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Biochemical and secondary metabolites changes under moisture and temperature stress in cassava (*Manihot esculenta* Crantz)

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Cassava's (*Manihot esculenta* Crantz) importance as a food security crop in Sub Saharan Africa is enhanced by its special traits such as tolerance to drought and high yields under drought stress. However, full understanding of tolerance mechanisms under hydrothermal stress in cassava is a key in developing highly tolerant varieties with increased yield. In our study, the effects of low soil moisture and increased temperature on cassava physiology were investigated. Twenty (20) cassava varieties were evaluated in a Randomized Complete Block Design in western Uganda. Hydrothermal stress was described as a period of no rainfall for a period of eight weeks leading to low soil moisture (contents between 28 to 35%) and average daily temperatures of ≥35°C. The average daily relative humidity during this period was considerably low (≤40%) further complementing already enhanced stress conditions. As such, the contents of important biochemicals and secondary metabolites in the plants were altered in a bid to counteract the effects of stress. Significant differences occurred in accumulation of main biochemicals such as soluble proteins ($P<0.05$), free reducing sugars ($P<0.05$) and bound reducing sugars ($P<0.05$) while reductions in the total starch yield by 70 to 100% of the original composition before stress were observed in all the test varieties. Changes in pigment properties were also observed with a decrease in the total carotenoid content (~65%) and chlorophyll a (*Chla*) (~40%) but no significant changes were observed for chlorophyll b (*Chlb*). Secondary metabolites such as phenolics and tannins too depicted varied but non-significant changes and they existed in low quantities. There were also significant changes in the phenotype (foliar portion of the plant) with at least two mechanisms of tolerance identified. The study showed the importance of carbohydrate and nitrogen cycle related metabolites in mediating tolerance in cassava by affecting their phenotypic expression in the plant.

Key words: Hydrothermal stress, bio-chemicals, pigments, secondary metabolites, cassava.

INTRODUCTION

Changing and unpredictable climate patterns have resulted into crop losses leading to food insecurity and poverty in a number of African economies. One of the solutions to this changing climate is the use of improved

crop varieties with tolerance to drought and ability to give a decent harvest despite the unexpected changes in climate (Sagoe, 2006). Cassava (*Manihot esculenta* Crantz) is one of such crops being the third most important source of calories in the tropics (FAO, 2010) and depicting various tolerance mechanisms to hydro-thermal stresses. Due to its versatile nature, it has been referred to as the "drought, war and famine crop" to much of the countries in sub-Saharan Africa, Asia and South America (Pearce, 2007) where it is already a major staple crop for a number of people (Okgbenin et al., 2010). It therefore has the potential of being considered as part of the solution to improving food security being experienced as a result of changes in rainfall and temperature patterns occurring in various parts of the world (Liu et al., 2008). It can grow on relatively poor soils, is easily propagated, requires little cultivation and can tolerate periodic incidences of drought (Hillocks, 2002). These traits increase its versatility in production and need to further understand their mechanisms so as to develop superior varieties adaptable to the changing conditions.

Alves and Setter (2000) have reported a number of traits and responses including those dependent on morphological, biochemical and physiological behavior that contribute to the resilience of cassava to moisture stress. These induced responses are due to activation of various metabolic pathways resulting into re-establishment of cellular homeostasis as well as structural protection of membranes (Lokko et al., 2007). They are expressed in form of biochemical manifestation like increased enzyme activity and levels of secondary metabolites such as phenolics and tannins, osmotically active solutes such as proline, antioxidant enzymes such as catalase and peroxidase, hormones such as absiscic acid and pigments such as chlorophylls and carotenoids. As a result, the cassava plant is enabled not only to recover after the stress (Okogbenin et al., 2010) but also to regain capacity to restore its normal metabolic activities in a shorter time and produce decent yield.

Owing to its metabolic efficiency under marginal conditions, cassava produces more energy per unit area than other crops under conditions of water stress (El-Sharkawy, 1993). Consequently, the growing of cassava in conditions of minimal rainfall has flourished. It has also been realized that the principal mechanisms that may control tolerance to drought in cassava include its sensitivity and response to changes in atmospheric humidity and soil water status (Fregene and Setter, 2007). Such mechanisms include among others tight regulation of stomatal opening and the ability to retain photosynthetic activity under prolonged water stress. In addition, the crop has a deep root system which enables it to reach water from lower soil layers under extended periods of hydro-stress. Some of the other water conservation mechanisms in cassava include reducing light interception, reducing leaf canopy and size, leaf fall and heliotropic responses (Alves and Setter, 2004). These measures in addition to responses at biochemical level such as increased activity of growth regulators such as absiscic acid and proteins, both regulatory and enzymatic allow cassava to tolerate a range of hydrothermal related stresses.

In as much as the drought tolerance mechanisms in cassava have been partly elucidated, cassava variety specific differences have been observed with a more mixed complex set of mechanisms being expressed in different cassava varieties (Okogbenin et al., 2013). This makes selection for drought tolerant cassava varieties difficult as it would require additional information concerning the levels of tolerance and how these affect the final yield of the plant. Thus, it is important to characterize the various mechanisms displayed by cassava and develop tools that will allow selections to be easily made under certain specific mechanisms. In addition, most of the studies have been focused on understanding the plants reaction to physical stress such as moisture stress (Turyagyenda et al., 2013, Utsumi et al., 2012), moisture and cold stress (Zhang et al., 2010) and stress due to low soil water status (El-Sharkawy, 2007) under controlled conditions that are different from field conditions. There is, therefore, need to characterize the level and type of tolerance under field stress to allow for specific selections in particular environments. In this study, biochemical properties of nitrogen and carbon metabolic pathways and secondary metabolites from the products of these pathways were used to confirm selections for drought tolerant cassava varieties grown under field conditions. The differences in the levels of individual biochemicals and secondary metabolites were used to elucidate traits governing tolerance. These were important in the understanding of the differences in cassava varieties within a certain group that display different phenotypic mechanisms.

MATERIALS AND METHODS

Variety selection and establishment of experimental plots

Twenty varieties of cassava were selected based on known parameters of dry matter content, resistance to Cassava Mosaic Disease (CMD) and farmer preference and established in a Randomized Complete Block Design (RCBD) in Kasese, Western Uganda. The trial consisted of two experimental and two control blocks in $81M^2$ plots, with up to 81 plants per plot. The weather and plant response to available conditions were monitored and changes in main metabolites such as free reducing sugars (mainly as glucose), carbohydrates, chlorophylls and secondary metabolites

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varieties at 6-8 weeks post stress (peak stress)

Plate 1. Contrasting phenotypes dependent on physiological mechanism for tolerance to drought at 8 weeks post critical stress. **A.** Stay green variety. **B.** Susceptible variety. **C.** Early recovering variety.

such as cyanide, phenolics and tannins were recorded. Weather, soil and water characteristics were also recorded during the trial period using the location specific weather station with capacity for determination of precipitation, moisture and temperature characteristics. This allowed the determination of a critical hydrothermal stress period in which samples were picked on a biweekly basis. To maintain their metabolic state, picked samples were preserved in liquid nitrogen and transported to the laboratory at the National Crops Resources Research Institute in Namulonge, where they were stored at -80°C. The samples were used to study the relationship between metabolites and physiological state of the plant.

