrations were not consistently positively correlated. We found that tuber cyanide differed among cultivars and between sites, when the same cultivar was available for comparison. The differences between sites were not, however, consistent across tissue types. For example, whereas cultivars 170 and 578 had double the amount of cyanide in tuber parenchyma of plants grown at Nampula compared with those at Liupo, the reverse trend was seen for leaves of cultivar 170. Thus, despite significant variation in tuber cyanogenic potential of cultivars between sites, cultivar by site interactions, known to be important for tuber yield,[50] did not affect cyanide concentrations of tuber parenchyma among the four northern cultivars, consistent with the findings of Bokanga et al.[50] By contrast, cultivar by site interactions were significant for the cyanide content of leaves and tuber peel. These results highlight the importance of growing the same cultivars in different locations to examine the suitability of cultivars in different environments and emphasize the need to sample all tissue types when precise information about nutritional quality is required (see also refs 32, 61, and 62). Furthermore, given that the environment can affect the association between leaf and root cyanide, and other aspects of leaf and tuber nutrition, the utility of leaf cyanide concentration as an indicator of tuber traits may be limited to large-scale screenings for which only a broad indication of relative cultivar toxicity is required.[32,50,61,62]

Variation in tissue cyanide concentration between sites and among cultivars in this study was not explained by variation in tissue nitrogen concentrations. Despite the well-known effects of nitrogen supply on cyanogenic glycoside concentration in other crop species (see, e.g., refs 63−65), the effects of soil nitrogen supply on plant nitrogen and cyanogenic glycoside concentrations have received surprisingly little systematic attention in cassava.[23,66] In this study, despite significant variation in both nitrogen and cyanide contents between sites and among cultivars, no correlations between nitrogen and

cyanide concentrations were detected for either leaves or tuber parenchyma. Fertilization studies of cassava have focused on yield and growth and, typically, few aspects of plant chemistry. For example, one study in which cyanide was not measured reports an increase in tuber nitrogen concentration with nitrogen addition;[67] other fertilization studies in which tissue cyanide but not nitrogen was measured report both increases and decreases in tuber cyanide associated with a range of nitrogen additions, in combination with various other agronomic practices (e.g., tilling, mulching) and nutrient additions (see, e.g., refs 68−70). Whereas a positive relationship between nitrogen supply (soil and/or foliar nitrogen) and foliar cyanide content has been reported in several studies of species with cyanogenic foliage (see, e.g., refs 49, 63, and 71), leaf cyanogenic glycoside concentration and soil or foliar nitrogen are not always correlated (see, e.g., refs 72−75). Studies of nitrogen allocation in species with cyanogenic roots are limited; root cyanogenic glycoside concentration was insensitive to nitrogen supply in the highly cyanogenic fibrous roots of the tropical species *Prunus turneriana*.[73] Given the importance of nitrogen supply for cassava growth and yield,[69] the substantial cultivar and tissue-specific variation in cyanide concentrations across sites found here, and the established importance of nitrogen in affecting plant growth and biochemical responses to both drought (see, e.g., ref [76]) and elevated CO_2,[77,78] further systematic study of factors governing nitrogen allocation to cyanogenic tissues in cassava is warranted.

Micronutrient Nutrition of Cassava. Cassava products are an important source of energy, and the leaves are an important source of protein and some minerals and vitamins for consumers in rural communities of developing countries.[31] However, the mineral and vitamin content can be reduced during processing and cooking,[2,79] leading to dietary deficiencies if not supplied from other foods. One study[80] in rural communities of Kenya and Nigeria found that over half of the children (2−5 years of age) for whom cassava is a staple food experience inadequate intake of iron, zinc, and vitamin A. In the present study we found that the concentrations of iron in tubers from all cultivars (range = 8−24 mg/kg) were at the low end of the published range for cassava (3−140 ppm), whereas the range of tuber zinc concentrations, 8−19 mg/kg, straddled the published standard for cassava of 14 ppm.[2] Concentrations of micronutrients in tubers varied less between sites and among cultivars than leaf micronutrient concentrations, and with the exception of low foliar zinc content at the two northern sites, foliar zinc and iron were typically greater than the published range of values for cassava.[2] Such variation among cultivars in the concentrations of both iron and zinc highlights the potential for breeding programs to improve the micronutrient density of cassava (see, e.g., ref 81). Furthermore, the importance of local environmental conditions also needs to be taken into consideration. For example, for cultivar 578 the concentration of iron was higher in the leaves when grown at the Nampula field site than at the Liupo field site; however, the reverse was true for zinc. Interestingly, this same cultivar also had some of the highest cyanide concentrations observed in this study. This example serves to highlight not only the need to undertake breeding experiments at multiple locations but also the risk of failing to consider a range of nutritional parameters in plant breeding programs.

