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Rebecca Vandegeer, Rebecca E. Miller, Melissa Bain, Roslyn M. Gleadow and Timothy R. Cavagnaro

Drought adversely affects tuber development and nutritional quality of the staple crop cassava (Manihot esculenta Crantz)

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Title: Drought adversely affects tuber development and nutritional quality of the staple crop cassava (*Manihot esculenta* Crantz)

Authors: Vandegeer R[1], Miller RE[1,2], Bain M[1], Gleadow RM[1], Cavagnaro TR[1,2]

[1]School of Biological Sciences and [2]The Australian Centre for Biodiversity Monash University, Clayton, Victoria, Australia, 3800.

Corresponding author: timothy.cavagnaro@monash.edu

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Summary

Cassava is a staple for over 850 million people, but it is toxic unless properly processed. A monotonous cassava diet often coincides with outbreaks of diseases such as konzo, especially during droughts. The concentration of cyanogenic glucosides in young tubers was 4-fold higher when plants were water-stressed, but was lower following re-watering. We conclude that any expansion of cassava into new areas must be accompanied by knowledge of appropriate methods for detoxification, especially in areas increasing in aridity due to climate change.

Abstract

Cassava (*Manihot esculenta* Crantz) is the staple food source for over 850 million people worldwide. Cassava contains cyanogenic glucosides and can be toxic to humans, causing paralysing diseases such as konzo, and even death if not properly processed. Konzo epidemics are often associated with times of drought. This may be due to a greater reliance on cassava as it is drought tolerant, but it may also be due to an increase in cyanogenic glucosides. Episodic droughts are forecast to become more common in many cassava-growing regions. We therefore sought to quantify the effect of water-stress on both yield and cyanogenic glucoside concentration (CNc) in the developing tubers of cassava. Five month-old plants were grown in a glasshouse and either well-watered or droughted for 28 days. A subset of droughted plants was re-watered half way through the experiment. Droughted plants had 45% fewer leaves and lower tuber yield, by 83%, compared to well-watered plants. CNc was 2.9-fold higher in the young leaves of droughted plants, while CNc in tubers from droughted plants was 4-fold greater than in tubers from well-watered plants. Rewatered plants had a similar biomass to control plants, and lower CNc than droughted plants. These findings highlight the important link between food quality and episodic drought.

Introduction

Cassava (*Manihot esculenta* Crantz) is the sixth most important crop in terms of global annual production, and is the main staple crop of approximately 850 million people world-wide (FAOSTAT 2011). It is very hardy and can be grown under a wide range of environmental conditions (Burns *et al.* 2010). It is consumed widely in South America, Asia and the Pacific Islands, but is of particular importance in sub-Saharan Africa (Nhassico *et al.* 2008, FAO 2009, Montagnac *et al.* 2009). Although consumption is widespread, cassava can be toxic to humans because it contains the cyanogenic glucosides linamarin and lotaustralin, which break down to release toxic hydrogen cyanide (HCN) in sufficient concentrations to be toxic (Cliff *et al.* 1985, McKey *et al.* 2010, Nzwalo and Cliff 2011).

All parts of cassava are cyanogenic, but the concentrations are highest in the leaves and the periderm (or peel) of the tuberous root. The starchy parenchyma (or flesh) of the tuberous roots, the part most commonly eaten, is much less cyanogenic (Jørgensen *et al.* 2005; Montagnac *et al.* 2009). Despite their toxicity, the leaves of cassava are also eaten in some countries, including Madagascar and Mozambique (Cardoso *et al.* 2005), because they are higher in protein than the tuberous roots. If not properly processed, the consumption of cassava can directly cause serious illness or death. In addition to acute toxicity, a monotonous cassava diet is associated with chronic diseases such as konzo and tropical ataxia (Cliff *et al.* 1985). Konzo epidemics are more common during times of drought or when access to alternative food is limited by social or environmental factors (Ernesto *et al.* 2002, Nhassico *et al.* 2008; Nzwalo and Cliff 2011). The association between konzo epidemics and drought was first identified in 1980 when there was a major drought in the Nampula region of Mozambique. The increased incidence of konzo at this time was associated with higher urinary thiocyanate levels, indicating cyanide intoxication. The people were highly dependent on cassava during this drought, but the underlying reason for the link was unclear (Cliff *et al.* 1985). Later epidemics of konzo were also associated with a monotonous cassava diet (Cliff *et al.* 1994). Subsequent studies found that flour produced from cassava tubers in drought years in northern Mozambique contained, on average, three times as much cyanide compared with years when rain was adequate (Ernesto *et al.* 2002; Cardoso *et al.* 2005). This could either be a consequence of less water available for processing cassava to remove cyanogens,