Determination of changes in primary metabolites (carbohydrate and protein contents)

The changes in the yield and level of carbohydrates were assessed by determining starch and reducing sugar contents before and during the hydrothermal stress period. This was meant to determine the effect of hydrothermal stress on the level of stored carbohydrate as the stress time increased. Starch yield was a measure of total extractable starch from the root portion (100 g) as a percentage of total wet mass (Nuwamanya et al., 2011). The starch content was determined after ethanolic extraction of free and bound reducing sugars from 0.1 g starch. Enzymatic hydrolysis of the pure starch (Nuwamanya et al., 2011) and determination of the subsequent released reducing sugars were done (Dubois et al., 1956). Free reducing sugars were taken to be the water extractable sugars at 30°C. Bound reducing sugars were taken to be the hot ethanol extractable sugars at 70°C for 5 min minus the free reducing sugars. Total leaf protein was determined under different experimental conditions using the Bradford assay after extraction using phosphate buffered saline (Hajiboland and Amirazad 2010).

Determination of pigment concentrations and secondary metabolites

Quantitative measurements for chlorophyll a (*chla),* chlorophyll b *(chlb)* and carotenoids were determined spectrophotometrically by taking their absorbencies at the following wavelengths respectively; 662, 644 and 445 nm (Wettshtein, 1957). Total leaf phenolics were extracted from 1 cm diameter leaf disc cut out of the 5th fully expanded leaf which was placed in 1.4 mL of a methanol:HCl solution (99:1 v/v) and allowed to extract for 48 h at -20°C (Mazza et al., 2000). Absorbance of extracts was read in a spectrophotometer at 725 nm (Dai and Mumper, 2010). The cyanogenic content of the fresh peels and the peeled cassava root was determined after an initial extraction for 3 min of 10 g material in 0.1 M phosphoric acid by hydrolysis followed by reaction with chloramine-T pyridine barbituric acid (Konig Reaction) as developed by Bradbury et al. (1991). The cyanide content of the peelings was also determined in the same way except that the extraction was carried out for 10 min to allow for complete extraction at extended times.

Data handling and results analysis

The data collected was analyzed using GenStat Discovery Edition (2012). Mean values for each cassava variety/accession were recorded and relationships between different parameters were determined. Microsoft excel software was used to study the trends in the different properties of the plant studied. Trends in the subsequent moisture, rain and related weather data were also determined and related to the observed changes in plant phenol-type. Selections for drought tolerance were confirmed by chloro-phyll content, accumulation of sugars and loss of carbohydrates, accumulation or loss of proteins and ability to recover earlier.

RESULTS

Based on observed phenotypic characteristics, varieties were ranked according to their response as shown in Plate 1. The groupings included varieties that maintained a moderately high Leaf Area Index (LAI) during hydrothermal stress or stay green varieties (SGV) (Plate 1A). These included varieties such as NASE 2, NASE 3, 0686, MH/0067 and the local variety Magana. However, some varieties completely lost leaves as stress progressed and even the remaining leaves during stress were dechloropyllated and yellowed signifying losses in chlorophyll and related pigments hence little or no capacity to photosynthesize. These varieties showed little or no capacity

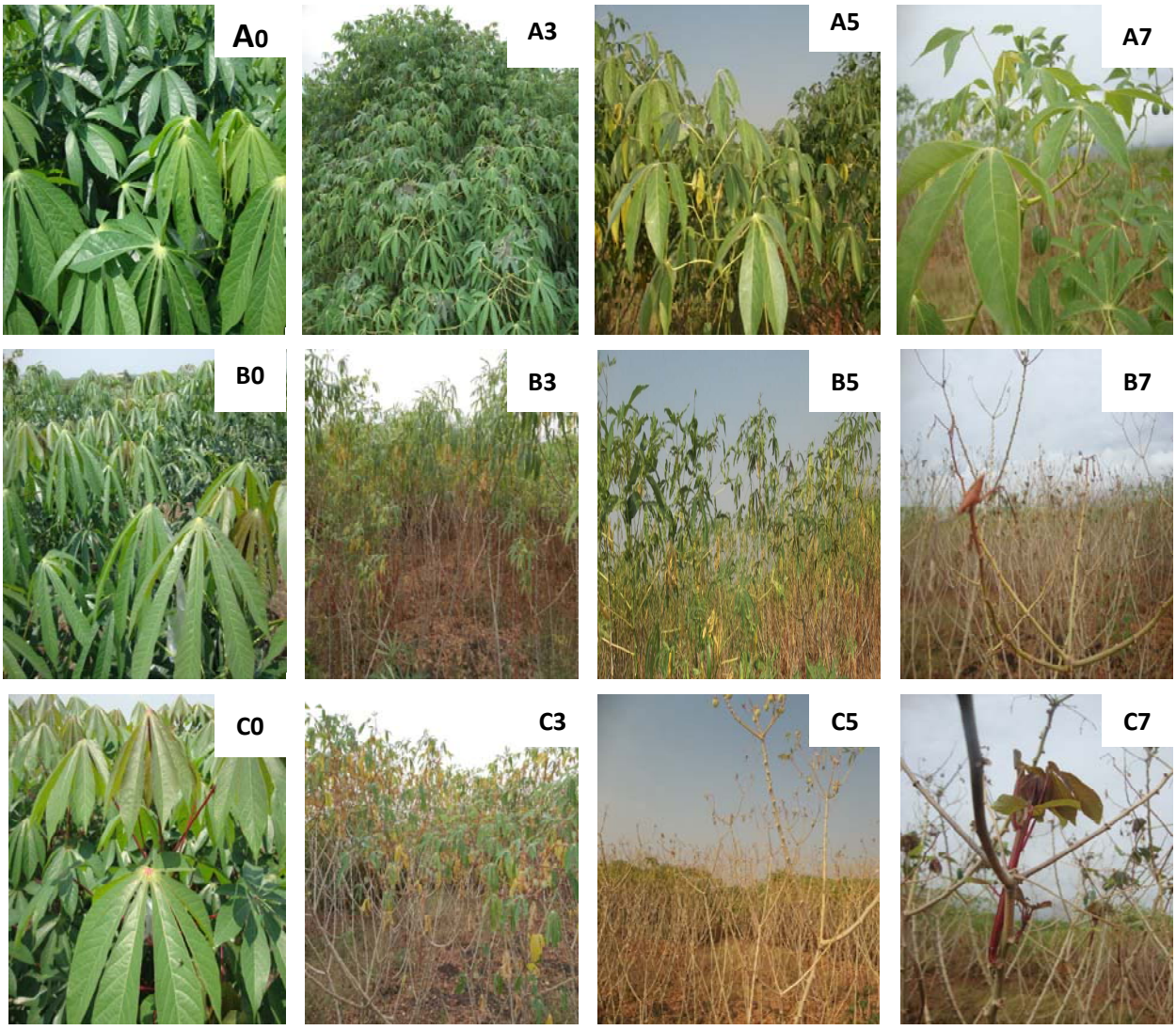

Plate 2. Physiological differences in the stay green (A), susceptible (B) and early recovering (C) varieties pre-stress (0), 3 weeks post stress (3), 5 weeks post stress (5) and 7weeks post stress (7).

to recover easily after stress and they were labeled susceptible varieties (SV) (Plate 1B). They included varieties such as NASE 1 and the local variety Rugogoma and Mercury. The other grouping included those that lost all their leaves immediately after onset of stress only to recover immediately with increase in relative humidity or early recovering varieties (ERV) (Plate 1C).