Like most plants, cassava forms arbuscular mycorrhizas (AM); however, in the case of cassava the plants are highly

4952

dx.doi.org/10.1021/jf2047288 | *J. Agric. Food Chem.* 2012, 60, 4946−4956

dependent upon these associations to sustain normal growth.[82,83] Given the importance of AM in plant acquisition of nutrients[84] and the limited access of many of the world's farmers to synthetic fertilizers, AM have been identified as playing an important role in achieving food security,[85] especially in the developing world.[23,81,86] In the present study, the roots of cultivars were well colonized by arbuscular mycorrhizal fungi (AMF), as would be expected in such low-input farming systems (see, e.g., ref [83]). The difference in colonization rates is likely a reflection of differences in the fungal inoculum potential of the different soils and may in part help to explain the higher concentrations of some nutrients in the plants at Liupo (i.e., N in tubers and leaves and Zn and P in leaves; see the Supporting Information) compared to the plants grown at Nampula. Although this is speculative, and deserves further attention, these data provide good support for the potential for AMF to be considered in efforts seeking to improve the nutrition of plants in developing world farming systems.

Cassava will continue to be an important staple food crop due to its production of starchy storage roots and its ability to grow in marginal agro-ecosystems. It is necessary for cassava breeding programs to continue to develop new cultivars that are adapted to variation in environmental conditions, particularly as global climatic patterns change and, thus, the suitability of cassava-growing areas also changes.[6] To this end more studies are required to examine the interactive effects of temperature, water and nutrient availability, and atmospheric CO_2 concentrations on the yield and quality of cassava cultivars.[23] Whereas cassava is a remarkable crop, and is certain to continue to play a major role in achieving global food security, we contend that the expansion of cassava production must be accompanied by knowledge of effective detoxification methods[46,87,88] and the health effects of cyanide poisoning from inadequately processed cassava,[47] especially where growers are unaware of the risks associated with consumption of incorrectly prepared cassava-based foodstuffs.[89] To this end, the use of portable cassava cyanide testing kits,[33] as used in this study, is a simple development activity that could significantly reduce the risk of cyanide poisoning in existing and new cassava-growing areas.

The present study is one of only a few that have examined both the nutritional composition and cyanogenic potential of cassava (see, e.g., ref [90]), both of which are important in consideration of the overall nutritional quality of this food crop and therefore its suitability as a strategy for achieving food security. The nutritional quality of cooked cassava products, particularly from the tubers, is relatively low compared with that of cereals, soybeans, and animal products.[2] Furthermore, cassava leaves and tubers contain very low amounts of the sulfur-containing amino acids methionine and cysteine,[2,24,25] which are important for the detoxification of cyanide.[26,27] Recent work by Fauquet and colleagues[91] has increased the total protein content of tuberous cassava roots in transgenic plants and, concomitantly, reduced the concentration of cyanogenic compounds by half in both roots and leaves. Transgenic cassava with reduced cyanide has been developed independently by two groups.[40,81] Further characterization is required, however, before it is clear whether yields in these crops are maintained.[40] Moreover, acceptance of ultralow cyanide cultivars by small landholders in southern Africa remains uncertain.[86,92] Further development of low cyanide and/or biofortified cassava is likely to be advantageous to cassava consumers; however, manipulation of these traits needs to be considered in light of other plant traits such as yield, water use efficiency, pest and disease resistance, taste, and cooking consistency.[93]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Concentrations of macro- and micronutrients in cassava leaves and tuberous roots sampled at three agricultural field stations in Mozambique. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: timothy.cavagnaro@monash.edu. Phone: +61-3-99055793. Fax: +61-3-99055613.

Present Address

§Department of Zoology, School of Life Sciences, La Trobe University, 3086 Victoria, Australia.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Nassar, N. M. A.; Ortiz, R. Cassava improvement: challenges and impacts. *J. Agric. Sci.* **2007**, *145*, 163−171.

