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The Utility of Biomarkers in Selection for Hydrothermal Stress Tolerance in Cassava (*Manihot esculenta* Crantz).

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ABSTRACT: To understand the usefulness of biomarkers in selection for hydrothermal stress tolerance traits in cassava varieties.Completely randomized design experiments were set up in the screen house where four varieties including the stay green genotype NASE 3, the early recovering variety NASE 16 and the susceptible variety NASE 1 were studied in comparison to the known stay green variety MH96/0686 as the stress tolerant reference.The study was carried out at the Makerere University Agricultural Research Institute Kabanyolo (MUARIK) for a period of four months between August and December 2013. Leaf samples were collected from the four cassava varieties at four months after planting for analysis of leaf growth rate characteristics, total leaf protein, changes in chlorophyll and carotenoid contents, and changes in peroxidase and catalase (antioxidant enzymes) activities. Reduction (up to 40%) in the growth rate for morphological parameters was observed for early recovering variety NASE 16 compared to stay green varieties which coincided with loss of biomass after stress. Total leaf protein content was adversely reduced in susceptible variety by 45% while increments at two and three weeks after stress were observed for early recovering (30%) and stay green varieties (25%) as moisture decreased. Under thermal stress, leaf protein content reduced by over 20% in NASE 16 and NASE 3 compared to the control. Total chlorophyll values taken for moisture and thermal stress treatments indicated losses in chlorophyll with up to 38% in heat susceptible varieties and 15% in resilient varieties. Variety NASE 16 displaying an avoidance mechanism was highly sensitive to heat as indicated in the reduction of the amount of chlorophyll compared to stay green varieties. Besides, the carotenoid content also reduced along the stress period and up to 60% reductions were observed in the stay green varieties NASE 3 and MH96/0686. Surprisingly, a rise and fall trend was observed for peroxidase activity under moisture stress while catalase activity reduced with time in both treatments. These results show distinct differences in tolerance mechanisms displayed by the stay green and the susceptible varieties. The ability to maintain high proteins and pigments (chlorophylls and carotenoids) in cassava leaves may be used as a screening tool for hydrothermal tolerance traits. While high protein contents coupled with high antioxidant enzyme activities (catalase and peroxidase) was associated with the avoidance mechanisms displayed by the early recovering variety, NASE 16, the high protein content coupled with high pigment contents is associated with the stay green trait, in NASE 3 and MH96/0686.
Key words: Cassava, Leaf protein, Chlorophyll, Antioxidant enzymes, Early-recovering, Stay-green

INTRODUCTION

Water and temperature stress are the prime factors that pose negative impacts on agricultural production in sub Saharan Africa. The effects of these two are more pronounced during crops' reproductive and bulking period (Alves *et al*, 2004) resulting into significant losses in both yield and biomass (Turyagyenda *et al*., 2013). The intervention areas in such a case are limited to the selection of varieties with characteristics that enable them to survive amid stress (Shanker and Venkateswarlu, 2011, Frank 1996) and the use of genetic tools such introgression of genes conferring stress tolerance traits in development of particular varieties (Okgbenin *et al*.,

2013). Physiologically, plants respond to hydro and thermal stress by deploying various mechanisms that help in the alleviation of both cellular hyperosmolarity as well as ionic disequilibrium (Parida *et al*., 2007). Some of the physiological responses include: a) the increased production of chlorophyll and carotenoids for improved photosynthetic efficiency, b) protection of various physiological components against oxidative damage as a result of stress using antioxidant enzymes, c) deployment of a number of proteins such as transport protein and heat shock proteins that help in improving the stress tolerance mechanisms for the plant. However, a system involving the interaction between proteins and pigments, when well-managed, would confer improved growth characteristics to the plant which in turn would define a tolerance criterion for the imposed stress.

As a matter of fact, photosynthetic acclimatization to stress has been attributed to incremental chlorophyll accumulation (Rolland *et al*, 2002) allowing the plant to offset the effects of stress. However increase in chlorophyll content can easily be offset by prolonged stress especially during drought, and hence reduces the photochemical efficiency of the plant through PSII with consequent reduction in both chlorophyll a and b content (Sthapit *et al*, 1993). High leaf retention which describes the stay green phenotype (Nuwamanya *et al*., 2014a) is associated with high chlorophyll content, improved yield and transpiration efficiency (Borell *et al*., 2000). Thus the sensitivity of chlorophyll to low moisture and high temperature stress varies from variety to variety and can be used as a basis of selection. In addition, proteins interact with photosynthetic pigments to effect tolerance to environmental stress. Typically, metabolic proteins such as water channel proteins, key enzymes for osmolyte biosynthesis, detoxifying enzymes, and transport proteins are produced in plants under stress (Amudha and Balashbrumani, 2011). Other proteins produced are involved in the regulation of carbohydrate metabolism, stress-defense response, as well as membrane transport (Wang *et al*., 2010, Amudha and Balashbrumani, 2011, Wahid *et al*., 2007). Owing to the above activities, stress-induced gene expressions are indicated by increase in transcription levels for proteins involved in stress tolerance such as key enzymes (catalase and peroxidase). Such stress induced proteins participate in protection of cells and in regulation of genes involved in stress response (Mir *et al*., 2012). From the foregoing, it can be observed that the physiological responses present an outstanding opportunity for selection of stress tolerant cassava varieties using changes in protein contents.

In order to select stress tolerant varieties from the available Ugandan cassava germplasm, a specific selection criteria has to be developed. Such a criterion should also be able to accommodate easily applicable methods based on phenotypic and biochemical observations which can be easily applied in the field. Notably, biochemical markers would offer a valuable tool in selection of cassava varieties for particular stresses. Biochemical markers including pigments and proteins are comparatively simple to analyze and are thus very key in describing various stress related phenomena in response to particular environmental effects. In this study, the utility of biochemical markers in selection for heat and water stress tolerant varieties were explored. Their variations in particular cassava varieties under specific stress conditions have been studied in order to highlight their importance in selection of stress tolerant cassava genotypes.

MATERIALS AND METHODS

Four cassava varieties including the drought tolerant MH96/0686 (Turyagyenda *et al*., 2013) as the test genotype, stay green (SG) NASE 3, early recovering (ER) (NASE 16) and drought susceptible (DS) (NASE 1) were used in this study. All the varieties are improved cassava varieties obtained from the cassava breeding program at National Crops Resources Research Institute (NaCRRI), Uganda. Previous field based phenotypic selections involving 20 cassava varieties in Uganda confirmed that MH96/0686 was tolerant to drought by exhibiting the popular stay green trait with high leaf retention as was NASE 3.The early recovering variety NASE 16 which responds to drought by avoidance mechanisms by shedding off all the leaves and recovering early enough as stress was offset was established. NASE 1 displayed no mechanism of tolerance and was among the genotypes adversely affected by hydrothermal stress (Nuwamanya *et al*., 2014a). For the determination of effect of low moisture, the temperature of the glasshouse were set at 25–30 °C during the day (Alfredo and Setter 2004), night-time temperatures ranging between 15 and 20 °C, and humidity typically at ~65–80 %. In the determination of the effect of heat (thermal) stress, the glasshouse conditions during the day and night were set at 40±2 ^{0}C and humidity typically at between 50-85% for 48 hours keeping all other factors constant. Cassava cuttings (25-30 cm in length with at least four live nodes) were grown in twenty liter (20L) plastic buckets in a completely randomized design (CRD) replicated three times. Before planting, each bucket was filled with about 18 kg of sterilized soil (forest soil: river sand in a 1:1 (v/v) respectively). Two stem cuttings were planted vertically in the soil in each of the buckets. To determine the effect of water stress on biochemical markers, plants were exposed to water stress by reducing soil moisture content to 25-30% using a soil moisture meter (Model 1820, Luster leaf Rapitest, Illinois, USA). A similar number of plants remained watered at field capacity to act as control. In order to investigate the

effect of increased temperature, a new set of plants (6 plants per treatment for each variety) were exposed to increased temperature (40±2 ^{0}C) for 36 hours keeping all factors constant. Prior to these, all plants were watered with 0.5 L/day until 60 days after planting (DAP). Thereafter, the plants were subjected to gradual thermal stress conditions until the soil moisture content was approximately 25 % after which observations were taken for a total of 21 days. For temperature stress, the plants in the controlled temperature plant growth chamber were exposed to immediate temperature increase up to 40^{0}C for 36hours. Control plants (well-watered and plants not exposed to high temperatures) were maintained at soil moisture contents higher than 80% by applying 0.5L of water daily and at room temperatures (25^{0}C).