or of increased toxicity of the cassava itself, or both (Santisopasri *et al.* 2001; Okogbenin *et al.* 2003; Nzwalo and Cliff 2011).

Water-stress is a reality in most rain-fed agricultural systems. Climate predictions for southern Africa forecast an increase in episodic droughts and evapo-transpiration (IPCC, 2007). Although there are many studies on the effect of drought on the yield and productivity of cassava (e.g. Baker at al. 1989; El-Sharkawy 2003; Alves and Setter 2000), there has been little research regarding the effect on cyanogenic glucosides and all of those have been field-based, with a wide range of other variables (Bokanga *et al.* 1994; Santisopasri *et al.* 2001; Okogbenin *et al.* 2003; El-Sharkaway 2003). El-Sharkaway (2003), for example, found that prolonged water-stress resulted in an increased concentration of cyanogenic glucosides in the tubers at harvest. Given that cassava is able to tolerate a wide range of growing conditions (Burns *et al.* 2010), it is not clear how stressed the plants actually were, and whether there were other differences in nutrient supply. Studies of other cyanogenic species have also found that water-stressed plants contain higher concentrations of cyanogenic glucosides, at least in the leaves (Nelson 1953; Gleadow and Woodrow 2002; Woodrow *et al.* 2002). The effect of drought on the concentration of cyanogenic glucosides in cassava has not, to our knowledge, been investigated under controlled conditions. Furthermore, it is also not known whether the cyanogenic glycoside content will change if re-watered after a period of drought, or whether the effects of water-stress on roots and leaves are similar or not.

Here we present results of a study in which we grew cassava under controlled conditions to determine the effect of drought and recovery from drought on plant growth and chemistry, independent of temperature or nutrient supply. Growth, biomass partitioning and chemical composition were measured, including concentrations of cyanogenic glucosides. Our hypothesis was that if cyanogenic glucosides are constitutive, then any increase in concentration resulting from water-stress will persist after re-watering. On the other hand, if cyanogenic glucosides are labile, as suggested by Møller (2010), then any increase in cyanogenic glucosides associated with drought will be transient and re-watered plants will have the same toxicity as plants that received water for the duration of the experiment.

Methods

Plant material and growing conditions

Twenty-four cassava plants (*Manihot esculenta* Crantz cv. MCol 1468) were propagated clonally in coarse sand from a single parent plant. Each cutting had at least two nodes and was ca. 50 mm in length. Cuttings arising from different parts of the parental stem were distributed evenly across all treatments to account for potential differences in growth due to cutting origin (Jørgensen *et al.* 2005). After sprouting, the cuttings were transferred to 140 mm diameter (1.3 L) plastic, free-draining pots, containing 0.9 kg of a commercial potting mix ('Potting mix', Richgro, Australia), and transferred to a glasshouse. One hundred days after planting, the plants were then transferred to 250 mm diameter (8 L) plastic, free-draining pots, containing 5.5 kg of a 50:50 mixture of commercial potting mix (as above) and washed, coarse river sand. This mixture, which is referred to as "soil" hereafter, provided a growth media which has uniform drainage characteristics, and allowed for ready extraction of roots at the time of harvest. To each pot, a 10 mm layer of small, white polystyrene beads was placed on the soil surface, to minimise evaporative water loss from the soil. All plants were grown in a glasshouse on the Clayton campus of Monash University, Australia (March–October 2010). Mean day/night temperature (measured at 10 min intervals) was 18.8 ± 0.20°C / 16.9 ± 0.03°C. Day length was extended to 18 h, beyond the normal photoperiod using sodium lamps (MK-1 Just-a-shade, Ablite Australia). Mean daily photon load was 495.1 ± 108.6 mol quanta m^{-2}. Plants were watered twice a week with tap water, and once a week with liquid nutrient solution ('THRIVE', Yates, Australia), from propagation through to the commencement of different watering regimes.