(2) Montagnac, J. A.; Davis, C. R.; Tanumihardjo, S. A. Nutritional value of cassava for use as a staple food and recent advances for improvement. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8*, 181−194.

(3) FAO; IFAD. *The World Cassava Economy: Facts, Trends and Outlooks*; Food and Agriculture Organization of the United Nations and International Fund for Agricultural Development: Rome, Italy, 2000; p 59.

(4) Balagopalan, C. Cassava utilization in food, feed and industry. In *Cassava: Biology, Production and Utilization*; Hillocks, R. J.,Thresh, J. M., Bellotti, A. C., Eds.; CABI Publishing: Oxon, U.K., 2002; pp 301−318.

(5) FAOSTAT. Food and agricultural commodities production (http://www.faostat.fao.org) (accessed July 9, 2010).

(6) Liu, J. G.; Fritz, S.; van Wesenbeeck, C. F. A.; Fuchs, M.; You, L. Z.; Obersteiner, M.; Yang, H. A spatially explicit assessment of current and future hotspots of hunger in Sub-Saharan Africa in the context of global change. *Global Planet. Change* **2008**, *64*, 222−235.

(7) Lobell, D. B.; Burke, M. B.; Tebaldi, C.; Mastrandrea, M. D.; Falcon, W. P.; Naylor, R. L. Prioritizing climate change adaptation needs for food security in 2030. *Science* **2008**, *319*, 607−610.

(8) Dahniya, M. T. An overview of cassava in Africa. *Afr. Crop Sci. J.* **1994**, *2*, 337–343.

(9) De Tafur, S. M.; El-Sharkawy, M. A.; Cadavid, L. F. Response of cassava (*Manihot esculenta* Crantz) to water stress and fertilization. *Photosynthetica* **1997**, *34*, 233–239.

(10) El-Sharkawy, M. A. Cassava biology and physiology. *Plant Mol. Biol.* **2003**, *53*, 621–641.

(11) Lebot, V. *Tropical Root and Tuber Crops: Cassava, Sweet Potato, Yams and Aroids*; CABI: Wallingford, U.K., 2009.

(12) Alves, A. A. C. Cassava botany and physiology. In *Cassava: Biology, Production and Utilization*; Hillocks, R. J., Thresh, J. M., Bellotti, A. C., Eds.; CABI Publishing: Oxon, U.K., 2002; pp 67–89.

(13) Cliff, J.; Coutinho, J. Acute intoxication from newly-introduced cassava during drought in Mozambique. *Trop. Doct.* **1995**, *25*, 193–193.

(14) Cliff, J.; Nicala, D. Long term follow-up of konzo patients. *Trans. R. Soc. Trop. Med. Hyg.* **1997**, *91*, 447–449.

(15) Mlingi, N.; Poulter, N. H.; Rosling, H. An outbreak of acute intoxications from consumption of insufficiently processed cassava in Tanzania. *Nutr. Res. (N.Y.)* **1992**, *12*, 677–687.

(16) Nartey, F. Studies on cassava, *Manihot utilissima* Pohl—I. Cyanogenesis: the biosynthesis of linamarin and lotaustralin in etiolated seedlings. *Phytochemistry* **1968**, *7*, 1307–1312.

(17) Poulton, J. E. Localization and catabolism of cyanogenic glycosides. In *Cyanide Compounds in Biology*; Evered, D., Harnett, S., Eds.; Wiley: Chichester, U.K., 1988; pp 67–91.

(18) de Bruijn, G. H. The cyanogenic character of cassava (*Manihot esculenta*). In *Chronic Cassava Toxicity*; Monograph IDRC-010e; Nestel, B., MacIntyre, R., Eds.; International Development Research Centre (Ottawa): London, U.K., 1973; p 162.

(19) Cardoso, A. P.; Mirione, E.; Ernesto, M.; Massaza, F.; Cliff, J.; Haque, M. R.; Bradbury, J. H. Processing of cassava roots to remove cyanogens. *J. Food Compos. Anal.* **2005**, *18*, 451–460.

(20) Bokanga, M. Distribution of cyanogenic potential in the cassava germplasm. *Acta Hortic.* **1994**, *375*, 117–123.

(21) FAO/WHO. Codex standard for sweet cassava. In *Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission*; Food and Agriculture Organisation and World Health Organisation of the United Nations: Rome, Italy, 2005; Vol. Codex Standard 238–2003.