Determination of total protein and antioxidant activities

Total protein content was determined immediately at onset of both water and thermal stress and thereafter throughout the stress period using the Bradford assay with modifications. Cassava leaves were picked from stressed and control plants and immediately transferred to an ice cooled container. The samples were then transferred to a freezer (-20^0 C) for temporary storage after which 5g of the sample was obtained and crushed in an ice cooled mortar using cold phosphate buffered saline (Hajiboland *et al*., 2007). The resultant homogenate was transferred to an Eppendorf tube and centrifuged at 13000 rpm for 10 minutes. The pellet was discarded and the resultant supernatant was used for quantification of proteins using the Bradford method where 100 µl sample volumes were picked from supernatant and added to an equal volume of NaOH followed by vortexing. The protein standard was prepared in the same way and to the resultant mixtures, 4.8 mL of Bradford reagent was added to make a final volume of 5 mL. This was followed by incubation at room temperature for 5 min after which the absorbance of the solution was taken using a spectrophotometer (BioChrome WPA, Biowave DNA Spectrophotometer, Harvard, USA). Protein concentration was then determined from protein standard curve constructed using Bovine serum Albumin (BSA) as a standard. The total protein content in mg/g of leaf samples was calculated using the modified formulae below (Luo and Huang, 2012)

Protein content of the sample (mg/g) $=(Pn) = \frac{C*VT}{(VS*WF*1000)}$

Where: C= Concentration at the Y- intercept for the standard curve, VT= total volume of the extracted liquid, VS=the added specimen during measurement, WF= wet weight of the sample

In order to verify the physiological changes in response to water and thermal stress, respectively, catalase and peroxidase activities were determined. The first and second fully expanded leaves (5 g) from control plants as well as experimental plants grown under moisture and heat stress were harvested at 0, 4, 8, 12, 16 and 20 days for moisture stress and at 0, 3, 6, 12, 18, 24, and 36 h and homogenized in 5 mL of potassium phosphate buffer (10 mM, pH7.0) containing 4% (w/v) polyvinylpyrrolidon (Mr 25 000). The homogenate was then centrifuged at 10,000 rpm for 15 min, and the supernatant obtained was used as the enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0-4°C. Catalase activity was determined as described by Chen et al. 1993. The reaction mixture contained 50 mM (1300µL) potassium phosphate buffer (pH7.0), 10 mM H_2O_2 (500 µL), and 200 µL of enzyme extract in a 2 mL volume. The reaction was initiated by addition of the enzyme extract and the degradation of H_2O_2 by catalase monitored spectrophotometrically for 10 minutes at 30 second intervals by measuring the change in the absorbance at 240 nm. For determination of peroxidase activity, the reaction mixture constituted 2.1 ml of pure water, 0.32 ml of 100mM phosphate buffer (pH, 6.0), 0.16 ml of 0.5 % hydrogen peroxide and 0.1ml of 0.5% guiacol solution. After mixing thoroughly, the mixture was allowed to equilibrate for 20min at 20^{0}C in the dark and the reaction was initiated by addition of 0.1 ml of enzyme extract. The peroxidase activity, measured as increase in absorbance at 420 nm, was recorded for 4 minutes at 5 second intervals using a spectrophotometer (BioChrome WPA, Biowave DNA Specrophotemeter, Harvard, USA). Enzyme activity was defined as the number of µmoles of hydrogen peroxide converted per mg protein/gram leaf. Total protein was determined by the Bradford method using BSA as the standard.

Determination of chlorophyll and carotenoid concentration

tal chlorophyll content for individual plants from both the control and stressed plots was also measured using a chlorophyll meter (SPAD-520, Spectrum Technologies, Inc., Plainfield, IL, U.S.). The percentage differences in chlorophyll content as determined by the chlorophyll meter between stressed and control plots were then used to determine the extent of effect of stress on different test plants while the rate of reduction in total chlorophyll content readings with time were used to elucidate the differences in stress susceptibility against the control (Percival *et al.,* 2008). In order to determine the concentration of carotenoids, leaf samples (5g) were immersed in a 70 % ethanol solution and placed in the dark for 48 hours to allow for the extraction of the two pigments. Quantitative measurements for carotenoids were taken as the spectrophotometric absorbance of the

ethanol extracts at 445nm (Wettshstein; 1957). Carotenoid concentrations were determined in µg/g of leaf sample from a modified formula by Arnon (1949).

Morphological characteristics

In order to correlate the effect of biomarkers on the observable phenotypic traits, the morphological characteristics such as leaf lobe number, leaf lobe height and width, petiole length and number of leaf scars were determined. Three plants per genotype from each replication for each treatment were measured immediately at onset of stress period and after every four days with increasing moisture stress. A portable field tape measure (Hans's instruments, Bayern International, Germany) was used to take the measurements while the number of leaves, leaf lobes and leaf scars were manually counted. Leaf lobe retention was then determined as the percentage proportion of accumulated leaf scars during the stress period. Average leaf expansion rate (AER) based on leaf lobe width and length was calculated using the formulae below.

$$(AER) \quad = \frac{100(N2 - N1)}{(N1)n}$$

Where N1= Numerical value of leaf property on initial measurement after stress imposition, N2= Numerical value of leaf property one week after to initial measurement, n=Total number of observation after initial measurement

The growth rate was determined by plotting the progression of plant height over time and calculating the rate of change from the equation of the line. Positive values were taken to infer growth progression with time while negative values pointed to dormancy or drying up of growth parts resulting into reduced plant height showing growth arrest under moisture stress .

RESULTS AND DISCUSSION

Utility of biochemical markers (total proteins and antioxidant enzymes) as selection tools for hydrothermal stress.

In order to investigate the utility of biochemical markers in the selection of hydrothermal stress tolerant cassava varieties, protein contents were determined as described in the materials and methods (section 1). In all varieties, an initial increase in protein content was observed within the first five (5) days of water stress. Thereafter, variations could be observed among varieties (Fig. 1). While total protein content reduced after five days of water stress among varieties NASE 1, MH96/0686 and NASE3, respectively, that of NASE 16 continued to rise significantly ($p = 0.48$) reaching peak levels at day 11 to 0.99 mg/g , before falling to 0.3 mg/g and remaining at this level throughout the stress period (Fig. 1 C). Even then, this lowest protein content for NASE 16 was still higher than the lowest for NASE 1 (0.1 mg/g), and MH96/0686 (0.05 mg/g), respectively (Fig. 1A & D). Another notable variation was observed in NASE 3, whereby, following a brief drop in protein content from 0.101 mg/g (at day 6) the lowest of 0.06 mg/g (at day 11), the protein content increased throughout the stress period, reaching the highest of 0.325 mg/g (Fig. 1 B).

In this respect, NASE 3 behaved quite differently from MH96/0686, both of which are known stay green varieties. This suggested that these two varieties, which share a common phenotype, may be employing slightly different mechanisms to maintain the green-leaf canopy. A slightly different behavior was also observed when plants from the four varieties were subjected to thermal stress for at most 36 hours as described in materials and methods (section 1) with notable differences observed among stressed plants. Under thermal stress, there was an initial increase in protein content (0.2 – 0.25 mg/g) among the susceptible variety plants (NASE 1) which lasted for 6 hours, followed by a sharp drop of over 50 % to 0.125 mg/g within the next 6 hours, after which the levels remained constant throughout the stress period (Fig.2 A).

