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DEVELOPMENTAL VARIABILITY IN SOME ENZYMIC ANTIOXIDANT PROPERTIES OF SOME VEGETABLES

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ABSTRACT

The present study focuses on assessing developmental variation in the levels of some enzymatic antioxidants, peroxidative levels and chlorophyll contents in selected vegetables commonly consumed in Nigeria, namely *Amaranthus caudatus (Tete), Celocia agentea (Soko funfun), Celocia argentea, pigmented (Soko olobe)* and *Chorchorus olitorius (Ewedu)*. There was no statistically significant difference ($p>0.05$) in catalase activities in *Amaranthus caudatus* within the first and second week of growth when compared to its activity during the third and fourth week of growth. However, a statistically significant increase ($p<0.05$) in catalase activity was observed in *Celocia argentea* pigmented and *Chorchorus olitorius* during the third and fourth week of growth. A statistically significant ($p<0.05$) as well as progressive increase in peroxidase activity was observed in *Amaranthus caudatus*, *Celocia argentea* and *Celocia argentea* pigmented throughout the experimented period while there was no significant difference in peroxidase activities in *C. olitorius* during the third and fourth week of growth. An increase, (though not statistically significant) was observed in superoxide dismutase activities in *A. caudatus* and *C. argentea* with age. There was a pronounced decrease in the level of lipid peroxidation with age in *A. caudatus* and *C. argentea* when compared to *C. argentea pigmented* and *C. olitorius*. The chlorophyll content, though lowered in *C. argentea pigmented and C.olitorius* was found to increase with age in all these vegetables The extent of lipid peroxidation as indicated by the levels of malondialdehyde formation at the end of four week period was more pronounced in *A. caudatus, C. argentea*, and *C. argentea pigmented* within the first and second weeks of growth than what was observed in *C. olitorius*.. An inverse relationship was observed between the level of malondialdehye formation (an index of lipid peroxidation) and chlorophyll loss (chlorosis) in these vegetables.

Keywords: Developmental variability, antioxidant, vegetables.

INTRODUCTION

Under natural conditions, plants are often exposed to various environmental stresses that decrease productivity (Inaki *et. al*., 1998). At the whole plant level, the effect of stress is usually perceived as a decrease in photosynthesis and growth which is often associated with alterations in carbon and nitrogen metabolism. At the molecular level, the negative effect of stress on leaves, may be in part, a consequence of the oxidative damage to important molecules, as a result of imbalance between production of activated oxygen and antioxidant defenses (enzymatic and non-enzymatic) which may play a critical role in preventing oxidative damage due to the generated activated oxygen in plants

(Bowler *et al.*, 1994; Iannelli *et al.*, 1999). In chloroplast for instance, superoxide radical (O^{-2}) is produced by photoreduction of O_2 at photosystem 1 (PS1) and photosystem II (PSII), and singlet oxygen is formed by energy transfer to 0_2 from triplet excited state chlorophyll (Asada and Takahashi, 1987). Hydrogen peroxide can originate, in turn, from the spontaneous or enzymatic catalyzed dismutation of superoxide radical (O^{-2}). During optimal conditions, plant leaves have been reported to be rich in antioxidant enzymes and metabolites (among which are ascorbate peroxidase (APX), ascorbate (ASC), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), carotenoids (CRD), peroxidase (POX), and α-tocopherol) which can cope with activated O_2, thus minimizing oxidative damage (Yasemin and Serpil, 2005). Most vegetables consumed by human contain a large proportion of indigestible fibrous materials but they are also good sources of minerals, vitamin and proteins. Leafy vegetables are generally used by Nigerians in the preparation of soup, therefore, the leaf protein content of most of them have been determined (Oke,1973; Olatunbosun *et.al*., 1972). Recently, antioxidant potential of some berries (Rani *et al*., 2004) and piper species (Karthikeyan and Rani, 2003) were reported. Though, fruits species and vegetables have been reported to be rich in compounds with antioxidants properties (Halliwell, 1994), however, such studies, to our knowledge have not included variation in the enzymatic antioxidant properties with growth in most commonly consumed vegetables. This study was therefore undertaken to evaluate variation in the level of some antioxidant during growth of some selected vegetables namely *Amaranthus caudatus* (A.C), *Celocia argentea* (C.A), *Celocia argentea pigmented* (C.A P), and *Corchorus olitorius* (C.O), in order to gain an insight into possible inherent protective capacity of the vegetables cells/tissues against oxidative stress.

MATERIALS AND METHODS

Vegetable materials

Seeds of *A. caudatus*(A.C), *C. argentea* (C.A), *C. argentea pigmented* (C.A P) and *C. olitorius* (C.O) were purchased from Ogun-State Agricultural Development (OGADEP) office, Kuto, Abeokuta, Nigeria. They were confirmed to be viable by following the method described by Adetiloye, *et al.* (1989). The vegetables were sown in a pot (60cm x 60cm) filled with soil under natural conditions within the farmland of the University of Agriculture, Abeokuta.