Drought treatments

This experiment included three experimental treatments. Plants were either droughted ('drought treatment', hereafter), droughted and then re-watered ('re-watered treatment', hereafter) or well-watered for the duration of the experiment ('control', hereafter). These treatments were applied in May 2010, 123 days after the transplanting (see above). The control treatment was established by watering plants to 100% of field capacity (FC; determined following Asghari and Cavagnaro, 2011) until the end of the experiment, 151 days after planting. The drought treatment was established following Khan *et al*. (2003) by withholding water from the plants (123 days after transplanting) until a soil moisture content of 25% of FC was achieved, and then maintaining the soil moisture content at 25% of FC until the end of the experiment (151 days after planting). The re-watered treatment was

established as for the droughted treatment (see above), except that 14 days after the drought treatment commenced (i.e. 137 days after transplanting), watering was resumed to achieve a soil moisture content that was 100% of FC until the time of harvest (151 days after planting). Plants in all treatments were weighed on a daily basis to monitor soil moisture content (Fig. 1). Each of the watering treatments was replicated eight times; although two replicates from the control treatment were excluded because one failed to establish from the cutting, and the other developed multiple stems, whereas all other plants had a single stem.

Sampling

Leaf samples were taken during the drought phase (starting 123 days after transplanting – see above) of the experiment to monitor changes in leaf chemistry (cyanogenic glucoside concentrations (CNc) see below), as follows. Two leaf disks (5 mm diameter) were excised from the middle of the centre lobe of the third fully-expanded leaf of each plant (avoiding the midrib), using a hole-punch, at midday on days 0, 14, and 28 of the drought phase of the experiment. At the same time the leaf disks were taken on days 14 and 28 of the drought phase of the experiment, half of the third fully-expanded leaf from each plant was removed, weighed and placed in a Petri dish of distilled water for 24 h, re-weighed to determine hydrated weight, and then dried at 60°C for 48 h for dry weight determination. Leaf relative water content was then calculated by dividing the difference between fresh weight and dry weight by the difference between hydrated weight and dry weight (Blomstedt *et al.* 1998). As a measure of plant physiological stress, Fv/Fm, the ratio of variable to maximum chlorophyll *a* fluorescence, was measured for the third fully expanded leaf using a chlorophyll fluorometer on day 28 (WALZ, PAM-210).

Harvest

Plants were destructively harvested 28 days after imposition of watering treatments (i.e. 151 days after planting). For measurement of leaf chemistry (see below), two leaf disks (5 mm diameter) were excised from the third fully expanded leaf, which had expanded during the drought phase of the experiment. All leaves were then removed from the plants, weighed, and leaf areas measured. The plant stems were cut at the soil surface and weighed. The roots were then carefully washed from the soil with water and separated into fine roots and tuberous

roots (>5 mm diameter). The tuberous roots were further separated into the outer pericarp layer (referred to as 'peel', hereafter) and the inner flesh layers (referred to as 'flesh', hereafter). A sample of tuber flesh (1 cm^3) from the largest tuber for each plant was excised from the widest point of the tuberous root, weighed and analysed chemically. For consistency, the largest tuber was selected, as this is the tuber that is most likely to be eaten by consumers. All remaining plant material was dried at 60°C for 72 h, weighed, and ground to a fine powder for later analysis.