They included NASE 16, NASE 19 and Bukalasa. Some varieties had both mechanisms but were not very pronounced in each case. The variety groupings described were based on phenotypic observations during stress period (Plate 2).

Defining the critical hydrothermal stress period and selection of tolerant varieties

The results for the average temperature, relative humidity, and related weather conditions during the critical field stress period are presented in Figure 1. The average weather conditions under critical hydrothermal stress were defined by undertaking hourly weather measurements during the stress period. There was no rain during this period with 0 mm of rain received across the two month period. This coincided with reductions in relative humidity by more than 25% with consequent reductions in the dew point up to 8°C. In addition day and night temperatures increased significantly with day temperatures increasing by up to 5°C from an average of 30 to about 35°C while night temperatures increased by 3°C from an average of 18 to 20°C up to 23°C. The increments in temperature resulted into increments in the heat index by about 4 points (Figure 1). Daily average temperatures were high and ranged from 30.7 to 34.9°C during critical stress period compared to the normal averages

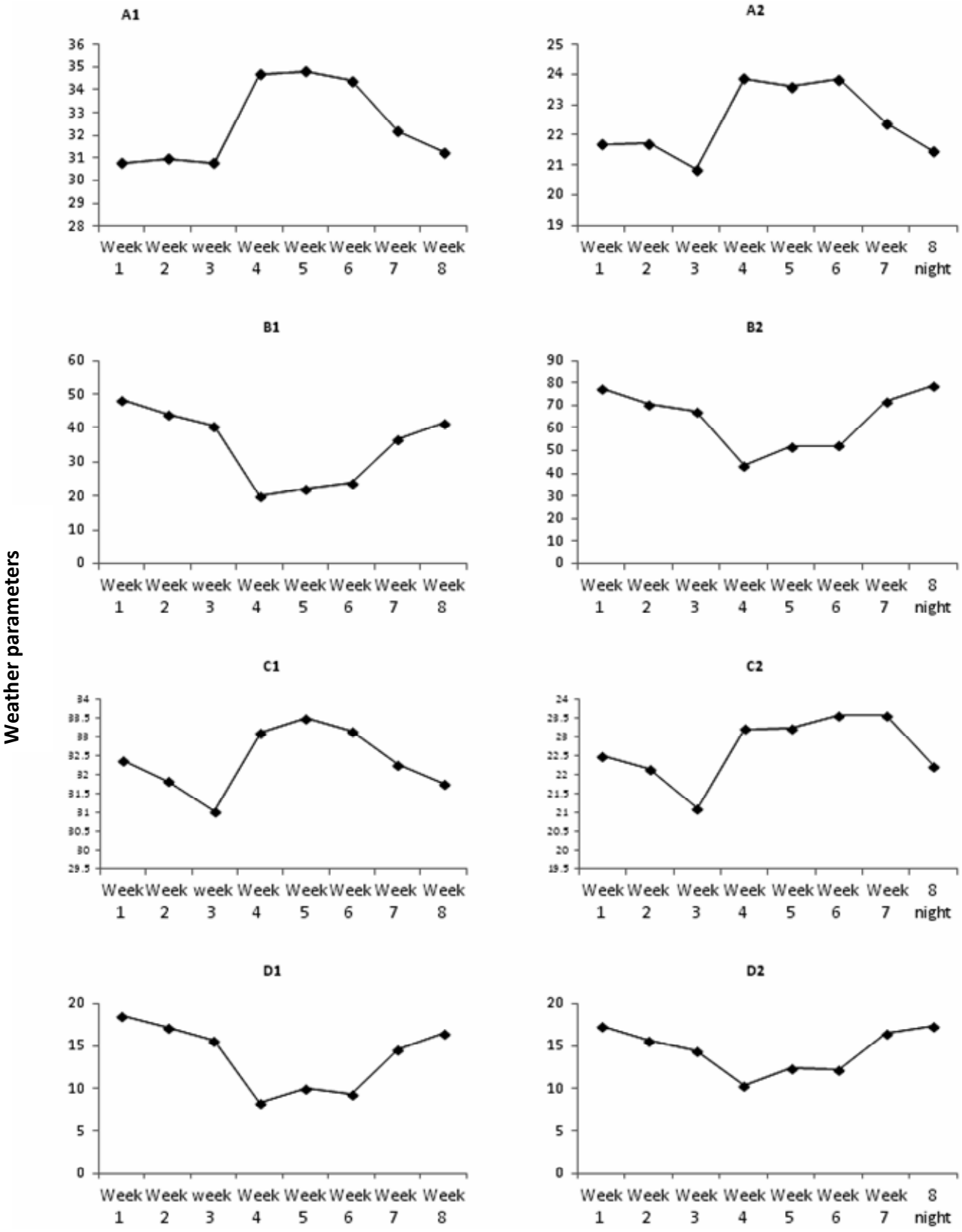

Figure 1. Average weekly changes in weather conditions during the critical hydrothermal stress period for both day and night conditions. A1=day temperatures; A2=night temperatures; B1=day relative humidity; B2=Night relative humidity; C1=Day heat index; C2=night heat index; D1=day dew point; D2=night dew point.

of 28.5 to 30.7°C. Week three during critical stress, the temperature increased from 30.5 to 34.9°C and stayed at an average of 34.5°C for three weeks dropping to 31.0°C at the end of the stress period (Figure 1A1). Night temperatures ranged from 20.5 to 23.8°C (Figure 1A2) up from an average of 16 to 18°C during normal conditions. Relative humidity during the day dropped significantly from about 78 to 85% during normal conditions to 22 to 50% in the stress period. The drop followed the same pattern as the temperature (Figure 1B1). At night the relative humidity dropped from 98% during normal conditions down to 48% during the critical stress period (Figure 1 B2).

The heat index described as the felt temperature followed the same pattern as the temperature and was important in making inferences on the effect of heat on the plant. Day heat index increased from 28.5 during normal conditions up to 33.8 during peak stress. At night, the heat index also increased from 18.5 to 23.5 (Figure 1C). High heat indices during the day lead to sun burns on the leaves resulting into increases in the rate of water loss on the plant surfaces. Besides that, heat index may have an effect on leaf aging and general drying of plant fragile organs such as stem tips and young leaves. Day and night dew point also dropped significantly with day dew point dropping from 18 to 22.5°C during normal conditions down to 8.4 to 9.0°C during peak stress period signifying an increase in dry atmospheric conditions and night dew point reducing significantly from 18 to 20°C during normal conditions to about 11 to 13°C during peak stress (Figure 1D). Reduction in mole fraction of water vapor in the atmosphere signified by low dew points indicates an increased evapo-transpiration from plant and soil surfaces. This intensified moisture and heat stress conditions were imposed on the plant during stress.