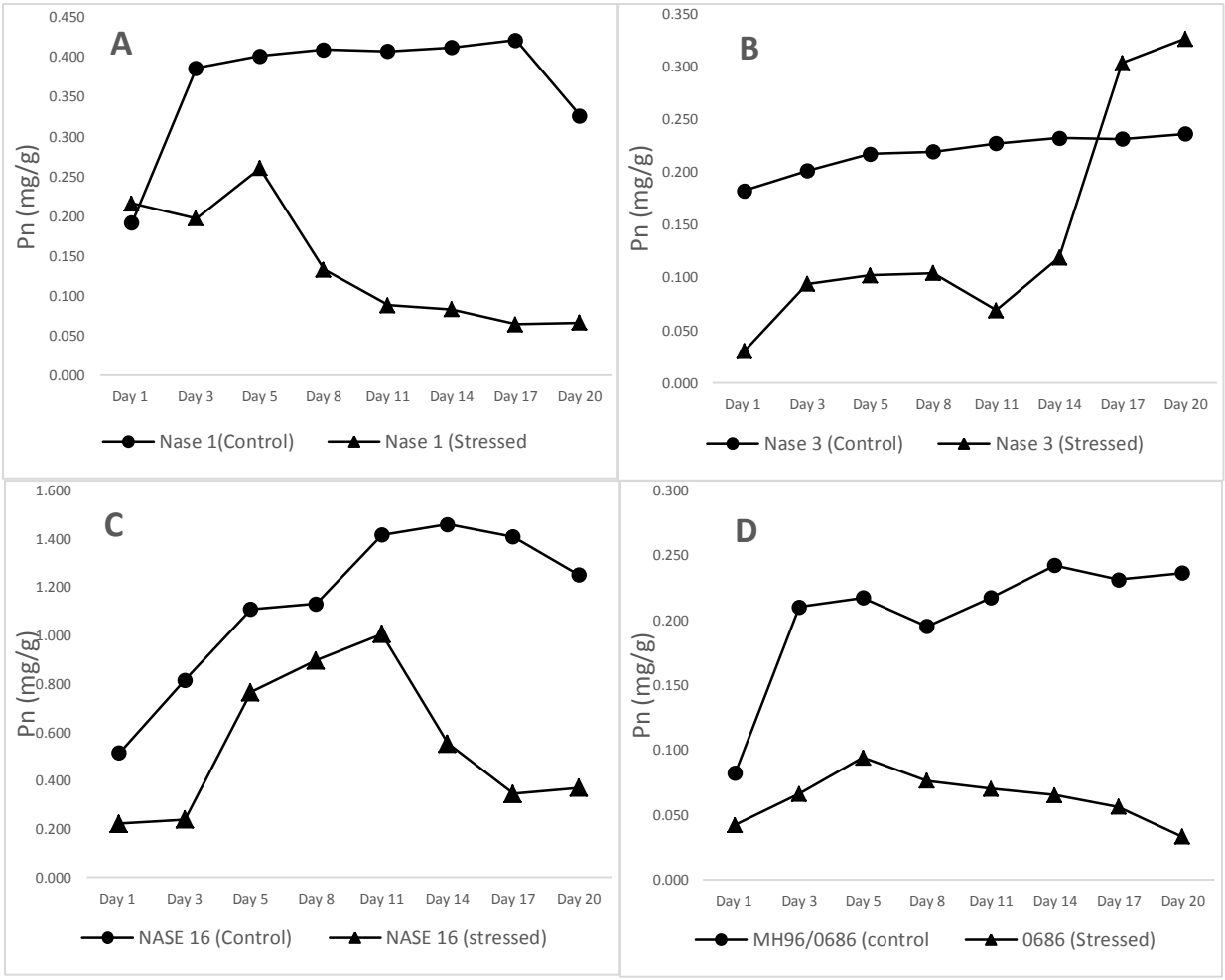

Figure 1. Changes in total protein content (mg/g) for the control and stressed treatments under water stress for the different varieties A=NASE 1, B=NASE 3, C= NASE 16 and D=MH96/0686.

Other varieties registered immediate reductions in protein content from the onset of thermal stress, with MH96/0686, registering a sharp drop within 3 hours (0.08 – 0.025 mg/g), whilst NASE 3 and NASE 16 took 12 hours for their protein contents to drop to the respective lowest levels of (0.2 – 0.025 mg/g) and (0.55 – 0.2 mg/g), respectively. Moreover, after the initial fall in protein contents among the three varieties, the protein levels either increased slightly, as observed for NASE3 and MH96/0686 or remained constant as observed for NASE 16. However, as observed under water stress, NASE 16 variety maintained higher protein content (0.2 mg/g) compared to other varieties i.e. 0.05 mg/g (MH96/0686); 0.12 mg/g (NASE 1) and 0.08 mg/g (NASE 3) post initial stress time, respectively (Fig.2).

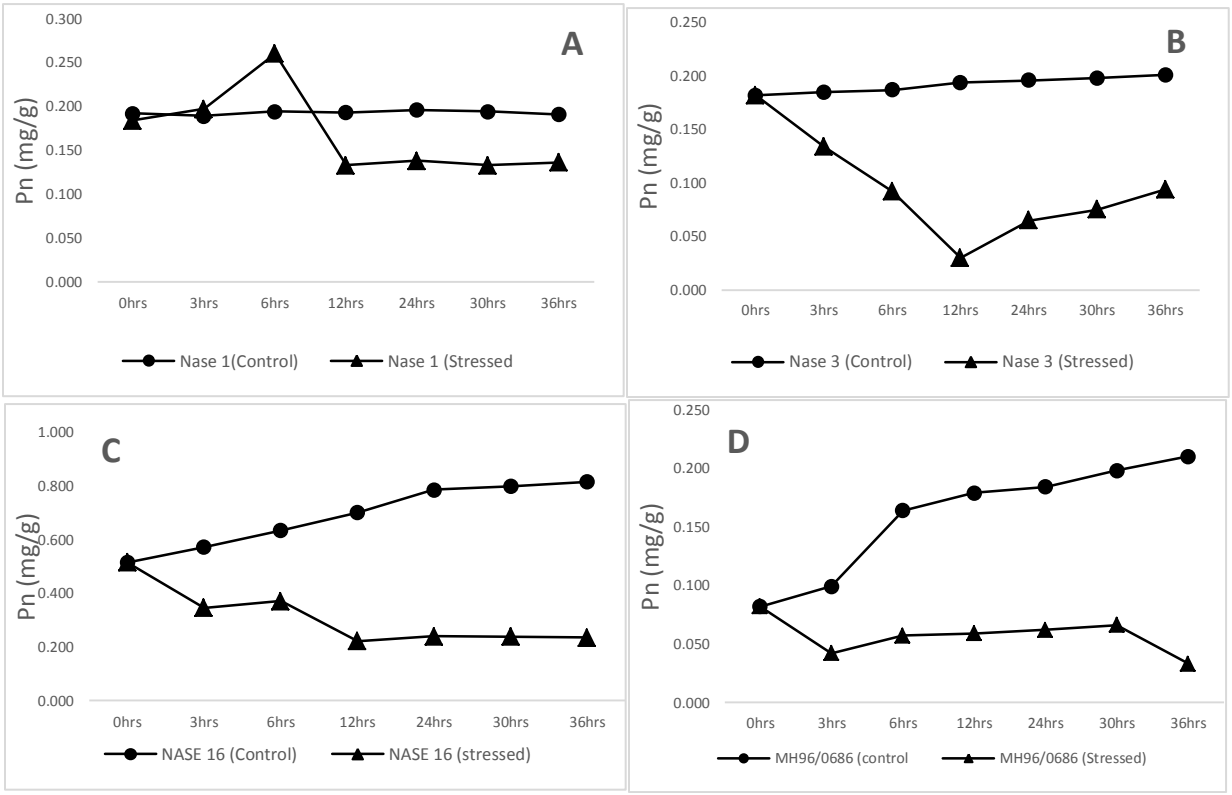

Figure 2. Changes in total protein content (mg/g) for the control and stressed treatments under thermal stress for susceptible variety NASE 1 (A), stay green variety NASE 3(B), early recovering variety NASE 16 (C), and the reference stay green variety (D).