(22) FAO/WHO. Codex standard for edible cassava flour. In *Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission*; Food and Agriculture Organisation and World Health Organisation of the United Nations: Rome, Italy, 1995; Vol. Codex Standard 176–1989.

(23) Burns, A.; Gleadow, R.; Cliff, J.; Zacarias, A.; Cavagnaro, T. Cassava: the drought, war and famine crop in a changing world. *Sustainability* **2010**, *2*, 3572–3607.

(24) Yeoh, H. H.; Truong, V. D. Protein contents, amino acid compositions and nitrogen-to-protein conversion factors for cassava roots. *J. Sci. Food Agric.* **1996**, *70*, 51–54.

(25) Diasolua Ngudi, D.; Kuo, Y.-H.; Lambein, F. Amino acid profiles and protein quality of cooked cassava leaves or 'saka-saka'. *J. Sci. Food Agric.* **2003**, *83*, 529–534.

(26) Westley, J. Mammalian cyanide detoxification with sulphane sulphur. In *Cyanide Compounds in Biology*; Evered, D., Harnett, S., Eds.; Wiley: Chichester, U.K., 1988; pp 212–218.

(27) Zagrobelny, M.; Bak, S.; Møller, B. L. Cyanogenesis in plants and arthropods. *Phytochemistry* **2008**, *69*, 1457–1468.

(28) Cavagnaro, T. R.; Gleadow, R. M.; Miller, R. E. Viewpoint: Plant nutrition in a high CO_2 world. *Funct. Plant Biol.* **2011**, *38*, 87–96.

(29) Bias, C.; Brunini, O.; Brunini, A. P. C.; de Carvalho, J. P.; Pádua, A. L. *Micro-zoneamento agroclimático como suporte à identificação de locais adequados para ensaios de campo e introdução de sistemas de produção*; Ministério da Agricultura, Republica de Moçambique, and FundAg – Fundação de Apoio à Pesquisa Agricola (Foundation for Support of Agricultural Research): Maputo, Mozambique, 2010.

(30) INAM. Instituto Nacional de Meteorologia, Mozambique (http://www.inam.gov.mz/) (accessed May 13, 2010).

(31) Achidi, A. U.; Ajayi, O. A.; Bokanga, M.; Maziya-Dixon, B. The use of cassava leaves as food in Africa. *Ecol. Food Nutr.* **2005**, *44*, 423–435.

(32) Cooke, R. D. An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). *J. Sci. Food Agric.* **1978**, *29*, 345–352.

(33) Bradbury, M. G.; Egan, S. V.; Bradbury, J. H. Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *J. Sci. Food Agric.* **1999**, *79*, 593–601.

(34) Bradbury, J. H. Cassava Cyanide Diseases and Neurolathyrism Network (CCDNN), http://online.anu.edu.au/BoZo/CCDN/three.html (accessed Oct 7, 2010).

(35) Gleadow, R. M.; Bjarnholt, N.; Jørgensen, K.; Fox, J.; Miller, R. M. Detection, identification and quantitative measurement of cyanogenic glycosides. In *Research Methods in Plant Science: Vol. 1. Soil Allelochemicals*; Narwal, S. S., Szajdak, L., Sampietro, D. A., Eds.; International Allelopathy Foundation, Studium Press (USA): Houston, TX, 2011; pp 283–310.

(36) Farquhar, G. D.; Ehleringer, J. R.; Hubic, K. T. Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 503–537.

(37) Giovannetti, M.; Mosse, B. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* **1980**, *84*, 489–500.

(38) Phillips, J. M.; Hayman, D. S. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **1970**, *55*, 158–161.

(39) Coakes, S. J.; Steed, L. G.; Ong, C. *SPSS: Analysis without Anguish*, version 17 for Windows; Wiley: Milton, Australia, 2010.

(40) Jørgensen, K.; Bak, S.; Busk, P. K.; Sørensen, C.; Olsen, C. E.; Puonti-Kaerlas, J.; Møller, B. L. Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. *Plant Physiol.* **2005**, *139*, 363–374.

(41) Koch, B.; Nielsen, V. S.; Halkier, B. A.; Olsen, C. E.; Moller, B. L. The biosynthesis of cyanogenic glucosides in seedlings of cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.* **1992**, *292*, 141–150.