Experimental Design

The experiment was designed as a randomized complete block with four replications of each vegetable for each harvest intervals. The vegetables were harvested weekly after germination for a period of four weeks and the leaves were used for analysis.

Plant Sample Extraction

Each of the four vegetable samples was prepared for enzyme assays according to procedure described by Rani *et. al.* (2004) except when otherwise stated. The sample were prepared by grinding one gram (1g) of fresh vegetable in 2ml of 50% ethanol, separately in a pre-chilled mortar and pestle and the extracts were centrifuged at 5,000g for 10min. The supernatants thus obtained were used immediately for vari-

ous enzyme assays following standard procedures using a Jenway 6405 UV/vis spectrophotometer (Jenway).

Assay of Catalase Activity

Catalase (EC 1.11.1.6) activity was spectophotometrically measured in the plant extract by following the procedures described by Aebi (1983). 150µl of crude extract was added to 3.0ml of substrate mixture containing 85µl of 30% H_2O_2 in 20ml of 50mM phosphate buffer, pH 7.0. The decomposition of H_2O_2 was followed as a decline in absorbance at 240nm for 3 min. One unit of activity was defined as the amount of enzyme that catalyses the decomposition of 1µmol of H_2O_2 per min

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Peroxidase Activity

The assay was carried out by the method of Addy and Goodman (1972). The reaction mixture consisted of 3ml of buffered pyrogallol (0.05M pyrogallol in 0.1M phosphate buffer, pH 7.0) and 0.5ml of 1% H_2O_2. To this was added 0.1ml enzyme extract and change in absorbance was measured at 430nm at 30 sec. interval for 2 min. The peroxidase activity was calculated using an extinction coefficicient of oxidized pyrogallol (4.5 litres/ ml). One unit of activity was defined as the calculated consumption of 1µmol of H_2O_2/min.

Superoxide Dismutase (SOD) activity

The assay of superoxide dismutase (EC1.15.1.1) was carried according to the procedure of Das *et al.* (2000) as described by Rani *et al*. (2004). In this method, 1.4ml aliquots of the reaction mixture (comprising 1.11ml of 50mM phosphate buffer pH 7.4, 0.075ml of 20mM, 0.04ml of 1% (v/v) Triton X-100, and 0.075ml of 10mM hydroxylamine hydrochloride and 0.1ml of 50mM EDTA) were added to 100µl of the sample extract and incubated at 30^{o}C for 5 minutes. 80µl of 50mM riboflavin were then added and the tubes were then placed into a light box, illuminated with 200 W-Philips fluorescent lamps for 10 min. After the exposure time, 1ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the colour formed was measured at 543nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions.

Lipid Peroxidation

Level of lipid peroxidation was estimated in terms of thiobarbituric acid reactive species (TBARS) formed, using malondialdehyde (MDA) as standard by the method of Buege and Aust (1978) as modified by Ohkawa, *et. al*. (1997). 1.0ml of the sample extract was added to 2.0ml of the trichloroacetic acid-thiobarbituric acid -hydrochloric acid [(TCA-TBA-HCl) reagent i.e. (15% w/v TCA, 0.375% w/v TBA and 0.25M HCl)]. The contents boiled for 15min, cooled and centrifuged at 5,000g to remove the precipitate. The absorbance of the supernatant was read at 535nm and the MDA concentration of the sample was calculated using extinction coefficient of $1.56x10^5M^{-1}$ $cm^{-1.}$

Chlorophyll Content

The chlorophyll content of each vegetables sample was extracted following the procedures described by Harborne (1993).To 0.01g of fresh vegetable leaves in 1.0ml of 80% acetone was added sea sand to aid extraction of chlorophyll. This

was then mesh mashed in mortar with pestle, with further addition of 5ml acetone, followed by filtration using whatman No1 filter paper into 10ml measuring cylinder. It was further eluted with addition of acetone and made up to 10ml mark with acetone. Absorbance of the extract was read at 645nm and 663nm while the total chlorophyll in the extract was estimated using the equation below (Harborne, 1993) .

Total Chl. (mg/l)=20.2 (A_{645})+8.02 (A_{663}).

Protein Concentration Determination (water soluble proteins)

The concentration of the water-soluble proteins in the supernatants of each vegetable extracts was estimated by the procedure of Bradford (1976), using bovine serum albumin (BSA) as standard. Each supernatant was assayed three times for both protein concentration and enzyme activities.

Statistical Analysis

The experiment was performed in a complete randomized design where differences among the antioxidants properties of vegetables were tested using SPSS statistical program. Statistical variance analysis of the data with four replicates (n=4) was performed using ANOVA and compared using Duncan multiple range test (1955).