In order to demonstrate the enzyme based physiological changes in response to water and thermal stress, respectively, catalase and peroxidase activities were determined as described in material and methods (section 1). Under moisture stress, high catalase activity was detectable by day four in the early recovering variety NASE 16 (0.0262 µg/g/h), while among the stay green varieties NASE 3 and MH96/0686, it was detectable at day 8 (0.295 µg/g/h) and day 12 (0.0192 µg/g/h), respectively. Surprisingly, catalase activity among the susceptible variety, NASE 1, increased continuously with time and had not reached the peak levels by day 21, the end of the test period (Fig 3A). The peroxidase activity followed a different pattern to that of catalase among the varieties. Notably, the high activity (0.227µg/g/h) observed by day 4 among NASE 16 plants, reduced thereafter with time to 0.06 µg/g/h by the end of the stress period. Peroxidase activities among the susceptible variety NASE 1, and the stay green varieties NASE 3, followed similar trend, reaching the highest level of activity of (0.14µg/g/h) by day 16, which dropped slightly to 0.09 µg/g/h and 0.12µg/g/h, respectively, by the end of the 21 day moisture stress period. As for MH96/0686, highest peroxidase activity (0.09 µg/g/h) was observed by day 12, which also dropped to 0.06 µg/g/h by the end of the 21 day moisture stress period (Fig. 3 C).

The effect of temperature stress on catalase activity is presented in Fig 3B. A rise and fall trend was observed for catalase activity in NASE 16 and the NASE 1 with peak activity after 3 hours at (0.034µg/g/h and 0.018 µg/g/h, respectively. Thereafter catalase activity dropped with time up to 0.008 µg/g/h in NASE 16 and 0.007µg/g/h in NASE 1. The stay green varieties NASE 3 and MH96/0686, however, accumulated catalase over the stress period and had peak activities 24 hours after stress imposition (0.026 µg/g/h for NASE 3 and 0.009 µg/g/h for MH96/0686), which later reduced to 0.004 µg/g/h by 36 hours (Fig 3B). Notable, though, is the observation that by the end of the stress period (36 h) catalase activity among all test varieties had greatly dropped to a range of 0.004 to 0.007 µg/g/h. Peroxidase activity under thermal stress reached the peak after 12 hours in NASE 16 (0.125 µg/g/h) and NASE 1 (0.095 µg/g/h) while it reached a peak after 24 hours in the stay green varieties NASE 3 (0.006 µg/g/h) and MH96/0686 (0.035 µg/g/h). Final peroxidase activity was high in NASE 16 (0.135 µg/g/h) while it was low in NASE 1 (0.001 µg/g/h).

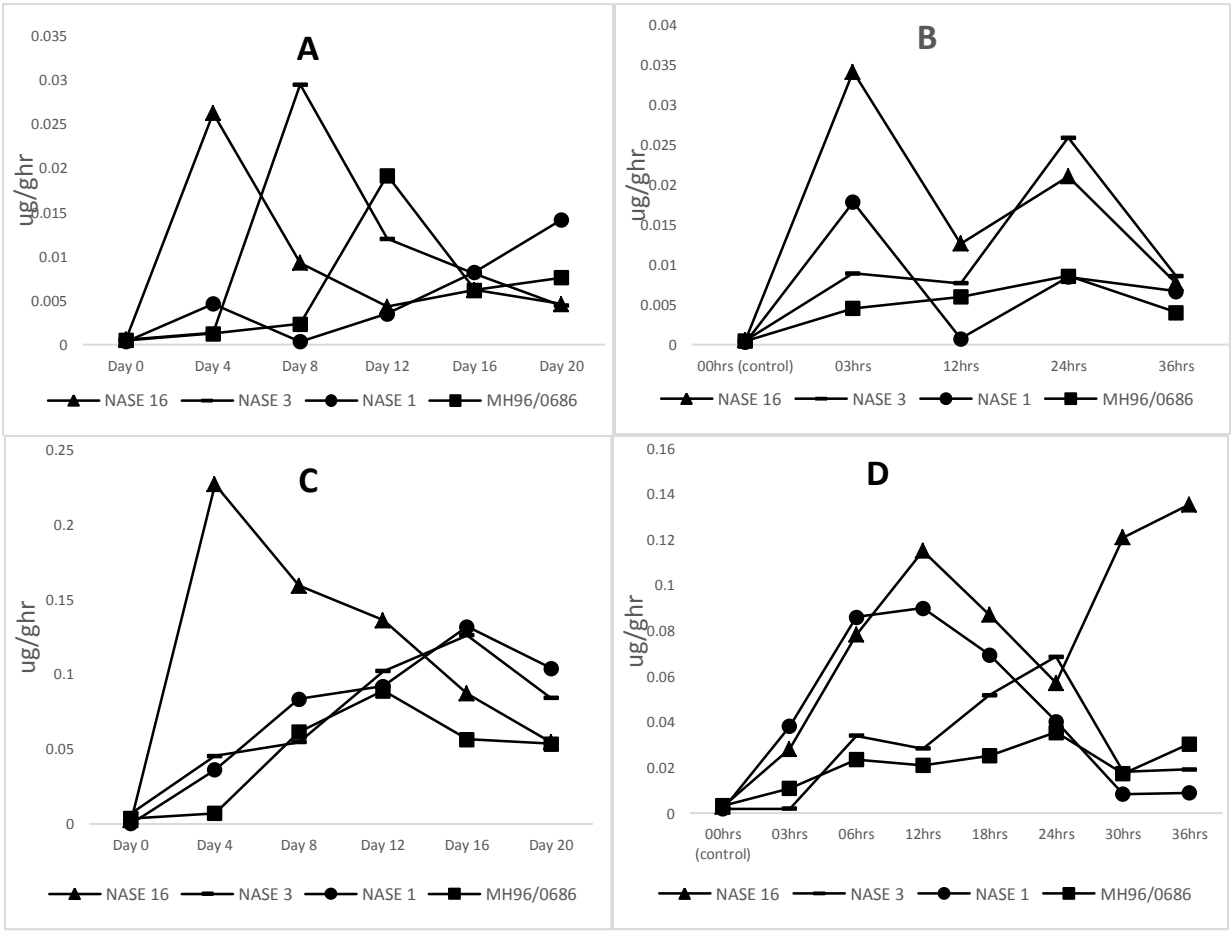

Figure 3. Response of antioxidant enzymes to hydrothermal stress among different cassava varieties. A=Catalase response to moisture stress, B=Catalase response to thermal stress, C=Peroxidase response to moisture stress, D=Peroxidase response to thermal stress. Note: Zero (0) denotes the control.