Plant chemical analysis: $\delta^{13}C$ and cyanogenic glucosides

Carbon isotope discrimination ($\delta^{13}C$, a measure of water-stress) was determined on dried and ground samples of the third fully expanded leaf of each plant at harvest, with an on-line mass spectrometer (Isochrom, VG Microtech, UK) after combustion in an elemental analyser (Carlo Erba 1110, ThermoQuest, Australia). Cyanogenic glucosides were measured as cyanide (CNc) evolved from fresh leaf disks and root samples (ca. 10 mg; see above) incubated in a 0.1 M phosphate buffer (pH 6.5) in sealed vials (Gleadow *et al.* 2011). Cyanide captured in an internal well containing 1 M NaOH was determined using a colorimetric assay, with NaCN as a standard (Woodrow *et al.* 2002). Tissue was then rinsed and dried for 24 h at 60°C and CNc determined on a dry weight basis.

Calculations and data analysis

Harvest index was calculated by dividing the total tuber dry weight by the total plant dry weight. The original stem cuttings used to establish the clones were excluded from biomass determinations. Growth characteristics and chemical concentrations were analysed by ANOVA (Zar 2010). Plant biomass plotted against total plant cyanide was analysed by regression analysis. Log transformations were performed where necessary and Tukey's tests (P = 0.05) were used post-hoc to compare significantly different means. Data analysis was performed in R (R development core team 2009) and JMP 9 (SAS Institute Inc.).

Results

Biomass, morphology and physiology

The RWC of leaves (Table 1) 14 and 28 days after the commencement of the drought experiment was significantly lower in plants in the droughted treatment (mean RWC = 86.0% throughout the experiment) than those in the control treatment (mean RWC = 90.5% throughout the experiment). The final leaf RWC of plants that were initially droughted and then re-watered was similar to that of the control plants. Consistent with this, $\delta^{13}C$ values for leaves that had expanded during the drought phase of the experiment (Table 1), were significantly higher (i.e. less negative) in the droughted treatment than the control and re-watered treatments. No differences in the ratio of leaf FW to DW, or in the chlorophyll fluorescence parameter Fv/Fm, were detected between any of the treatments at the final harvest either (data not shown).

At the final harvest, the total biomass (DW) of droughted plants was almost half that of control plants, with re-watered plants intermediate (Table 2). Droughted plants had significantly fewer leaves than the control and re-watered treatments, and lower overall shoot biomass than plants in the control treatment; however, no significant difference in specific leaf area or root: shoot ratio was detected between treatments (Table 2). While fine root biomass of droughted plants was similar to re-watered and control plants, droughted plants had fewer tuberous roots and a lower overall tuberous root biomass (Table 2). Further, two droughted plants did not produce any tubers at all. As a result, the harvest index (i.e. tuberous root mass as a proportion of total biomass) of droughted plants was less than the harvest index of both control and re-watered plants (Table 2).

The number of leaves per plant at the time of harvest in the drought treatment was half that of those in the (well-watered) control treatment (Table 2). The number of leaves on re-watered plants was marginally, albeit not significantly, less than in the control treatment, and significantly higher than on plants in the drought treatment. The reduction in leaf number in the drought treatment was due to both an increase in the number of fallen leaves, and fewer new leaves developing during the drought phase of the experiment (data not shown). Across all leaves, mean leaf size of droughted plants was similar to control plants, but the mean leaf

size of re-watered plants was 41% higher compared to that of droughted plants (P=0.0303; data not shown).

Plant chemical composition

In order to determine the impact of drought on the distribution of cyanide within the plant, cyanide concentration (CNc) of the third fully expanded leaf of all plants was measured over time (0, 14 and 28 days) following initiation of the watering treatments. At day 0 there was no significant difference in CNc between plants assigned to different watering treatments ($F_{2,19}$=1.73, P=0.204, Fig. 2). Fourteen days after the imposition of watering treatments, foliar CNc of all droughted plants was more than double that of control plants ($F_{2,22}$=6.76, P=0.0057). At day 28, leaf CNc was significantly higher in droughted plants than in both re-watered and control plants ($F_{2,18}$=8.98, P=0.002), and was 189% higher than the initial (day 0) foliar CNc of droughted plants ($F_{1,13}$=16.8; P=0.001). This difference in CNc between treatments is not a consequence of differences in leaf size, as the mean leaf area of the third fully expanded leaf at harvest was similar (data not shown).