Changes in levels of primary metabolites (carbohydrates)

Primary metabolites like carbohydrates and sugars which are products of photosynthesis are easily affected by changes in leaf characteristics brought about by physical changes in the environment. Thus, this study changes in carbohydrate contents were determined in order to understand the efficiency of phenotypic mechanisms observed in selection of drought tolerant varieties and how they relate to stored and metabolisable carbohydrate. These changes are presented in Table 1. Reductions in starch levels were observed with progression of stress time while reducing sugars increased. There was an average decline in starch yield over the stress period in all the varieties although it was well pronounced among the susceptible varieties and the stay green varieties compared to the early recovering varieties (Table 1). The rate of drop in starch yield measured as the gradient was -1.424 for the stay green (Nase 2, NASE 3, I/92/0686 and I/92/00062) and -2.356 for susceptible varieties (Rugogoma and NASE 1) respectively, accounting for approximately 25 to 40% reduction in starch yield. However, in the early recovering varieties, there was a drop of about 25% of the original starch yield after three weeks of the critical stress period with an increase observed by the 5th week of peak stress with an overall positive change of 0.073 accounting for 7.0% increase compared to the original starch yield prior to stress. The differences observed in percentage starch yield in different varieties indicated the use of different mechanisms of tolerance by the different cassava varieties and the dependence of some mechanisms on stored reserves of the plant.

The starch content reduced progressively with time in line with the drop in starch yield observed. A high rate of reduction was observed for stay green varieties (-0.024) compared to susceptible varieties (-0.017) and early recovering varieties (-0.014) for starch content. By the fifth week, the critical stress period of the starch content had reduced to an average of 0.073 g/g of fresh root from an earlier average of 0.15 g/g of fresh root in all varieties although high reductions were observed for stay green varieties. No significant differences ($p>0.05$) were observed for starch content variations across the different varieties, although significant differences were observed within variety groups with the stay green varieties posting higher losses. However, the levels of bound reducing sugars increased with increase in the stress time explaining the possible losses in starch yield and content observed. Reducing sugars were also negatively correlated to starch yield ($r = -0.685$) although they had no significant correlations with starch content. Carbohydrate (starch content and reducing sugar) (Table 1) profile changes revealed an interesting phenomenon in the reaction of cassava plants to stress especially in three broad groups cassava. While starch content was not significantly correlated to either starch yield, free reducing sugars, or bound reducing sugars, it was positively correlated to the cyanide content ($r = 0.269$). Such negative/positive correlation shows that increases in cyanide content do not interfere with stored forms of carbohydrates such as starch though products of carbohydrate metabolism are important in mediating the production of secondary metabolites such as cyanide which are important in plant defense against stress. The starch content was also negatively correlated to the protein content although the correlation was weak ($r = -0.245$). On the other hand, reducing sugars increased with increase in stress and the increments were well pronounced among the stay green, susceptible and early recovering variety groups rather than individual varieties within these groups. There was a general increase in total reducing sugars for all the varieties and variety groups between week one and week three in the critical stress period with the lowest percentage increase observed for the early recovering varieties (12.3%) compared to stay green varieties (44.17%) and susceptible varieties (26.0%). However, initial reducing

Table 1. Changes and rate of change (r) in the contents of starch and free reducing sugars over the eight (8) week stress period.

Group (Δ)	SC0	FRS0	BRS0	SC3	FRS3	BRS3	SC5	FRS5	BRS5	SC7	FRS7	BRS7	rSC	rFRS	rBRS
Stay green	0.133[a]	0.134[a]	0.363[a]	0.177[a]	0.240[a]	0.397[a]	0.075[a]	0.182[a]	0.455[a]	0.077[a]	0.231[a]	0.412[a]	-0.023	0.023	0.110
Early recovering	0.106[a]	0.171[b]	0.348[b]	0.111[b]	0.195[b]	0.787[b]	0.071[a]	0.166[a]	0.276[b]	0.075[a]	0.142[b]	0.278[b]	-0.014	-0.012	-0.072
Susceptible	0.106[a]	0.168[b]	0.330[c]	0.134[c]	0.227[c]	0.521[c]	0.070[a]	0.170[a]	0.613[c]	0.071[a]	0.239[a]	0.667[c]	-0.017	0.018	0.02
LSD	0.031	0.025	0.045	0.01	0.152	0.058	0.023	0.053	0.031	0.022	0.114	0.084			
P value	0.461	0.031	0.018	0.018	0.068	0.0001	0.99	0.067	0.001	0.323	0.034	0.001			

0, 3, 5, and 7 correspond to values of different carbohydrate metabolites for week 1, 3, 5 and 7 during the critical stress period. SC=starch content; FRS=free reducing sugars; bound reducing sugars; R=rate of change in SC; FRS and BRS during the 8 weeks of stress. Values with different corresponding letters are different at $p<0.05$.

sugar contents were higher for early recovering varieties (0.171 mg/g) compared to stay green varieties (0.134 mg/g) and susceptible varieties (0.168 mg/g). After the fifth week, increases were only observed for stay green varieties (from 0.182 to 0.231 mg/g) and the susceptible varieties (from 0.17 to 0.239 mg/g). In contrast, reducing sugar content values decreased for all the early recovering varieties (from 0.166 to 0.142 mg/g) at the same rate as their increase in other varieties after the fifth week of stress (Table 1).

This is shown by the positive gradient (0.02 and 0.11) for stay green and susceptible varieties, respectively, while a negative gradient was observed for the early recovering at -0.072. Signi-ficant ($p<0.05$) changes were observed for free reducing sugar contents in all test varieties and their quantities were higher in stay green and susceptible varieties compared to early recovering varieties (Table 1). Consistently, free low reducing sugar values were observed across the stress time for early recovering varieties compared to stay green varieties and susceptible varieties up to the end of the stress period and into the recovery phase as shown in Table 1.

Bound reducing sugars had higher contents compared to free reducing sugars in all cases observed and increased with the stress time (Table 1) and ranged from 0.33 to 0.787 mg/g in different varieties at different times of the stress period. Among the stay green varieties, they increased linearly from 0.368 mg/g at the onset of the stress to about 0.46 mg/g by the end of the 5th week post stress. Among the susceptible varieties, the rate of increase in bound reducing sugars was high compared to the stay green varieties with increments from 0.33 mg/g at onset of stress to 0.667 mg/g by the end of the stress period. In contrast, increments in bound reducing sugars among early recovering varieties were observed in the first three weeks after peak stress onset from 0.348 to 0.787 mg/g. After this, bound reducing sugars reduced significantly to 0.274 mg/g by the 5th week post initial peak stress depicting an overall negative gradient throughout the stress period.