From the foregoing observations on protein content and enzyme activity changes under hydrothermal stress, it is reasonable to infer that the plants undergo physiological re-adjustments when faced with either water or thermal stress, yet the re-adjustments slightly differ. Among the physiological changes the following may be suggested: a) synthesis of stress-response proteins; b) shutting down of wasteful pathways; and c) giving priority to such metabolic pathways that will enable the plant to tolerate the stress without increasing the demand on energy. The initial rise in protein content at the onset of water stress suggests a physiological response whereby, plants synthesize stress-response proteins and their products. The increase in protein among the stress tolerating varieties suggests a physiological adjustment where plants use proteins as the major source of both carbon and energy. This appears to be the case since the rate of photosynthesis is greatly reduced (explained in detail below). The direct consequence of reduced photosynthesis would be the depletion of storage carbohydrates as well as the simple sugars, leaving proteins as the available source of carbon, nitrogen, phosphorous and sulphur. Thus, the plants would mobilize amino acids endogenously by breaking down endogenous proteins to supply the necessary elements as well as amino acids. While such elements as phosphorous and sulphur would be used in the synthesis of high energy compounds [ATP, GTP and ADP] and osmolytes [glutathione, proline], respectively, the amino acids are required to synthesize new stress-responsive proteins and enzymes. Further, the amino acid pool has also been shown to activate the nitrogen storage mechanisms, in preparation for stress recovery (Fujiki *et al*., 2000). Moreover, proteins which have important hydrophilic and colloidal properties that increase the water retention capacities of the cell (Luo and Huang, 2012) are also produced.

The initial rise was followed by a fall in protein content which seemed to suggest the shutdown of particular pathways that would result in continuous loss of energy by the plant amid stress. With compromised photosynthesis amidst water stress, increased respiration is inevitable resulting into loss in the available carbon and energy resources. This in turn leads to the use of alternative energy sources that involve the remobilization of

structural and storage proteins, hence fall in total protein contents. On the other hand, protein break down comes with an added cost: harmful waste by-products such as reactive oxygen species (ROS), nitrous oxide (NO), among others, are released. If left unattended, these harmful by-products react with valuable structural proteins leading to protein hydrolysis and degradation. The degradation of these proteins result into production of reactive oxygen species still in particular, nitrous oxide which have detrimental effects on membranes and other cell components. Even then plants respond to the buildup of such reactive radicals by productions of antioxidant enzymes such as catalase and peroxidase as observed in Figure 3. It is not surprising that high levels of these enzymes were observed under both water and thermal stress. The antioxidant enzymes remove the harmful products of degradative pathways such as reactive oxygen radicals and hence protect cell membranes from oxidative damage.

Differences observed among varieties after stress exposure may also point to differences in the deployed genetic mechanisms for stress with a different stress tolerance strategy pointing to variety specific stress tolerance strategies. Delayed responses to protein increase during stress for NASE 3 compared to NASE 16 is one such difference in the stress tolerance strategy where NASE 3 was stay green displaying a tolerance strategy and NASE 16 displayed an avoidant strategy. Lack of specific coping mechanisms in the susceptible variety NASE 1 was revealed by reductions in protein over the stress period.

Relationship between chlorophyll and carotenoid concentrations and stress resilience

Using the non-destructive method involving the chlorophyll meter (SPAD 502-Illnois-USA) (Percival *et al*., 2008), leaf chlorophyll contents were determined for both stressed and control plots. The total chlorophyll content (SPAD values) was high among the stay green varietes MH96/0686 (average relative chlorophyll content of 40.2) and NASE 3 (average average relative chlorophyll content of 38.1) while it was low in the early recovering variety NASE16 (average average relative chlorophyll content of 34.9) (Table 1). Generally total chlorophyll contents reduced with plant age particulary in the early recovering variety NASE 16, where the chlorophyll contents reduced by more than 20% reductions over the experimental period from a relative chlorophyll content 39.6 to 19.9 with a higher rate of reduction in the experimental plots (rate=-2.01/week) compared to the control (rate = -1.06/week) and representing an average decline of 32.87%. Earlier during onset of stress, the percentage reduction in chlorophyll content in this variety was at 27.93% while later on during the stress period, the chlorophyll content losses were averaged at 35.99% showing an incremental reduction over the moisture stress period.

Table 1. SPAD values (relative chlorophyll content) for the different treatments across the different varieties

Variety	Treatment	Day 1	Day 5	Day 10	Day 15	Day 20	Average
NASE 16	Stress	39.75±2.26	29.7±0.31	24.52±0.05	21.93±0.06	19.93±0.27	27.17
	Control	39.59±0.86	36.81±1.76	34.65±1.77	33.06±0.57	30.56±3.14	34.93
NASE 3	Stress	39.18±0.87	30.81±0.11	29.31±0.04	30.35±3.41	28.33±0.27	31.60
	Control	40.05±1.77	39.66±0.16	36.43±1.51	37.13±2.43	37.26±1.43	38.11
NASE 1	Stress	34.39±0.84	32.29±0.48	26.48±0.37	24.81±2.11	25.68±0.44	28.73
	Control	34.84±1.33	34.63±0.44	36.22±0.07	39.07±0.38	36.03±0.22	36.16
MH96/0686	Stress	41.67±2.51	33.85±0.76	28.93±3.02	27.83±2.07	28.58±0.05	32.17
	Control	43.27±0.76	42.27±2.45	39.78±0.98	38.42±1.06	37.26±1.43	40.20

In the stay green variety MH96/0686, the relative chlorophyll content reduced from 41.7 to 32.2 and the rate of reduction was -0.879/week in control experiment while in the experimental plots, the rate of reduction was -1.432/week. The average chlorophyll content was reduced by 23.70% in the experimental plots compared to the control plot with earlier reductions of 21.17% while it was 25.6% later during water stress time. In NASE 3, the chlorophyll content also reduced in the experimental plot from a relative chlorophyll content of 39.2 to 31.6 and the rate of reduction was lower in the control plots (-0.363/week) compared to the experimental plots (-0.938/week). In the susceptible variety, low chlorophyll contents were observed before on set of stress compared to other varieties. Reductions were also observed for the chlorophyll content in NASE 1 from a relative chlorophyll content of 34.4 to 25.8 while the average reductions over the experimental time was 24.42% with low reductions earlier during stress time (average 5.53%) and higher reductions later (33.13%).

The results for total leaf chlorophyll content under thermal stress are presented in Table 2. There was a general decline in chlorophyll content over the 36h thermal stress period. The stay green varieties (NASE 3 and MH96/0686) maintained a higher average relative chlorophyll content value (38.75) even through the experiment compared to the early recovering variety NASE 16 (33.6) and the susceptible variety (32.7). High percentage reduction in chlorophyll content was observed for NASE 16 (25% reduction) and the stay green varieties NASE 3 (12.8%) and MH96/0686 (16.7%) compared to the control. The susceptible variety NASE 1 presented much lower chlorophyll content values with even lower chlorophyll losses (11.7%). Variations were also observed among the test varieties in terms of rate of loss of chlorophyll over time. High reduction rates were observed for NASE 16 (-

0.325 relative chlorophyll content/h) and MH96/0686 (-0.286 relative chlorophyll content/h) compared to NASE 3 (-0.173 relative chlorophyll content/h) and NASE 1(-0.151 relative chlorophyll content/h).

Table 2. Leaf total chlorophyll content measurements for the treatment plots under thermal stress

Variety	00hrs	3hrs	6hrs	12hrs	24hrs	30hrs	36hrs
NASE 16	39.67±0.11	37.01±0.28	34.08±0.81	33.18±0.17	31.12±0.79	30.99±1.34	29.50±0.29
NASE 3	39.62±0.62	39.93±0.38	37.52±1.53	37.04±0.13	35.83±0.61	35.43±0.26	34.63±0.04
NASE 1	34.63±0.32	35.22±0.83	33.26±0.06	33.39±0.45	32.17±0.19	31.22±0.12	30.60±0.43
MH96/0686	42.47±1.13	43.17±1.27	41.36±2.24	39.06±0.91	38.33±1.52	35.08±0.46	35.52±1.11

Note: SPAD values at 00hrs are average values for the control plots for each of the varieties tested.

Depending on the percentage changes observed between the control and the stressed experiment, it was observed that a high rate of loss in chlorophyll over the stress period was for the susceptible variety (4.222 relative chlorophyll content/day) compared to the early recovering variety NASE 16 (3.4176 relative chlorophyll content/day) and the stay green varieties NASE 3 (1.6503 relative chlorophyll content/day) and MH/0686 (1.7322 relative chlorophyll content/day). The high losses per day for NASE 1 may further point to lack of specific coping mechanisms as earlier observed.