At the time of harvest, the CNc of dried flesh of the largest tuber was significantly higher in the droughted plants, than in plants from the re-watered and control treatments (Table 2). Because, on average, individual tubers in the droughted treatment were also significantly smaller than in control or re-watered treatments (data not shown), the relationship between tuber size (i.e. developmental stage) and tuber CNc was further investigated. The CNc of a subset of tubers within a smaller size class (<500 mg DW) was compared. This size class was selected based on a clear break in the distribution of tuber size, and also included the majority of tubers from droughted plants, with n=14-18 tubers from each treatment. Further, mean tuber size did not differ between treatments within this size class ($F_{2,48}$=2.08, P=0.14; data not shown). Despite similar mean tuber size, tuber CNc was significantly higher in small tubers from the droughted treatment, compared to those from the control treatment, with tubers from the re-watered plants having intermediate CNc, similar to both the droughted and control treatments (Table 2).

Discussion

Periodic early drought affected growth and CNc in the edible portions of the staple food crop cassava. Re-watering of droughted plants resulted in a recovery of the plants in terms of water content and cyanide concentration, and to a lesser extent, plant biomass. Together these results indicate that early drought can have a significant effect on the growth and nutritive value of cassava, but that cassava has some capacity to recover from an early drought of short duration. Results are discussed in the context of the physiological response of cassava to drought, and the potential consequences for growers and consumers of this important staple crop.

The droughting of cassava plants resulted in a reduction in leaf relative water content (RWC) and an increase in leaf $\delta^{13}C$ values. Interestingly, Fv/Fm (of the third fully expanded leaf), a measure of plant physiological stress (Maxwell and Johnson 2000), did not change with watering regime. This is consistent with the observation that following initiation of the drought treatment, the plants dropped leaves (reduced leaf number), and those leaves that were retained showed no clear indication of water stress (Fv/Fm or wilting) and had greater water use efficiency ($\delta^{13}C$). Our experiment was conducted using temperatures at the lower end of the range at which cassava is grown. Cassava is grown, for example, up to 1800m elevation in east Africa (Bokanga *et al*. 1994) and can tolerate temperatures as low as 10 °C. It is likely that higher temperatures would exacerbate the effect of drought and further controlled studies of the interactive effects of drought and temperature on CNc are warranted. Earlier studies have shown that cassava can decrease water loss through closing its stomata, which are very sensitive to changes in VPD and soil moisture (Setter and Fregene 2007), and decreasing leaf area through arrested development and abscission (Conner *et al.* 1981; Alves and Setter 2000; Burns *et al.* 2010). Our data also suggest that following drought, cassava would be able to quickly resume growth when conditions become more favourable. Such rapid recovery in growth and leaf canopy has been observed by others (e.g. El-Sharkawy 1993; Connor *et al.* 1981; Baker *et al* 1989; El-Sharkawy 1993).

The large reduction in yield can be largely attributed to the loss of photosynthetic area, as has been observed previously (Baker *et al.* 1989; Setter and Fregene 2007). Although re-watering of the plants resulted in a recovery of total plant biomass, the final tuber biomass (the main

edible part of the plants) of the re-watered plants was less than that of the well-watered control plants. The timing of the period of water stress (e.g. during tuber filling or initiation period) and the cultivar of cassava seem to influence recovery and the degree of compensatory growth (Conner *et al.* 1981; Santisopasri *et al.* 2001; El-Sharkawy 1993, 2003). Baker *et al.* (1989) found that there was an even greater impact on tuber yield when water was limited towards the latter part of the growing season. Here we focused on the early stages of tuber development, and stress that longer-term effects of periodic drought on plant growth also need to be taken into consideration.