(42) Bourdoux, P.; Seghers, P.; Mafuta, M.; Vanderpas, J.; Vanderpas-Rivera, M.; Delange, F.; Ermans, A. M. Cassava products: HCN content and detoxification processes. In *Nutritional Factors Involved in the Goitrogenic Action of Cassava*; Monograph IDRC-184e; Delange, F., Iteke, F. B., Ermans, A. M., Eds.; IDRC: Ottawa, Canada, 1982; pp 51–58.

(43) Cardoso, A. P.; Mirione, E.; Ernesto, M.; Massaza, F.; Cliff, J.; Haque, M. R.; Bradbury, J. H. Processing of cassava roots to remove cyanogens. *J. Food Compos. Anal.* **2005**, *18*, 451–460.

(44) Coursey, D. G. Cassava as food: toxicity and technology. In *Chronic Cassava Toxicity*; Monograph IDRC-010e; Nestel, B., MacIntyre, R., Eds.; International Development Research Centre (Ottawa): London, U.K., 1973; pp 27–36.

(45) Balagopalan, C. Cassava utilization in food, feed and industry. In *Cassava: Biology, Production and Utilization*; Hillocks, R. J., Thresh, J. M., Bellotti, A. C., Eds.; CABI Publishing: Oxon, U.K., 2002; pp 301–318.

(46) Cumbana, A.; Mirione, E.; Cliff, J.; Bradbury, J. H. Reduction of cyanide content of cassava flour in Mozambique by the wetting method. *Food Chem.* **2007**, *101*, 894–897.

(47) Nhassico, D.; Muquingue, H.; Cliff, J.; Cumbana, A.; Bradbury, J. H. Rising African cassava production, diseases due to high cyanide intake and control measures. *J. Sci. Food Agric.* **2008**, *88*, 2043–2049.

(48) Bokanga, M.; Ekanayake, I. J.; Dixon, A. G. O.; Porto, M. C. M. Genotype-environment interactions for cyanogenic potential in cassava. *Acta Hortic.* **1994**, *375*, 131–139.

(49) Gleadow, R. M.; Woodrow, I. E. Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*. *Tree Physiol.* **2000**, *20*, 591–198.

(50) Bokanga, M.; Ekanayake, I. J.; Dixon, A. G. O.; Porto, M. C. M. Genotype-environment interactions for cyanogenic potential in cassava. *Acta Hortic.* **1994**, *375*, 131−139.

(51) Elsharkawy, M. A. Drought-tolerant cassava for Africa, Asia and Latin-America. *Bioscience* **1993**, *43*, 441−451.

(52) Okogbenin, E.; Ekanayake, I. J.; Porto, M. C. M. Genotypic variability in adaptation responses of selected clones of cassava to drought stress in the Sudan savanna zone of Nigeria. *J. Agron. Crop Sci.* **2003**, *189*, 376−389.

(53) Wright, G. C.; Hubick, K. T.; Farquhar, G. D. Discrimination in carbon isotopes of leaves correlates with water use efficiency of field-grown peanut cultivars. *Plant Physiol.* **1988**, *92*, 130−135.

(54) Condon, A. G.; Richards, R. A.; Rebetzke, G. J.; Farquhar, G. D. Breeding for high water-use efficiency. *J. Exp. Bot.* **2004**, *55*, 2447−2460.

(55) Rajabia, A.; Obera, E. S.; Griffiths, H. Genotypic variation for water use efficiency, carbon isotope discrimination, and potential surrogate measures in sugar beet. *Field Crops Res.* **2009**, *122*, 172−181.

(56) Li, C.; Wu, C.; Duan, B.; Korpelainen, H.; Luukkanen, O. Age-related nutrient content and carbon isotope composition in the leaves and branches of *Quercus aquifolioides* along an altitudinal gradient. *Global Planet. Change* **2009**, *23*, 1109−1121.

(57) Tsialtas, J. T.; Maslaris, N. Leaf carbon isotope discrimination relationships to element content in soil, roots and leaves of sugar beets grown under Mediterranean conditions. *Field Crops Res.* **2006**, *99*, 125−135.

(58) Masle, J.; Farquhar, G. D.; Wong, S. C. Transpiration ratio and plant mineral content are related among genotypes of a range of species. *Aust. J. Plant Physiol.* **1992**, *79*, 709−721.