Different reactions were observed for the effect of moisture stress on the amount of carotenoids in cassava leaves in the different varieties (Fig 4A). A general decrease was observed for NASE 3 (from 0.58 µg/g to 0.37 µg/g at the end of stress period) and NASE 16 (from 0.55ug/g to 0.36 µg/g at the end of stress period) while in NASE 1 the carotenoid content reduced from 0.37 µg/g to 0.33 µg/g at the end of the stress period. However, an increase in the carotenoid content was observed in NASE 16 mid way the stress period at day 12 reaching the peak at 0.64 µg/g. This increment differentiated NASE 16 from other test varieties showing a different tolerance mechanism to stress. On the other hand, all other varieties reduced their carotenoid contents along the stress period. Under thermal stress carotenoid content differed among the different test varieties with a general initial increase in the carotenoid content followed by a reduction after three hours for NASE 3 (from 1.52µg/g to 0.2µg/g) and NASE 16 (from 1.16µg/g to 0.3µg/g) and after 6 hours for MH96/0686 (from 2.23µg/g to 0.43µg/g). In the susceptible variety NASE 1, the carotenoid content reached the peak after 12hours at 0.85 µg/g and there after dropped to 0.5 µg/g. In other varieties, the final carotenoid values were lower than the original carotenoid composition except for NASE 1 (Fig 4B). In addition, reductions of up to 45% percent were observed for NASE 3 and NASE 16 while reductions of up to 50% were observed for MH96/0686 compared to, slight reductions (10%) were observed in NASE 1.

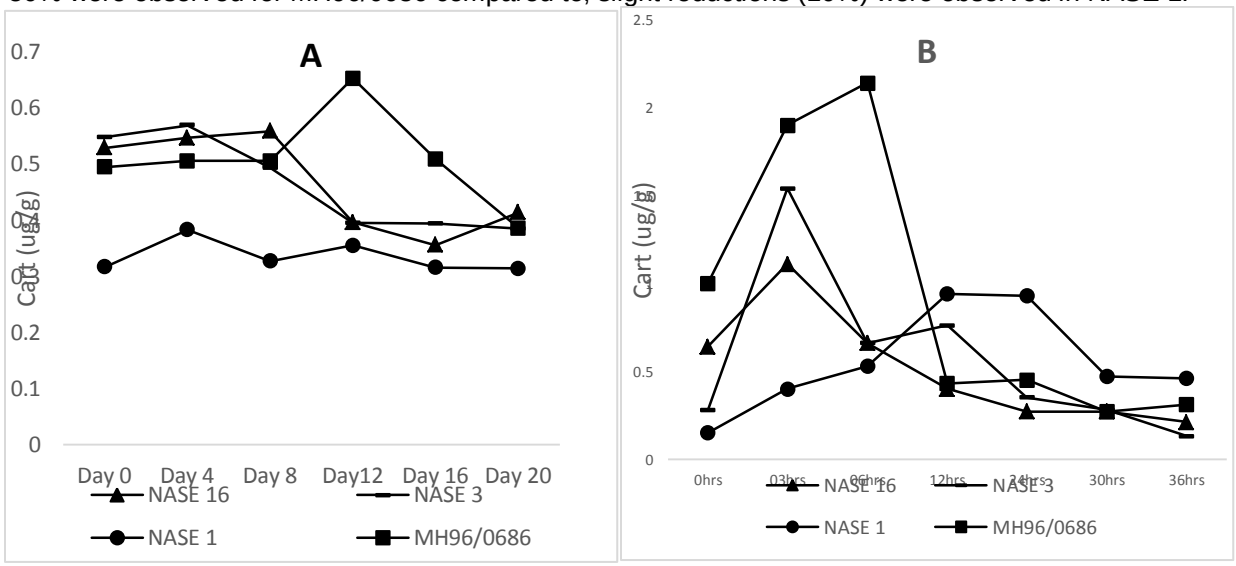

Figure 4. Effect of moisture stress (A) and thermal stress (B) on the carotenoid content of cassava leaves for the different test varieties at different times.

As already pointed out, the reductions in total chlorophyll content observed during stress also indicate a fall in photosynthesis, which in turn, affects glucose production and hence energy. Consequently, major physiological adjustments must be made if the plant is to survive the stress . The reduction in production of energy compounds would result in the use of proteins as both carbon and nitrogen sources as has been observed (Section1). This leads to adjustments in the phenotype such as the reduction in the photosynthetic surface due to reduced pigment

concentration. The adjustments help the plant to carry out basal metabolic reactions as a result of a shut down in major biosynthetic pathways that would involve the production of structural compounds such as chlorophylls and carotenoids. The relevance of such a course of action by the plant lies in the savings from such pathways which allow the plant to withstand periods of insufficent water supply or higher than normal temperatures. The continuous and steady reduction in chlorophyll content could also be attributed to the loss of actively photosynthesing leaves and the dechlorphyllation of already existing leaves along the stress period (Rolland *et al*., 2002). However, the immediate increase in carotenoid content on plant exposure to stress may explain the supplementary role of carotenoids and related compounds in protection of chlorophyll and other cell constituents against oxidative damage. In addition, the reduction in the carotenoid content over the stress period could be attributed to possible break down of pigments and poor re-synthesis/rejuvenation during stress (Alves *et al*., 2004). The high carotenoid contents observed before stress in the stay green and early recovering varieties compared to susceptible varieties points to use of these pigments as key selection tools for moisture stress tolerant varieties (Wahid and Ghanzfar, 2006).

Notably, the different reactions to thermal and water stress shown by cassava varieties were indicative of variety based differences in pigment content. Low reductions in relative chlorophyll content among the susceptible varieties under moisture stress could have been due to maintenance of low chlorophyll content leaves over time compared to the stay green and early recovering varieties which had higher chlorophyll content earlier on imposition of stress. Maintenance of high levels of chlorophyll in stay green varieties and early recovering varieties would thus be crucial in the selection for drought tolerance (Farshadfar, 2012). In addition, Nuwamanya *et al*., (2014b) has shown that the low chlorophyll contents in the leaves impact negatively plant tolerance moisture stress. Further, it has been established that moisture stress has significant effects on the plants photosynthetic apparatus even in varieties displaying particular tolerance characteristics. The results revealed a high response for both the stay green and the early recovering mechanism to moisture stress, an indicator of the ability of these varieties to respond earlier than the susceptible varieties.

In NASE 3, MH96/0686 and NASE 16, the observed reduction in the carotenoid content could be attributed to possible break down of pigments and poor re-synthesis during stress (Alves *et al*., 2004). However, high carotenoid contents observed before stress in the stay green and early recovering varieties compared to susceptible varieties suggested that carotenoids can be used as one of the selection tools for moisture stress tolerant varieties (Wahid and Ghanzfar, 2006). Furthermore, the delayed reduction in carotenoid content in MH96/0686 may have been due to its higher resilience towards stress mediated reduction of carotenoids. Such delayed response in MH96/0686 explains the stay green trait observed in this variety since carotenoids act as protective pigments for chlorophylls which are correlated to the stay green trait (Havaux, 1998). The low carotenoid levels observed in NASE 1 suggested that susceptible varieties are deficient in the protective role of carotenoid on chlorophyll and cell membrane components, specifically integral membrane proteins and other lipid components that make and support cell membranes. (Wahid *et al*., 2007). The reductions in chlorophyll and carotenoid content in NASE 16 may explain earlier loss of leaves in both the moisture and temperature treatments since these pigments are important in growth and development (Bray, 2002).