RESULTS AND DISCUSSION

The inevitable generation of Reactive Oxygen Species (ROS) and the oxidative damage in biological system have been reported to be counterpoised by an array of enzymatic defense system (Duncan, 1955; Uday *et.al.* 1990; Foyer *et.al.* 1994). The levels of antioxidants enzymes assessed in different vegetables weekly over a period of four (4) weeks after germination were presented in Tables 1 and 2.

The catalase (CAT) activities in pigmented *C. argentea* and *C.olitorius* were observed to be increasing progressively with age but in *A. caudatus* and *C. argentea,* it decreased in 2nd and 4th respectively. This enzyme activity was highest in A.C, lowest in C.A at the end of four weeks of experimental growth studies. A progressive as well as statistically significant increase ($p<0.05$) in the activity of peroxidase was observed in A.C and C.A over this growth period. A decline in the activity of this enzyme was observed in C.O within the 3rd and 4th week of growth (Table 2). Least activity of peroxidase (POD) was observed in C.A P within the first and second weeks after germination when compared to weeks three and four. There was no statistically significant difference ($p>0.05$) in the activity of SOD in A.C throughout the experimental periods. In C.A the SOD activity increased significantly during the 4th week of growth compared to the earlier growth periods while in C.O no statistically significant differences was observed in the SOD activities at the 2nd, 3rd and 4th week of growth (Table 2). The chlorophyll contents in the vegetables, though lowest in C.A.P was found not to be significantly different ($p>0.05$) in all vegetables during this experimental period.(Fig.1). More so, the level of MDA formed (an indicator of lipid peroxidation) was observed to decrease significantly ($p>0.05$) in A.C (except during the second week) and C.A with age while in C.A.P and C.O the variation was not consistent (Fig. 2). Regression

analysis between MDA and chlorophyll showed an inverse relationship (Fig. 3).

The increase in superoxide dismutase (SOD) activity is not unexpected since Dhinsa *et al.* (1981) have reported that right from the initial stage of senescence in leaves, SOD activity increased over time. It could therefore be said that, the increase observed in enzyme activities involve with oxidation reactions seems to be universal in that Bowler (1994), Rani *et al.* (2004) and Yasemin and Serpil (2005) had reported this phenomena for CAT, SOD and POD. Moreso, the increases are likely responses to increase in oxygen radicals' production in these vegetables which could have lead to yellowing if not curtailed. A balance in the activities of these enzymes is also critical to effect antioxidant activity and peroxide detoxification. For instance, if SOD activity far exceeds the capacity for POD to detoxify the H_2O_2 formed through SOD action on superoxide ions, then H_2O_2 can react with superoxide anions directly to produce singlet oxygen and OH^- radicals which are very active in lipid peroxidation. A decline in activities of antioxidant enzymes at some period in this study is in agreement with the report of Toivonem and Sweeney (1998) that a decline in antioxidant enzyme activity is often experienced during the period at which the tissue losses some of its self-regulatory capacity. The level of malondialdehyde MDA concentration formed within the 1st and 2nd weeks was observed in A.C and C.A to be higher than what was determined in 3rd and 4th weeks after germination. This could be attributed to a relatively low level of antioxidant enzymes activities within these periods thus, pave way for the formation of hydroxyl radicals that enhances lipid peroxidation. However, the MDA concentration within the 3rd and 4th weeks was quite lower than the 1st and 2nd weeks. The increase in chlorophyll content in these vegetables over time is of great importance to support photosynthetic activities within these vegetables. The observed relative lower chlorophyll content in C.A P could be attributed to the presence (at lower concentrations) of other pigments as evidence in the vegetable leaves color.

It is interesting to note that the vegetables used in these studies did not show visible signs of yellowing during and by the end of the experimental period, thus, confirming the role of this antioxidants system in controlling chlorophyll loss (yellowing) as a consequence of the systems ability to modulate other phenomena associated with endogenous capacity for survival.

In summary, this study has shown that among the enzymatic antioxidant studied in these vegetables, CAT and POD in A.C and SOD in C.O were predominant. The ability of the extracts of these vegetables in curtailing the in-vitro lipid peroxidation (especially at maturity, 4th week) with concomitant increase in antioxidant enzymes activities, points out the antioxidant potential of these vegetables, thereby emphasizing the importance of incorporating these vegetables as a regular components in diet. Furthermore, these vegetables could be exploited for commercial purification of specific antioxidants since they are quite present / available in them.