We found that leaf and tuber CNc were higher in droughted plants compared to well-watered plants. Furthermore, irrespective of the effects of drought on tuber size and development, the CNc of small tubers was higher under drought. Similarly, increases in leaf CNc in droughted plants observed here were not a consequence of differences in leaf size. The tuber results are consistent with those of Santisopasri *et al.* (2001) who found that the CNc of tuberous roots grown in the field was highest towards the end of the drought period but lowest at the beginning of the drought period (after the rainy period). This increase in CNc with drought is also consistent with findings for a diverse range of other species such as *Sorghum bicolor* (L.) Moench (Nelson 1953) and *Eucalyptus cladocalyx* F. Muell. (Gleadow and Woodrow 2002). Cyanogenic glucosides are both turned over and transported throughout the cassava plant (Møller 2010). Selmar (1994) and more recently, Siritunga and Sayre (2004) and Jørgensen *et al.* (2005) found that cyanogenic glucosides in cassava are synthesised almost exclusively in the leaves, and then transported to the roots for storage. High levels of leaf loss under drought, as observed in our study, may cause resources (including the remaining cyanide) to be drawn back from the senescing leaves and transported to other parts of the plant, such as the younger, more vulnerable leaves, and the tuberous storage roots (Munne-Bosch and Alegre 2004). Importantly, the tuber yield of re-watered plants was similar to the control plants and tuber CNc was less than in droughted plants. This again points to cassava having a highly plastic response to episodic drought, both in terms of growth and chemical composition.

The findings presented here provide a better understanding of the response of cassava to short episodes of early drought followed by water availability, which is common in natural

environments. The increased incidence of konzo during times of drought may be explained by the increased CNc in the plants, along with an increased reliance upon cassava (due to the failure of other less drought tolerant crops), and decreased availability of water for the detoxification of cassava foodstuffs. The findings of this study are relevant to efforts promoting cassava as a suitable crop in areas likely to become drier with climate change (El-Sharkawy 2003; IPCC 2007; McKey *et al.* 2010). We contend that any expansion of cassava must be accompanied by development activities that help to ensure that growers of cassava are aware of the need for, and appropriate methods to, detoxify cassava (Nhassico *et al.* 2008; Bradbury and Denton 2010), especially in times of drought.

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Table 1. Relative water content and carbon isotope signatures ($\delta^{13}C$) of third fully expanded leaf of cassava grown under drought, re-watered and well-watered (control) treatments. Relative water content (RWC) was measured on days 14 and 28 and carbon isotope signature ($\delta^{13}C$) at day 28. Re-watered plants were droughted for 14 days and then watered to field capacity until harvest. Means (± SE) with different letters are significantly different at the $P<0.05$ level (n=8 except for (well-watered) control plants where n=6).

	Droughted	**Re-watered**	**Control**
Relative water content (%)			
Day 14	86.0 ± 1.5^{a}	86.9 ± 1.1^{ab}	91.0 ± 0.7^{b}
Day 28	85.9 ± 1.3^{a}	88.8 ± 0.5^{ab}	90.4 ± 0.5^{b}
Leaf $\delta^{13}C$ (‰)			
Day 28	-22.2 ± 0.2^{a}	-24.4 ± 0.3^{b}	-24.1 ± 0.4^{b}

Table 2. Growth characteristics and tuber cyanogenic glucoside concentration at harvest of cassava in droughted, re-watered and well-watered (control) treatments. Re-watered plants were watered to field capacity on day 14. Means (± SE) with the same letter are not significantly different at the $P<0.05$ level (n=8 except for the (well-watered) control treatment where n=6).