Relationship between biomarkers and phenotypic characteristics

To establish the relationship between biomarkers and phenotypic traits, plant growth characteristics including plant morphological properties such as: growth rate, leaf fall and leaf area expansion were determined as described in materials and methods (Section 3.). Table 3 shows the observed morphological characteristics in response to hydrothermal stress. Under stress, the growth rate reduced and low positive rates were observed for the stay green varieties (2.04 cm/week for NASE 3 and 2.78 cm/week for MH96/0686) and the susceptible variety (3.9 cm/week) while the early recovering variety NASE 16 had negative growth rate (-0.28cm/week). Compared to the control, the growth rates were significantly reduced in the stress experiment (by between 60-80%) with more reductions observed for NASE 16 compared to the NASE 3, MH96/0686 and NASE 1 (Fig 5). The petiole length, an indicator of leaf extension away from the stem to allow maximum leaf area index and hence light capture (Thomas *et al*., 1999) was significantly (*P=.042)* reduced in stressed plants among all the varieties compared to the control. Due to leaf fall and compromised growth, negative incremental rates in petiole length were observed in NASE 16 (-0.175 cm/week), NASE 3 (-1.125 cm/week) and MH96/0686 (-0.347 cm/week), (Table 3).

Table 3. Growth rates per four days (changes in gradient) for the different morphological parameters.

Variety	Treatment	Plant height	Leaf lobes numbers	Petiole length	Leaf lobe length	Leaf Lobe Width	Leaf No.	Leaf scars
NASE 16	Control	11.13	0.55	0.47	-0.08	0.16	3.33	0.45
	Stressed	-0.28	0.23	-0.18	-0.55	-0.18	1.59	0.93
NASE 3	Control	6.75	0.54	0.47	0.33	0.30	1.99	0.37
	Stressed	2.04	0.24	-1.13	0.43	-0.08	1.02	0.53
NASE 1	Control	7.07	0.83	1.29	1.13	0.32	1.24	0.48
	Stressed	3.90	0.04	0.46	0.28	-0.07	0.43	0.68
MH96/0686	Control	6.70	0.81	0.26	-0.45	0.32	2.10	0.50
	Stressed	2.78	0.27	-0.35	-0.93	-0.16	1.47	0.93

The susceptible variety NASE 1, however, had no significant changes in petiole length. Differences in the rate of petiole length increments between the stressed and the control experiment were however higher in NASE 3 and NASE 1 compared to NASE 16 and MH96/0686.

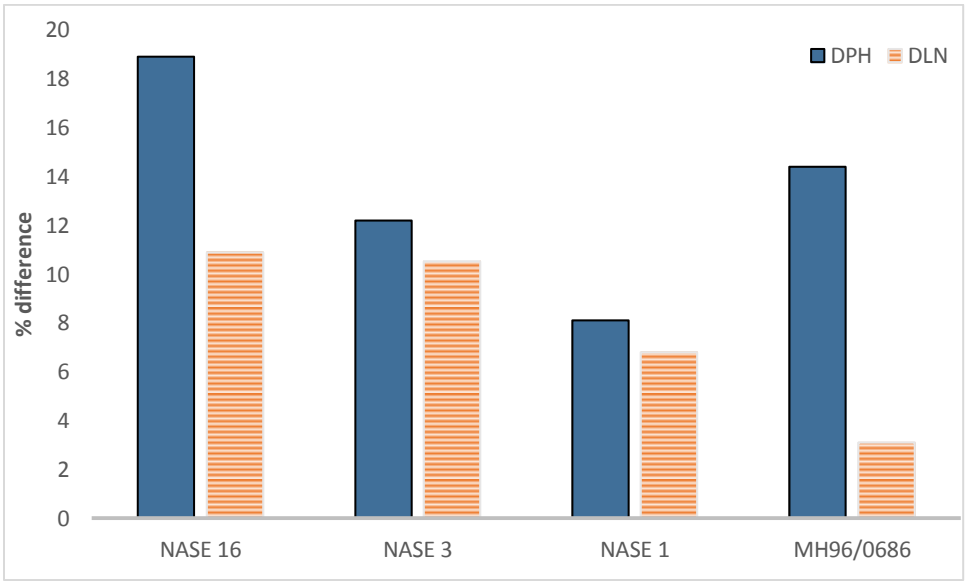

Figure 5. Average difference between stressed and control plots for plant height (DPH=% Difference in Plant Height) and leaf number (DLN=% difference in leaf number) among test varieties

Although the plants in all varieties exhibited the same growth pattern, differences were observed in the rate of accumulation of leaves between the varieties and between the stressed and control experiment (Table 3). As expected, accumulation of leaves over time was low among the stressed varieties., The rate of leaf accumulation was low in NASE 16 and NASE 1 (0.23 leaves/ week and 0.04 leaves/ week respectively) compared to the stay green varieties NASE 3 and MH96/0686 (0.24 leaves/ week and 0.27 leaves/week respectively). Differences were also observed among varieties for leaf loss where NASE 16 and MH96/0686 had higher rates of leaf loss (0.925leaves/ week and 0.933 leaves/ week respectively) compared to NASE 3 and NASE 1 (0.525 leaves/ week and 0.675 leaves/ week respectively). Leaf fall among the different test varieties was observed as leaf scars on individual plants under study. The number of leaf scars was high among the stressed plots (0.53-0.93 leaf scars on average) compared to the control (0.37-0.5 leaf scars on average) in all varieties and so did the rate of accumulation of leaf scars.

Leaf expansion rates due to increased cell division were measured as either leaf lobe length or width and used to determine the effect of stress on leaf development as earlier suggested (Thomas *et al*., 1999). In this study, reductions in the expansion rate where observed under stress for all varieties but especially for leaf lobe length (Table 4). Reduction in Leaf lobe length was low in the stay green varieties NASE 3 and MH96/0686 (-3.86 %/week and -6.11 %/week respectively) compared to the early recovering variety NASE 16 (-9.61 %/week). A high rate of reduction observed in NASE 16 may be due to loss of most leaves as stress time increased and maintenance of the only new small flag leaves (Table 4). Like leaf lobe length, significant (*P=.012)* reductions in leaf lobe width expansion were also observed in stress experiment compared to the control. This was more pronounced in NASE 16, MH/0686 and NASE 1 varieties (4.21 cm/week, 4.43 cm/week and 2.94 cm/week respectively) compared to

NASE 3 (-0.29 cm/week). Reductions in leaf sizes (length and width) under stress conditions may explain the avoidance strategy of stress tolerance, which involve reduction in the transpirational surface at the expense of reduced photosynthesis as earlier observed by Thomas *et al*., (1999). Reductions in leaf sizes under stress may also be due to physiologically mediated reductions in leaf area as observed in the stay green varieties, which maintain basal metabolism as the plant awaits for relief from stress. Such morphological adjustments; involving the reduction in total leaf area and/ or loss of all leaves, are important in reducing the available transpiration surface, which in turn, increases water use efficiency in the plant (Borell *et al*., 2000). It was also observed that moisture stress resulted into loss in the number leaf lobes, a distinctive property in cassava, which allows cassava plants to reduce the surface exposed to stress and hence clearly define the avoidance strategy as observed in NASE 16.

Table 4. Leaf expansion rate (%) and differences between leaf expansion in stressed and control experiments

		Average expansion rate %			Difference Stressed Vs Control		
Accession	Treatment	Petiole AER	Leaf length AER	Leaf width AER	Petiole Length	Leaf length	Leaf width
NASE 16	Control	3.68	0.86	5.50			
	Stress	-1.37	-9.61	-4.21	-5.05	-10.47	-9.71
NASE 3	Control	4.59	4.97	6.65			
	Stress	-4.57	-3.86	-0.29	-9.16	-8.82	-6.93
NASE 1	Control	7.96	10.91	8.01			
	Stress	2.74	2.19	-2.94	-5.22	-8.72	-10.96
MH96/0686	Control	3.55	-0.60	10.32			
	Stress	-2.42	-6.11	-4.43	-5.97	-5.52	-14.75

The losses in leaf lobe numbers are preceded by remobilization of nutrients (especially nitrogen) from the leaves to new growing points (Parida, 2007) and may explain the increase in protein observed in the early recovering variety earlier in the stress period. It was noted that although the plants maintained both the leaves and leaf lobes, the remobilization of nitrogen (protein) appeared not to have been affected, rather proteins appeared to serve as substitutive substrates for the generations of ATP as well as GTP for sustained growth. This partly explains the losses in protein observed in stay green and susceptible varieties. Moisture stress effects on leaf expansion rates are thus important in defining the biochemical response of cassava plants to stress as was earlier suggested by Thomas *et al*., 1999.