Changes in the fresh root and peel cyanide contents

During stress, cassava plants accumulate nitrogenous compounds such as proteins, cyanide and phenolic substances such as secondary metabolites which are important as defense compounds and mediate vast physiological responses. Results for the cyanogenic potential of the roots and peels through the peak stress period are presented in Table 2. The cyanide content was at least two times higher in the peel compared to the fresh portion of the root with tremendous increments at four weeks post peak stress. Significant variations ($p<0.05$) were observed among the different variety groups with low contents observed among the susceptible varieties (about 168 ppm) pre critical stress period which composed of most of the “sweet” and local varieties. With onset of peak stress, the peel cyanide content was significantly ($p<0.05$) high for early recovering varieties (about 602 ppm) compared to stay green varieties (about 393 ppm) and susceptible varieties (about 386 ppm). In the third week of the critical stress period, specific changes in peel cyanide content were observed for susceptible varieties rising up to about 510 ppm and no significant changes were observed for early recovering and stay green varieties. Furthermore by the 5th week of the critical stress period, increased cyanide levels were observed in the peels with over four times increase in varieties among the groups of susceptible varieties (about 1605 ppm) early recovering (1457 ppm) and stay green (984 ppm) varieties. However by the 7^{th} week of the critical stress period, the levels of cyanide in the peel (cortex) had reduced and by the end of the stress period,

Table 2. Changes and rate of change(r) in root and peel cyanide content over the stress period.

Variety group	CR0	CP0	CR3	CP3	CR5	CP5	CR7	CP7	CR8	CP8	rCR	rCP
Stay green varieties	0.393[a]	0.393[a]	0.309[a]	0.298[a]	0.549[a]	0.984[a]	0.347[a]	0.501[a]	0.356[a]	0.646[a]	-0.01	0.07
Early recovering varieties	0.301[a]	0.602[b]	0.245[b]	0.535[b]	0.642[a]	1.457[b]	0.246[a]	0.407[a]	0.087[b]	0.149[b]	-0.04	-0.10
Susceptible varieties	0.168[a]	0.386[a]	0.381[c]	0.510[c]	0.405[a]	1.605[b]	0.301[a]	0.667[a]	0.070[c]	0.286[c]	-0.03	-0.01
LSD	0.177	0.241	0.032	0.036	0.283	0.082	0.09	0.192	0.214	0.315		
P-Value	0.242	0.03	0.0310	0.011	0.076	0.01	0.317	0.330	0.001	0.001		

0, 3, 5, and 7and 8 correspond to values of different cyanide contents metabolites for week 1, 3, 5 and 7 during the critical stress period and week 8 during recovery. CR=Cyanide content for the root; CP=cyanide content for the peel; CR=cyanide content for root cortex; R=rate of change in CR and CP during the 8 weeks critical stress period. Values with different corresponding letters are different at $p<0.05$.

the cyanide content had reduced to 149 ppm for the early recovering varieties and 286 ppm for the susceptible varieties but it was still high in stay green varieties at 646 ppm. Root cyanide contents also increased with critical stress time at different rates among the different variety groups. It increased from 393 to 549 ppm among the stay green varieties and from 300 to 642 ppm among the early recovering varieties by the fifth week of the critical stress period. In susceptible varieties, significant increments were observed by the third week from 168 to 510 ppm. Decrease in cyanide content was observed within the recovery phase 5 weeks post initial peak stress dropping specifically in early recovering (from 642 to 87 ppm) and susceptible (from 405 to 70 ppm) varieties. However no significant drops were observed for stay green varieties. Significant ($p<0.05$) differences were observed for root cyanide content among the variety groups at all times during critical stress period. Among variety groups, rate of change in the fresh peeled root was higher for stay green varieties (-0.043) and susceptible varieties (0.028) throughout the stress period. High negative changes in the cyanide content of the peel were observed for the ERVs (Table 2). The relationships between cyanide content and primary metabolites were studied and the results are presented in Figure 2. It was established that increments in cyanide content coincided with increments in protein content and reductions in main metabolites such as starch and sugars. In fact, drops in total carbohydrate content three weeks into the critical stress period coincided with increments in the cyanide content. The same applied to main pigments chlorophyll and carotenoids which also reduced as the protein and cyanide content increased (Figure 2). In the recovery phase, significant drops in cyanide content coincided with a positive gradient for total carbohydrate although the protein content was not affected. This phenomenon was the same for all the varieties although differences were observed in the contents of these metabolites for each variety and within the variety groups.

Changes in total protein and phenolic compounds

The production of moisture and heat responsive proteins in plants plays a key role in plant tolerance to stress and thus in critical stress times, plants change the amount and number of proteins within stress responsive organs such as leaves (Quietsch et al, 2000). The understanding of the alterations in leaf protein content was thus undertaken and the results for leaf protein contents are presented in Table 3. An increment in total protein content was observed for all the variety groups up to the 5th week of the stress period after which a drop was observed in seventh week of the stress period. There were significant differences ($p<0.05$) among varieties for protein contents at the onset of stress and later within the critical stress period with cumulative increments observed for all varieties between the 3rd and 5th week in the critical stress period. High leaf protein contents were observed in susceptible varieties and ranged from 0.34 to 0.5 mg/g while the least were observed for early recovering varieties and ranged from 0.21 to 0.26 mg/g. Five weeks post stress, the protein concentration was higher in stay green varieties (0.56 mg/g). In the fifth week of stress and at the onset of increments in relative humidity, protein content increased two fold with high increments observed for both stay greens and susceptible varieties and ranged from 0.34 to 0.5 mg/g while the least were observed for early recovering varieties and ranged from 0.21 to 0.26 mg/g. Five weeks post stress, the protein concentration was higher in stay green varieties (0.56 mg/g). In the fifth week of stress and at the onset of increments in relative humidity, protein content increased two fold with high increments observed for both stay

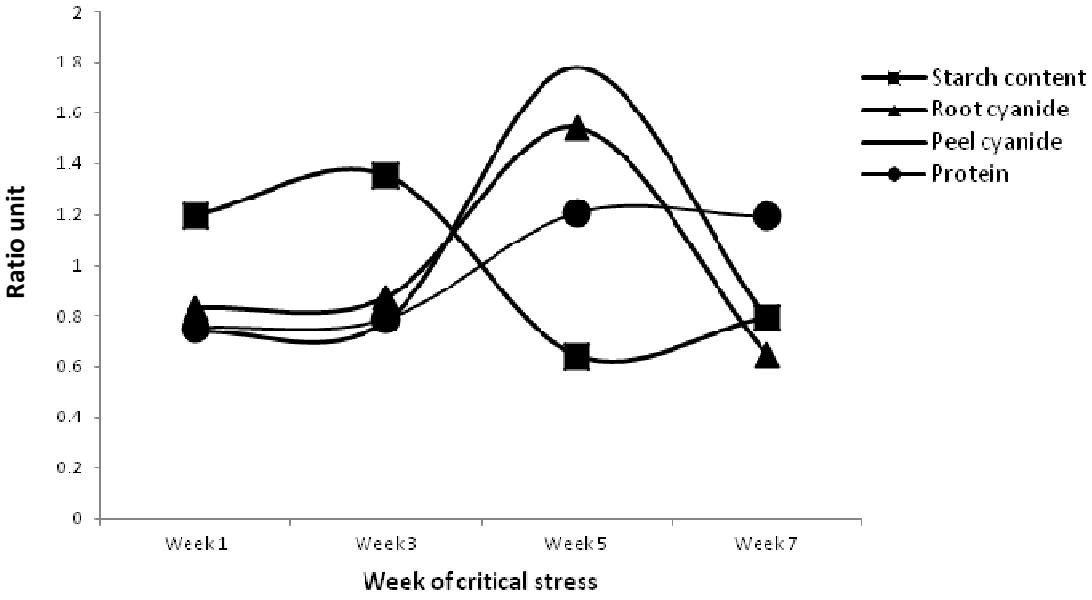

Figure 2. Relative changes in main nitrogenous cassava plant metabolites compared to starch content presented as the ratio of concentrations of different metabolites at different times in relation to the final values at the end of the critical stress period.