	Drought	Re-watered	Control
Plant growth characteristics			
[2]Total plant (g dw)	14.5 ± 2.2 [a]	23.7 ± 2.2 [b]	29.3 ± 3.8[b]
[2]Aboveground biomass (g dw)	7.0 ± 1.5 [a]	14.0 ± 1.0 [ab]	16.1 ± 1.1[b]
[1]Leaf number	8.0 ± 1.8 [a]	13.4 ± 0.8 [b]	15.7 ± 1.4[b]
[3]Specific leaf area (cm^2 g^{-1})	85.0 ± 13.6 [a]	68.1 ± 1.3 [a]	70.0 ± 1.1[a]
[4]Number of tubers per plant	2.6 ± 0.7 [a]	3.8 ± 0.5 [ab]	5.8 ± 0.7[b]
Total tuber mass (g dw)	1.4 ± 0.7 [a]	5.1 ± 1.4 [ab]	8.3 ± 2.1[b]
Fine roots (g dw)	6.1 ± 1.3 [a]	4.6 ± 0.5 [a]	4.9 ± 0.8[a]
Root: shoot	1.4 ± 0.3 [a]	0.7 ± 0.1 [a]	0.8 ± 0.1[a]
Harvest index (%)	7.6 ± 2.7 [a]	19.2 ± 3.9 [ab]	26.3 ± 3.4[b]
Tuber CNc (mg g^{-1} dw)			
Largest tuber flesh	1.24 ± 0.22 [a]	0.50 ± 0.19[b]	0.29 ± 0.20[b]
[5]Small tuber flesh	2.26 ± 0.44 [a]	1.48 ± 0.33[ab]	0.85 ± 0.16[b]

[1]Leaf number = number of attached leaves at harvest; [2]Unattached leaves at harvest are not included; [3]SLA was measured on all attached leaves at harvest; [4]Tubers are defined as roots with a diameter >5 mm; [5]The CNc of a subset of tubers within a smaller size class (<500 mg DW) was compared (see text for details).

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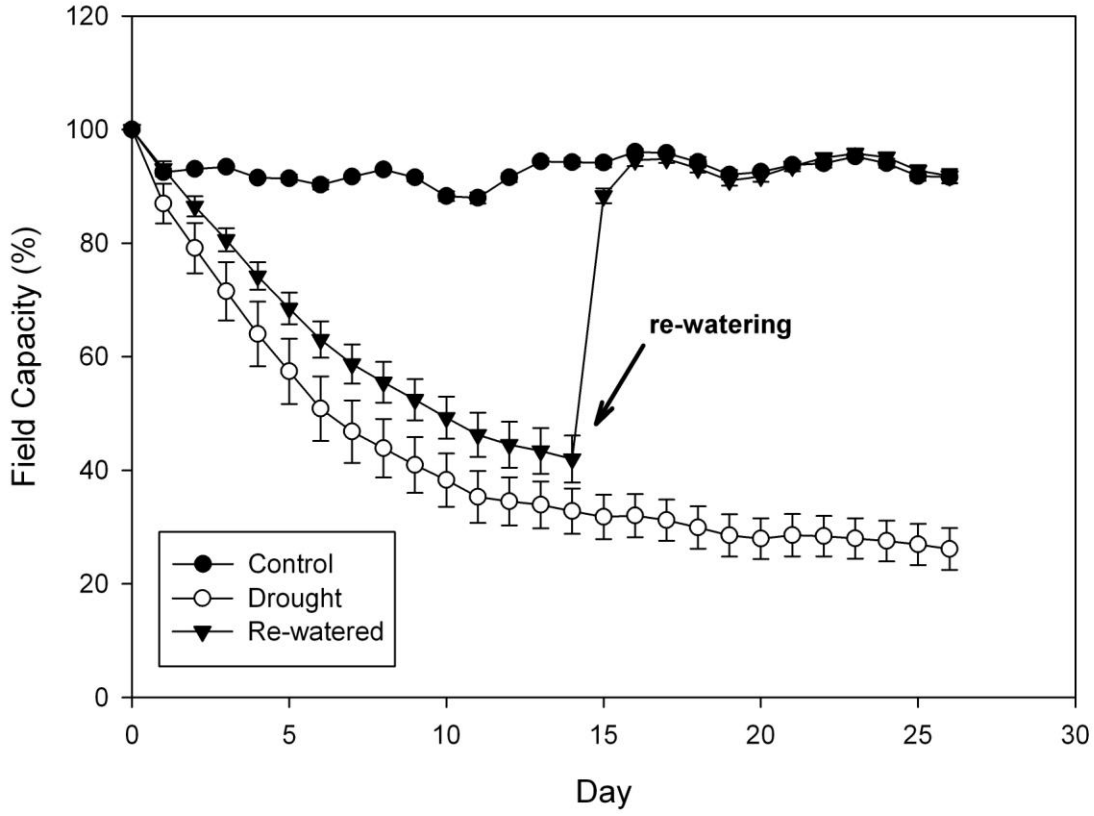