(59) Merah, O.; Deleens, E.; Monneveux, P. Grain yield, carbon isotope discrimination. mineral and silicon content in durum wheat under different precipitation regimes. *Physiol. Plant.* **1999**, *107*, 387−394.

(60) Sparks, J. P.; Ehleringer, J. R. Leaf carbon isotope discrimination and nitrogen content for riparian trees along elevational transects. *Oecologia* **1997**, *109*, 362−367.

(61) Mahungu, N. M. Relationships between cyanogenic potential of cassava and other agronomic traits. *Acta Hort.* **1994**, *375*, 125−129.

(62) Cooke, R. D.; Howland, A. K.; Hahn, S. K. Screening cassava for low cyanide using an enzymatic assay. *Exp. Agric.* **1978**, *14*, 367−372.

(63) Forslund, K.; Jonsson, L. Cyanogenic glycosides and their metabolic enzymes in barley, in relation to nitrogen levels. *Physiol. Plant.* **1997**, *101*, 367−372.

(64) Busk, P. K.; Møller, B. L. Dhurrin synthesis in sorghum is regulated at the transcriptional level and induced by nitrogen fertilization in older plants. *Plant Physiol.* **2002**, *129*, 1222−1231.

(65) Wheeler, J. L.; Hedges, D. A.; Archer, K. A.; Hamilton, B. A. Effect of nitrogen, sulphur and phosphorus fertiliser on the production, mineral content and cyanide potential of forage sorghum. *Aust. J. Exp. Agric. Anim. Husb.* **1980**, *20*, 330−338.

(66) Gleadow, R. M.; Evans, J. R.; McCaffery, S.; Cavagnaro, T. R. Growth and nutritive value of cassava (*Manihot esculenta* Cranz.) are reduced when grown in elevated CO_2. *Plant Biol.* **2009**, *11*, 76−82.

(67) Howeler, R. H.; Cadavid, L. F. Accumulation and distribution of dry matter and nutrients during a 12-month growth cycle of cassava. *Field Crops Res.* **1983**, *7*, 123−129.

(68) Cadavid, L. F.; El-Sharkawy, M. A.; Acosta, A.; Sanchez, T. Long-term effects of mulch, fertilization and tillage on cassava grown in sandy soils in northern Colombia. *Field Crops Res.* **1998**, *57*, 45−56.

(69) Susan John, K.; Ravindran, C. S.; George, J. *Long Term Fertilizer Experiments: Three Decades Experience in Cassava*; Central Tuber Crops Research Institute: Kerala, India, 2005; p 83.

(70) Susan John, K.; Venugopal, V. K.; Manikantan Nair, M. Dry matter production, nutrient uptake, tuber quality and soil nutrient status as influenced by substitution of common salt for muriate of potash for cassava in an oxisol of Kerala. *J. Root Crops* **2007**, *33*, 20−25.

(71) Graham, C. J. Nonstructural carbohydate and prunasin composition of peach seedlings fertilized with different nitrogen sources and aluminium. *Sci. Hortic.* **2002**, *94*, 21−32.

(72) Briggs, M. A. Chemical defense production in *Lotus corniculatus* L. I. The effects of nitrogen source on growth, reproduction and defense. *Oecologia* **1990**, *83*, 27−31.

(73) Miller, R. E.; Gleadow, R. M.; Woodrow, I. E. Cyanogenesis in tropical *Prunus turneriana*: characterisation, variation and response to low light. *Funct. Plant Biol.* **2004**, *31*, 491−503.

(74) Webber, B. L.; Woodrow, I. E. Chemical and physical plant defence across multiple ontogenetic stages in a tropical rain forest understorey tree. *J. Ecol.* **2009**, *97*, 761−771.

(75) Simon, J.; Miller, R. E.; Woodrow, I. E. Variation in defence strategies in two species of the genus *Beilschmiedia* across nutrient and rainfall gradients. *Plant Biol.* **2007**, *9*, 152−157.

(76) Gleadow, R. M.; Woodrow, I. E. Defense chemistry of cyanogenic *Eucalyptus cladocalyx* seedlings is affected by water supply. *Tree Physiol.* **2002**, *22*, 939−945.

(77) Gleadow, R. M.; Foley, W. J.; Woodrow, I. E. Enhanced CO_2 alters the relationship between photosynthesis and defence in cyanogenic *Eucalyptus cladocalyx* F. Muell. *Plant Cell Environ.* **1998**, *21*, 12−22.