greens and susceptible varieties. As products of the both carbohydrate and aromatic amino acid path ways, phenolic compounds constitute a range of metabolites such as phenylpropanoids and flavonoids that are important as plant secondary metabolites, defense compounds and as stores of nitrogen. They are affected by changes in plant carbohydrate and amino acid metabolisms and are important indicators of plant response to environmental change. During the critical stress period, phenolic compounds were monitored and the changes in leaf phenolic contents are presented in Table 3. Phenolic compounds concentrations increased at different rates in all the varieties but the increase was more pronounced among susceptible varieties from 0.123 µg/g at onset of stress to about 0.42 µg/g by the 7th week post peak stress. In stay green and early recovering varieties considerable increments were observed five weeks post initial stress with significant differences ($P<0.05$) among the variety groups observed later in the critical stress period. Susceptible varieties accumulated phenolic compounds throughout the stress period even after recovery of other varieties. All varieties had high concentrations of phenolic compounds five weeks post peak stress which reduced with increase in relative humidity during the recovery period except in the susceptible varieties where continuous increments were registered.

Changes in main plant pigments (chlorophyll and carotenoids) during critical stress

Plant pigments are important in various physiological processes such as photosynthesis in addition to plant defense. Understanding the effect of stress on these pigments allows us to get insights into the plants' mode of resilience to stress and its ability to uphold certain physiological processes such as photosynthesis under optimal stress conditions. Variations observed for leaf pigments mainly chlorophylls and caretonoids across the variety groups and at different times during the peak stress period are presented in Table 4. An overall reduction in the amount of carotenoids was observed during the stress period. Significant differences ($P<0.05$) were observed among variety groups for carotenoid content at the 3rd and 7th week of critical stress. Among the early recovering varieties, carotenoid content remained the same at about 0.345 µg/g at the start of the peak stress period only to increase to about 0.421 µg/g during the recovery phase. In stay green varieties, reductions were observed where carotenoid content reduced from 0.456 µg/g at the start of the peak stress period to 0.356 µg/g by the end of the peak period. No significant increments were observed during the recovery phase for these varieties. In susceptible varieties, the carotenoid contents dropped from 0.511 to 0.378 µg/g by the end of the stress period. Slight increments were observed during the recovery phase for these varieties to about 0.442 µg/g of carotenoids (Table 4). In particular, a drop was observed in total carotenoid content with significant drops in stay green varieties (gradient, -0.041) while in susceptible varieties, a drop was observed between week 1 and 3 post-stress and as well as between week 5 and 7. Minor reductions were observed for carotene content (gradient -0.014) throughout the stress period for susceptible varieties. For early recovering varieties, increments were

Table 3. Change in main nitrogenous compound (protein and phenolics) during the eight week critical period.

Variety group	Protein 1	Protein 3	Protein 5	Protein 7	Phenolic 1	Phenolic 3	Phenolic 5	Phenolic 7
Stay green varieties	0.027[a]	0.029a	0.056a	0.018a	0.015a	0.018ab	0.019	0.015
Early recovering varieties	0.034[b]	0.031a	0.049a	0.026b	0.012a	0.014b	0.033	0.042
Susceptible varieties	0.021c	0.026a	0.022b	0.018a	0.015a	0.026a	0.012	0.014
LSD	0.003	0.01	0.0198	0.004	0.021	0.018	0.016	0.012
P-Value	0.001	0.492	0.010	0.01	0.235	0.167	0.045	0.01

1, 3, 5, and 7 correspond to values of different metabolite contents for week 1, 3, 5 and 7 during the critical stress period and week 8 during recovery
Values with different corresponding letters are different at $p<0.05$

Table 4. Changes in the pigment content with stress time during the critical stress period.

Parameter	Chla1	Chla3	Chla5	Chla7	Chlb1	Chlb3	Chlb5	Chlb7	Cart1	Cart3	Cart5	Cart7
Stay green varieties	0.201	0.197	0.198	0.246	0.094	0.101	0.073	0.109	0.456	0.479	0.350	0.362
Early recovering varieties	0.122	0.152	0.218	0.252	0.061	0.068	0.086	0.106	0.335	0.377	0.373	0.421
Susceptible varieties	0.227	0.112	0.182	0.228	0.108	0.052	0.08	0.100	0.511	0.312	0.378	0.442
LSD	0.028	0.044	0.038	0.053	0.027	0.018	0.014	0.025	0.173	0.082	0.113	0.114
P-Value	0.001	0.02	0.009	0.004	0.001	0.01	0.151	0.015	0.153	0.029	0.755	0.034

1, 3, 5, and 7 correspond to values of different cyanide contents metabolites for week 1, 3, 5 and 7 during the critical stress period and week 8 during recovery. CR=Cyanide content for the root, CP=Cyanide content for the peel R=Rate of change in CR and CP during the 8 weeks critical stress period.

observed overall (gradient, 0.025) with a slight drop at week 5 and further increments by week 7 at onset of new leaves. A slight drop observed at week 5 coincides with onset of recovery and end of peak stress where only very young leaves were available on most plants.

Chlorophyll a (*chla*) exhibited a different pattern from carotenoids in most variety groups during the stress time. Among the early recovering varieties, *chla* increased from 0.112 µg/g to twice as much as (0.218 µg/g) by the end of the stress period reaching a peak of 0.252 µg/g after recovery and onset of new leaves. However in stay green varieties, *chla* values dropped from 0.204 µg/g at the onset of peak stress to about 0.198 µg/g a rather less significant drop. A major decrease was observed in susceptible varieties where the concentration of *chla* dropped from 0.227 to 0.11 µg/g by the third week post peak stress (Table 4).

Much as *chlb* did not change significantly during stress time, a drop in the concentration of *chlb* was observed in the other variety groups except the early recovery varieties where no specific changes were observed during stress with averages of about 0.068 µg/g only to increase to about 0.106 µg/g during the recovery phase. Among the stay green varieties, concentrations of *chlb* reduced from 0.084 µg at the onset of peak stress to about 0.073 µg/g at the end of stress. The same pattern was observed for susceptible varieties where *chlb* contents dropped by almost half the original value from 0.108 to 0.052 µg/g by the third week of critical stress. At onset of recovery, increments in *chlb* content were observed for both stay green and susceptible varieties (Table 4).