443

444 **Fig. 1.** Soil moisture content (% field capacity) under drought (open circles), re-watered
445 (black triangles) and well-watered (control, black circles) conditions. Values are Means (±
446 SE) of n=8 pots except for the (well-watered) control treatment where n=6. Re-watered plants
447 were watered to 100% field capacity on day 14, as indicated by the arrow.

448

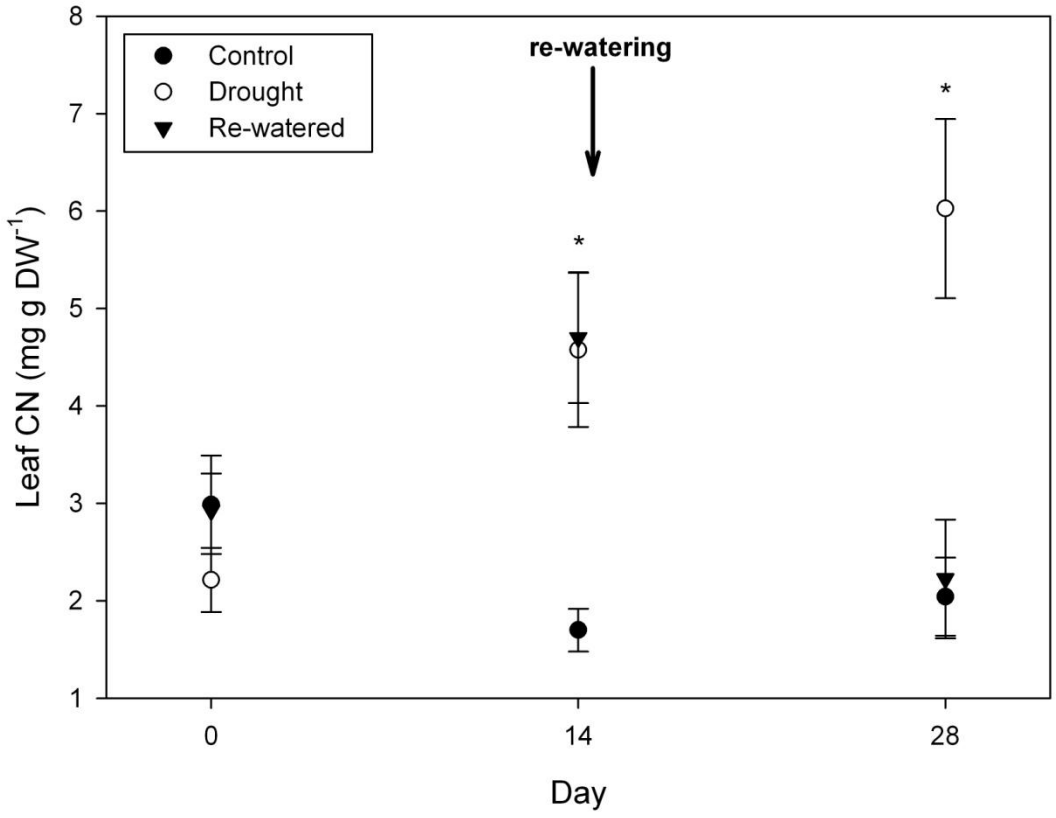

Fig. 2. Cyanide concentration (CNc, mg CN g^{-1} dry weight) of the third fully expanded leaf of cassava grown in drought (open circles), re-watered (black triangles) and well-watered (control, black circles) treatments for 28 days (±SE, n=6-8). Re-watered plants were watered to 100% field capacity on day 14, after cyanide sampling, as indicated by the arrow. * indicate significant differences between treatments at $P<0.05$ at each time point.