(78) Nowak, R. S.; Ellsworth, D. S.; Smith, S. D. Functional responses of plants to elevated atmospheric CO_2 − do photosynthetic and productivity data from FACE experiments support early predictions? *New Phytol.* **2004**, *162*, 253−280.

(79) Achidi, A. U.; Ajayi, O. A.; Maziya-Dixon, B.; Bokanga, M. The effect of processing on the nutrient content of cassava (*Manihot esculenta* Crantz) leaves. *J. Food Process. Preserv.* **2008**, *32*, 486−502.

(80) Gegios, A.; Amthor, R.; Maziya-Dixon, B.; Egesi, C.; Mallowa, S.; Nungo, R.; Gichuki, S.; Mbanaso, A.; Manary, M. J. Children consuming cassava as a staple food are at risk for inadequate zinc, iron, and vitamin A intake. *Plant Food Hum. Nutr.* **2010**, *65*, 64−70.

(81) Sayre, R.; Beeching, J. R.; Cahoon, E. B.; Egesi, C.; Fauquet, C.; Fellman, J.; Fregene, M.; Gruissem, W.; Mallowa, S.; Manary, M.; Maziya-Dixon, B.; Mbanaso, A.; Schachtman, D. P.; Siritunga, D.; Taylor, N.; Vanderschuren, H.; Zhang, P. The BioCassava Plus Program: biofortification of cassava for Sub-Saharan Africa. *Annu. Rev. Plant Biol.* **2011**, *62*, 251−272.

(82) Carretero, C. L.; Cantos, M.; Garcia, J. L.; Azcon, R.; Troncoso, A. Growth responses of micropropagated cassava clones as affected by *Glomus intraradices* colonization. *J. Plant Nutr.* **2009**, *32*, 261−273.

(83) Straker, C. J.; Hilditch, A. J.; Rey, M. E. C. Arbuscular mycorrhizal fungi associated with cassava (*Manihot esculenta* Crantz) in South Africa. *South Afr. J. Bot.* **2010**, *76*, 102−111.

(84) Smith, S. E.; Read, D. J. *Mycorrhizal Symbiosis*, 3rd ed.; Academic Press: Cambridge, U.K., 2008.

(85) Cardoso, I. M.; Kuyper, T. W. Mycorrhizas and tropical soil fertility. *Agric. Ecosyst. Environ.* **2006**, *116*, 72−84.

(86) McKey, D.; Cavagnaro, T. R.; Cliff, J.; Gleadow, R. M. Chemical ecology in coupled human and natural systems: people, manioc, multitrophic interactions and global change. *Chemoecology* **2010**, *20*, 109−133.

(87) Bradbury, J. H. Simple wetting method to reduce cyanogen content of cassava flour. *J. Food Compos. Anal.* **2006**, *19*, 388−393.

(88) Bradbury, J. H.; Denton, I. C. Rapid wetting method to reduce cyanogen content of cassava flour. *Food Chem.* **2010**, *121*, 591−594.

(89) Burns, A. E.; Bradbury, J. H.; Cavagnaro, T. R.; Gleadow, R. M. Total cyanide content of cassava food products in Australia. *J. Food Compos. Anal.* **2012**, *25*, 79−82.

(90) Bradbury, J. H.; Holloway, W. D. *Chemistry of Tropical Root Crops: Significance for Nutrition and Agriculture in the Pacific*; ACIAR: Canberra, Australia, 1988.

(91) Abhary, M.; Siritunga, D.; Stevens, G.; Taylor, N. J.; Fauquet, C. M. Transgenic biofortification of the starchy staple cassava (*Manihot esculenta*) generates a novel sink for protein. *PLoS One* **2011**, *6*.

(92) Chiwona-Karltun, L.; Mkumbira, J.; Saka, J.; Bovin, M.; Mahungu, N. M.; Rosling, H. The importance of being bitter − a

qualitative study on cassava cultivar preference in Malawi. *Ecol. Food Nutr.* **1998**, *37*, 219−245.

(93) Wilson, W. M.; Dufour, D. L. Why "bitter" cassava? Productivity of "bitter" and "sweet" cassava in a Tukanoan Indian settlement in the northwest Amazon. *Econ. Bot.* **2002**, *56*, 49−57.