DISCUSSION

The selection of drought tolerant cassava varieties has been the most elusive and challenging among many cassava breeders. However, the use of combined selection tools both in areas of genomics and biochemistry could provide a lot of insights into possibilities that will help in selection. In particular biochemical tools can be easily determined and used for faster selection. In this study, changes in major biochemical compounds in the

Table 5. Correlation analysis for contents of different metabolites mid critical stress period.

CnPP	1											
CnPR	**0.834**	1										
RS	0.116	-0.047	1									
BRS	**-0.339**	**-0.418**	**0.518**	1								
Cart	0.098	0.038	-0.032	**-0.496**	1							
Chla	-0.051	0.188	**0.583**	-0.020	0.382	1						
Chlb	0.235	0.296	0.367	**-0.442**	**0.659**	**0.752**	1					
FRS	0.175	-0.008	**0.725**	0.627	**-0.346**	0.084	-0.001	1				
Phe	0.163	0.305	0.425	0.319	-0.189	**0.531**	0.091	0.116	1			
Prt	-0.154	**-0.236**	**0.873**	**0.664**	-0.006	**0.596**	0.268	**0.663**	**0.567**	1		
SCa	0.269	0.183	0.042	0.151	**-0.597**	**-0.308**	**-0.425**	-0.071	0.162	**-0.245**	1	
SY	0.087	-0.006	-**0.685**	-0.097	-0.219	**-0.858**	**-0.736**	-0.152	**-0.432**	**-0.614**	0.041	1
	CnPP	*BRS*	*Cart*	*Chla*	*Chlb*	*FRS*	*Phe*	*Prt*	*Sca*	*SY*	*CnPR*	*RS*

CnPP=Cyanogenic potential for root cortex; CnPR=cyanogenic potential for the fresh root; RS=reducing sugar; BRS=bound reducing sugars; Cart=carotenoid; Chla=chlorophyll; Chlb=chlorophyll b; FRS=Free reducing Sugars; Phe=phenolics content; Prt=protein content; SCa=starch content; SY=starch yield.

main carbohydrate and nitrogen metabolic pathways were investigated and their variations highlighted in different varieties under optimal stress in the field. Changes in carbohydrates were observed during the stress period as their levels decreased with time since they act as a resource for the production of most of the protective proteins and other bio-molecules required in the reversing of effects of stress in plants (Kheder et al., 2003). Loss in total starch yield in all varieties which was observed, is an indicator of the process of remobilization of stored resources in a plant to cater for survival during stress (Setter and Fregene, 2007). In fact, loss in starch was negatively correlated to total reducing sugars (r=-0.685), an indicator that increments in reducing sugars such as glucose where as a result of losses in starch. It was also negatively correlated to *chla* (r=-0.835) and *chlb* (r=-0.736) while it was positively correlated to phenolic content (r=0.432) and the protein content (r=0.614) (Table 5). This shows that losses in starch may be due to shut down of major photosynthetic processes due to loss of photosynthetic pigments but also results into accumulation of important stress metabolites such as proteins and phenolics. In essence, there was reverse translocation of metabolisable sugar resources from the root for leaf and stem growth and maintenance among the stay green varieties accounting for the decline in starch yield as seen in the first three weeks of peak stress (Table 1) have been suggested by (Mir et al., 2012). However, the losses in starch yield differed with susceptible varieties losing more of the starch at any time and faster compared to the stay green and early recovering varieties. For the stay green varieties the remobilized sugars may allow for the maintenance of a decent leaf area index throughout the stress with minimum photosynthesis. This may explain the low drop in sugar contents observed compared to early recovering varieties that had lost all the leaves by 5th week post peak stress. Much as the susceptible varieties maintain a number of leaves, the leaves were usually dechlorophyllated with a low potential for any photosynthetic activity and hence their survival depended on remobilized resources from the root. The early recovering mechanism allowed the plants to shade off all the leaves hence reducing the transpiration rate (Borrell et al., 2000) and the burden of maintaining less photosynthesizing leaves (Tsukaguchi et al., 2003) amid stress and maintaining stored resources. The drop in starch yield in first two weeks explained the coping mechanism by these cassava varieties as they counteracted the effects of the stress. The slight increment in starch yield observed after six weeks coincided with the increase in relative humidity and the reestablishment of young vigorous leaves which started to carry out photosynthesis. The difference in these varieties lies in the fact that while stay greens maintained old but less efficient leaves for photosynthesis, the early recovering varieties easily regain their leaf potential with young vigorous leaves that increased the level of starch deposits in the root immediately after the stress period. Loss of leaves early in stress period by early recovering varieties reflected the ability of these plants to maintain a high level of stored carbohydrate by shutting down most of the metabolic processes carried out by the growing points of the plant during stress. Such plants are better suited for stress management compared to the stay green varieties. Significant losses observed for susceptible and stay green varieties which maintain a high leaf area index points to the fact that the maintenance of a high leaf area index requires more remobilization and hence considerable losses in starch. These varieties are not so well suited for stress management since severe stress will lead to depletion of storage starch from the root hence affecting plant productivity.

Increase in reducing sugar content observed (Table 1) can be attributed to the breakdown of starch and other storage carbohydrates during the stress period. However, the rate of breakdown is an indicator of the level of remobilization of these starches by the different varieties. Sudden increases in reducing sugars content for early recovering varieties (Table 1) is possibly due to the stress shock and the need for more energy for driving a number of processes in the bid to counteract the various stresses. However, the drop in reducing sugars signifies a drop in metabolic activity by these plants conserving both energy and resources in this instance. A linear trend observed for the increase in reducing sugars for the susceptible varieties meant that they do not have any coping mechanisms and hence use their storage reserves to accommodate the effect of the stresses observed at the different time points with increasing stress resulting into continued remobilization. These varieties may also exhibit a high metabolic rate induced by stress that would drain all the available resources putting the plants into a continuous catabolic state.

Meanwhile, the high concentration of bound reducing sugars during peak stress point to an internal genetic mechanism by which plants increase freely metabolizable carbohydrate in order to tolerate various stresses. Consistent increase in bound reducing sugars over the stress time (Table 1) points to continuous remobilization of the starch resources over time as the plant struggles to survive (Mir et al., 2012). In particular, the reduction in the recovery phase may be due to the utilization of the sugars (mainly glucose) for generation of energy that would be used in the anabolic pathways that synthesize plant defensive compounds such as cyanogenic glucosides (Jorgensen et al., 2005) among others. In this study, it was noted that a negative correlation occurred between bound reducing sugars and the cyanide contents for both peels (r=-0.418) and fresh root portion of the plant (r=-0.339) (Table 5). This supports the notion that such secondary metabolites are synthesized using the available sugars. The negative correlation between the cyanogenic potential and protein (r= -0.236) also points to the utilization of amino acids as the nitrogen source in production of cyanide used as a stress management strategy. Although, the differences in the rate of increase of these sugars (Table 1) were differed across variety groups. Such differences may be used in categorizing tolerance in these groups where for instance a high rate of reduction in stay green varieties compared to the early recovering ones was consistent with observed losses in starch yield and described the mechanisms used in these different sets of stay green and early recovering varieties. High remobilizations were required in stay green varieties to maintain the leaf area index and the unshed leaves during stress period. Variations observed in carbohydrate levels also point to the importance of carbohydrate metabolism in mediating stress response. The different metabolic pathways for carbohydrates production and utilization provided the much required precursors for stress responsive metabolites which in turn mediate observed patterns of tolerance or susceptibility. Genes and their products in these pathways therefore could be used to make valid inferences on what happens during carbohydrate metabolism (Fujiki et al., 2000).