As earlier observed, these loss in plant morphological properties, especially leaf size, were negatively correlated to chlorophyll content(r=-0.659), and positively correlated to leaf antioxidant enzyme (Catalase [r=0.876] and peroxidase [r=0.569]) activity. These correlations show that the chlorophylls and antioxidant enzymes are important factors that determine the ability of the plant to maintain required photosynthetic and transpiration surface that are essential for carrying out basal metabolic processes during stress. The reductions in chlorophylls henceforth results into reduced leaf size and reduced plant metabolism, while the increase in the levels of antioxidant enzymes appeared to promote resilience and growth amidst hydrothermal stress by improving and protecting basal metabolic processes. Thus the pigment and antioxidant enzymes are vital biomarkers that influence the observed phenotype in cassava. In addition, the losses observed in biomass (plant height and leaf numbers) may also be due to losses in total chlorophyll content, which would explain the positive relationship (r=.569) observed between plant biomass (leaf numbers) and chlorophyll content. In particular, the reductions observed in plant height, especially in NASE 16, were partly due to loss of most of the floral parts as the chlorophyll content reduced. It was also observed that in the early recovering variety, buds and growing nodes were dormant and did not show any signs of growth with time and thus the low growth rates observed were due to significant arrest of growth due to water stress and due to dormancy of growing portions of the plant during stress time in addition to loss of leaves. As the leaf numbers and sizes reduced the carotenoid contents increased in the early recovering varieties, while in the stay green varieties the carotenoid content reduced. In the early recovering varieties, the protective role of carotenoids is demonstrated, while in the stay green varieties the carotenoid contents appeared not to influence leaf maintenance on the plant. Thus, unlike chlorophylls which appeared to play a vital role of influencing leaf growth and development, the role of carotenoids as a biomarker appeared to be largely protection of new growing points. It was further noted that varieties exhibiting marked differences in leaf growth and yet with the ability to maintain high carotenoid levels, employ the avoidance strategy of stress tolerance.

CONCLUSION

The high accumulation of leaf based biomass including high leaf numbers, high leaf lobe numbers and leaf retention among the stay green phenotypes (NASE 3 and MH96/0686) was related to increased chlorophyll content, a low rate of reduction in leaf protein content, and reduced rate of antioxidant enzyme activity. Such properties were descriptive of a stress tolerant genotype of the stay green varieties. The reduced biomass along hydrothermal stress time was positively correlated to high activities of antioxidant enzymes, high carotenoid content and negatively correlated to chlorophyll concentrations in early recovering varieties (NASE 16). However, the leaf protein content in these varieties, which was initially high, reduced significantly in leaves with hydrothermal stress period. This, in turn, correlated strongly to nitrogen sequestration processes resulting from leaf loss that were also important in stress recovery mechanisms. The loss of proteins from leaves may also be a confounding factor that results into leaf loss as observed. All of these properties defined an avoidance strategy displayed by the early recovering varieties. The lack of such coordinated responses in the susceptible varieties makes them more prone to stressful conditions. Since the properties named above are also easily quantifiable, they can be important biomarkers for selection against hydrothermal stress in cassava.

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Competing interests

No competing interest arose through the carrying out and publication of this work.

Author contributions

Yona Baguma, Samuel Kyamanywa and Ssetumba Mukasa, designed the work and took the leading role in the implementation of the work. Ephraim Nuwamanya and Yona Baguma carried out research and wrote the manuscript. Patrick. Rubaihayo and Joseph Hawumba reviewed the manuscript and regularly advised on the implementation of the work.

REFERENCES

Alves AC, Setter TL.2004. Abscisic acid accumulation and osmotic adjustment in cassava under water deficit. *Environmentaland Experimental Botany (*51):259–271.

Amudha J, Balasubramani G. 2011. Recent molecular advances to combat abiotic stress tolerance in crop plants Biotechnology and Molecular Biology Review. 6(2) 31-58,

Borrell AK, Hammer GL, Henzell RG, Douglas AC. 2000. Genetic variation in transpiration efficiency of stay green and senescent sorghum hybrids grown under post anthesis drought. Crop Science

Buchanan-Wollaston V. The molecular Biology of leaf senescence. Journal of Experimental Botany,(48), 307,pp.181-199,

Farshadfar E. 2012. Application of integrated selection index and rank sum for screening drought tolerant genotypes in bread wheat International Journal of Agriculture and Crop Sciences. IJACS (4) 325-332.

Fujiki Y, Ito M, Nishida I, Watanabe A. 2000. Multiple signaling pathways in gene expression during sugar starvation. Plant Physiol. (124), 1139–1148.

Grover A, Kapoor A, Lakshmi O, Agrawal S, Sahi C, Katiyar-Agarwal S.2001. Understanding molecular alphabets of the plant abiotic stress responses. CurrSci 2001(80):206–216

Hajiboland R, Amirazad H. *2010.* Drought tolerance in Zn-deficient red cabbage (*Brassica oleracea* L. var.capitataf. rubra) plants. *Horticultural Science. (37): 88–98.*

Havaux M. 1998. Carotenoids as membrane stabilizers in chloroplasts. Trends in Plant Science (3):147-151.

Luo X, Huang Q.2012. Studies on cold resistance in cassava. J. Agricscie. 2012(4)6: 104-119.

Mir RR, Zaman-Allah M, Sreenivasulu N, Trethowan R, Varshney R. 2012. Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. TheorAppl Genet (1)

Parida A. 2007. Alterations in photosynthetic pigments, protein and osmotic components in cotton genotypes subjected to short-term drought stress followed by recovery. Plant Biotechnol .Rep. (1), 37–48

Percival CG. Keary IP, Noviss K 2008. The Potential of a Chlorophyll Content SPAD Meter to Quantify Nutrient Stress in Foliar Tissue, Arboriculture & Urban Forestry 34(2):89–100.

Rolland F, Moore B, Sheen J. 2002. Sugar Sensing and Signaling in Plants. The Plant Cell, S185–S205, Supplement, www.plantcell.org

Schoffl F, Prandl R, Reindl A. 1999. Molecular responses to heat stress. In: Shinozaki, K., Yamaguchi-Shinozaki, K. (Eds.), Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. Landes Co, 81–98.

Sthapit BR. 1993. Chlorophyll fluorescence analysis (CFA) for assessing cold tolerance at anthesis in Nepalese indigenous rice genotypes. IRRN (18): 1.

Thomas D, Eamus D, Bell D.1999. Optimization theory of stomatal behavior. I. A critical evaluation methods patterns of carbon assimilation, stomatal conductance and of calculation. J. Exptal Bot (50),235-246

Turyagyenda L, Kizito EB, Ferguson M, Baguma Y, Agaba M, Harvey J. 2013. Physiological and molecular characterization of drought responses and identification of candidate tolerance genes in cassava. *AoB PLANTS*: doi: 10.1093/aobpla/plt007

Wahid A, Gelani S, Ashraf M, Foolad MR. 2007. Heat tolerance in plants: An overview. Environmental and Experimental Botany (61) 199–223

Wahid A, Ghazanfar A. 2006. Possible involvement of some secondary metabolites in salt tolerance of sugarcane. J. Plant Physiol. (163), 723–730.

Wang J, Ruan S, Wu W, Xu X, Wang Y, Han Z. 2010. Proteomics approach to identify differentially expressed proteins induced by iron deficiency in roots of *malus. Pak. J. Bot*, 42(5): 3055-3064.