Wide variations in the concentration of cyanogens among cultivars of cassava, ranging from 10 to 2,000 ppm hydrogen cyanide equivalent were observed. Similar variations have been observed among cassava varieties growing under moisture and salinity stress where by increased accumulation of cyanide was prevalent (Cardoso et al., 2005). Their accumulation follow a similar trend within the fresh root and the peel with the root accumulating more of the cyanide at any particular time and hence acting as the first line of defense. Cyanogenic glycosides have been shown to play an important role in nitrogen storage in some species (Møller, 2010), which would explain the increments observed away from normal suggestive of the initiation of plants mechanism for nitrogen storage. The drop in cyanide concentration of about 200 ppm observed after stress also points to the importance of nitrogen in growth and development during the recovery phase as suggested by (Lechtenberg, 2011). Moreover, it may also be related to higher yields observed in early recovering varieties as a result of improved nitrogen-use-efficiency and reduced herbivory (Møller, 2010; Lechtenberg, 2011). Significant correlations (r=-0.236) between cyanide and protein content confirmed the increased nitrogen use efficiency in cassava. An increase in cyanide and protein content in relation to the decrease in bound and free sugars (Figure 2) suggests a gene expression mechanism meant to synthesize the relevant stress factors at the expense of carbohydrate metabolism for energy generation. Cyanide accumulation may also suggest roles of increased nitrogen use efficiency by cassava plants during stress which may play important roles during recovery and continued re-growth since it also acts as a sugar storage mechanism (Lechtenberg, 2011). The role of cyanide as the main secondary metabolite in mediating stress tolerance in cassava has been echoed by a number of research initiatives (Yi, 2012; Møller, 2010; Lechtenberg, 2011). Increases in levels of cyanide observed midway the critical stress period followed by the decrease in the amount of free and bound reducing sugars in the 3rd week of stress (Figure 2). This showed that increase in the level of this metabolite allowed the utilization of sugars produced and hence presents a stress management option in the cassava plant (Poulton, 2001). It also indicated an intricately organized pattern where stress recognition mechanisms in the plant allowed for production of free sugars which were then utilized by the plant later in production of stress responsive metabolites including sugars as osmolytes and secondary metabolites such as phenols and tannins (Lechtenberg, 2011;

Yi et al., 2012; Morant et al., 2007). Møller (2010) reported that the role of cyanide in mediating stress was not well known in cassava but it was thought to be a protective agent against other biotic constraints that might have effects on the plant. However, the results from this study suggested that the level of accumulation during stress was important in allowing the selection of better suited varieties for stress resistance. It was also observed that the accumulation of cyanide in different parts of the roots varied among varieties, another factor showing that cyanide accumulation is part of the stress management strategy. Besides, accumulation in the peel appears to be important for protection of the plant against herbivory especially on the root which acts as a storage organ for nutrients during the stress period.

Cassava differentially accumulates proteins in leaves compared to other parts of the plants (Montagnac et al., 2009). Proteins mediate photosynthetic, growth and regulatory functions in the plant with some mainly being enzymes. Plants under severe stress undergo changes which affect the acquisition of nutrients hence affecting the production of storage proteins (Gleadow et al., 2009). However, stress responsive proteins are produced in the plants which occur as secondary metabolites and or osmolytes helping the plant to absorb the limited water from ground (Harding et al., 2003). The enzymes are also important in counteracting the effects of reactive oxygen species observed in many stressed organisms including plants (Apel and Hirt, 2004). The positive correlation between protein contents and pigments such *chla* (r=0.596) also explain their role as protective agents to these important biomolecules. This may explain low protein contents observed at onset of peak stress and their increase with increase in stress time (Table 3) up to a time when the relative humidity had appreciably setting in the recovery phase of the plant. It also coincided with increase in cyanide content (Figure 2) especially for peel from 298 to 984 ppm, an important factor in defending the recovering plant against other biotic stresses like herbivory and pests. Therefore, proteins supplement the role of cyanide as nitrogen storage reserves and secondary defense metabolites for plants during stress helping plants also to go through to the recovery phase using stored resources. The source of these proteins is invariably, the free glucose derived from starch remobilization as shown by the positive correlations between protein and total sugars (r=0.873) and also between protein and bound reducing sugars (r=0.664) (Table 5). Like proteins, phenolic compounds are also important as stress responsive secondary metabolites. They also act as important stores for nitrogen and as a sugar storage mechanism (Wahid et al., 2007). They increase with increments in protein and they were positively correlated to the protein content (r=0.567) and plant pigments (r=0.531) showing that increments in phenolic compounds resulted into increments in pigments. This shows that these compounds act as protective molecules for pigments and other related compounds. Like cyanide, phenolics as nitrogenous secondary metabolite are also important defenses against herbivory, though their specific role in stress tolerance may be as UV screens (Chalker-Scott, 2002) and as osmolytes allowing plants to sustain particular levels of metabolic water (Havaux, 1998). Thus, the role of proteins as secondary metabolites and enzymes and other metabolites such as phenolics during stress in cassava are to maintain a decent nitrogen and carbon source for the plant to use during the period of recovery after stress and also to protect the plant against injury by stress especially for membranes and other important plant structures.

Conclusion

This study identified main mechanisms of tolerance to drought depending on biochemical characteristics displayed by the plant. These included starch remobilization (reduction of storable carbohydrate resources) and increase in reducing sugar contents (increase in usable carbohydrate metabolites) with consequent negative effects on starch yield. Another mechanism involves the increase in the level of proteins, secondary metabolites like cyanide contents during the stress period and their utilization in form of nitrogen during the recovery phase of the plant. These mechanisms were linked to observed phenotypic differences during stress and where distinct in the three broad phenotypic manifestations observed. Significant relationship between stay green phenotype and *chl* content suggested role playing by *chl* in mediating stress response. The interplay between carbohydrate and nitrogen metabolism in mediating the stay green phenotype was observed. This involved the apparent use of carbohydrate metabolism derived resources in maintaining nitrogen metabolism pathway and its products. In the early recovering varieties, shut down of some if not all elements of carbohydrate metabolism after shedding off of leaves, allowed for reduced metabolism hence more productivity. The results also show that selection for stress tolerance should be done at the right time in the plant growth cycle. Selection for tolerance at a later stage in the plant growth cycle allows the breeders to select for mechanism that maintain a decent yield for the farmer. This also allows the selection of other drought related trait depending on the end user preference especially where the above ground biomass is involved.

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