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Table 1:Catalase and Peroxidase enzymes activities in different vegetables within four weeks of growth

	CATALASE (units\mg)				PEROXIDASE (units\mg)			
	1	2	3	4	1	2	3	4
Weeks vegeta-bles								
A.C	0.24 ± 0.002^{a}	0.16 $\pm1.85\times10^{-3a}$	0.86 ± 0.035^{b}	1.27 $\pm6.67\times10^{-3c}$	0.36 $\pm5.77\times10^{-4a}$	1.24 $\pm2.67\times10^{-3b}$	2.17 $\pm3.49\times10^{-2c}$	2.35 $\pm2.88\times10^{-2c}$
C.A	0.021 $\pm5.7\times10^{-3a}$	0.026 ± 0.00^{ab}	0.031 ± 0.00^{b}	0.026 $\pm3.0\times10^{-3b}$	0.17 $\pm3.3\times10^{-4a}$	0.267 ± 0.00^{b}	0.31 ± 0.00^{c}	0.915 ± 0.00^{d}
C.A P	0.015 ± 0.00^{a}	0.054 $\pm3.3\times10^{-4b}$	0.21 $\pm8.8\times10^{-4c}$	0.27 $\pm8.81\times10^{-3c}$	0.082 $\pm1.67\times10^{-3a}$	0.077 $\pm1.15\times10^{-3a}$	0.57 $\pm5.2\times10^{-3b}$	0.57 $\pm2.73\times10^{-3b}$
C.O	0.022 $\pm2.33\times10^{-3a}$	0.064 $\pm3.6\times10^{-3a}$	0.236 $\pm2.5\times10^{-2b}$	0.254 $\pm2.51\times10^{-3b}$	0.254 $\pm2.03\times10^{-3a}$	0.258 $\pm5.24\times10^{-3a}$	0.11 $\pm1.2\times10^{-3b}$	0.11 ± 0.00^{b}

Values in the same row with the same superscript are not statistically significantly different at $p<0.05$

Table 2: Superoxide dismutase activity in vegetables over a period of four week of growth

	Superoxide Dismutase (units\ mg)			
Weeks / vegetables	1	2	3	4
A.C	0.039±0.003[a]	0.102±0.0003[a]	0.107±0.003[a]	0.117±0.0035[a]
C.A	0.086±0.003[a]	0.12±0.0067[a]	0.112±0.0011[a]	0.127±0.0003[a]
C.A.P	0.137±0.033[ab]	0.123±0.067[ab]	0.127±0.028[b]	0.19±0.035[b]
C.O	0.163±0.024[a]	0.132±0.001[b]	0.422±0.017[b]	0.263±0.012[b]

Values in the same row with the same superscript are not statistically significantly different at $p<0.05$

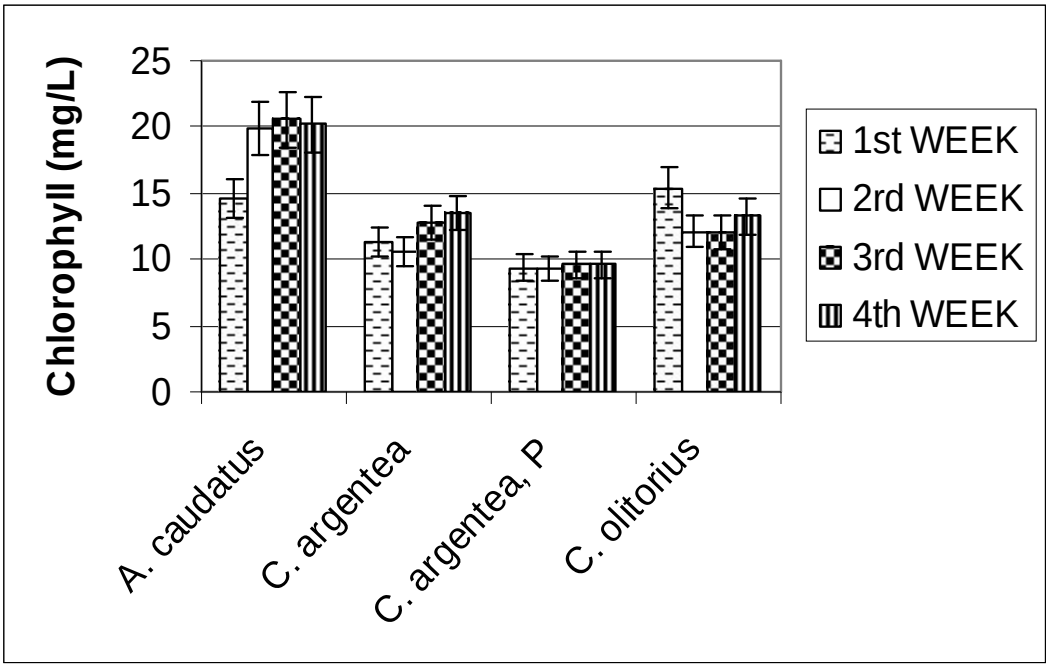

Figure 1:Variation in chlorophyll levels in different vegetables over a period of four weeks

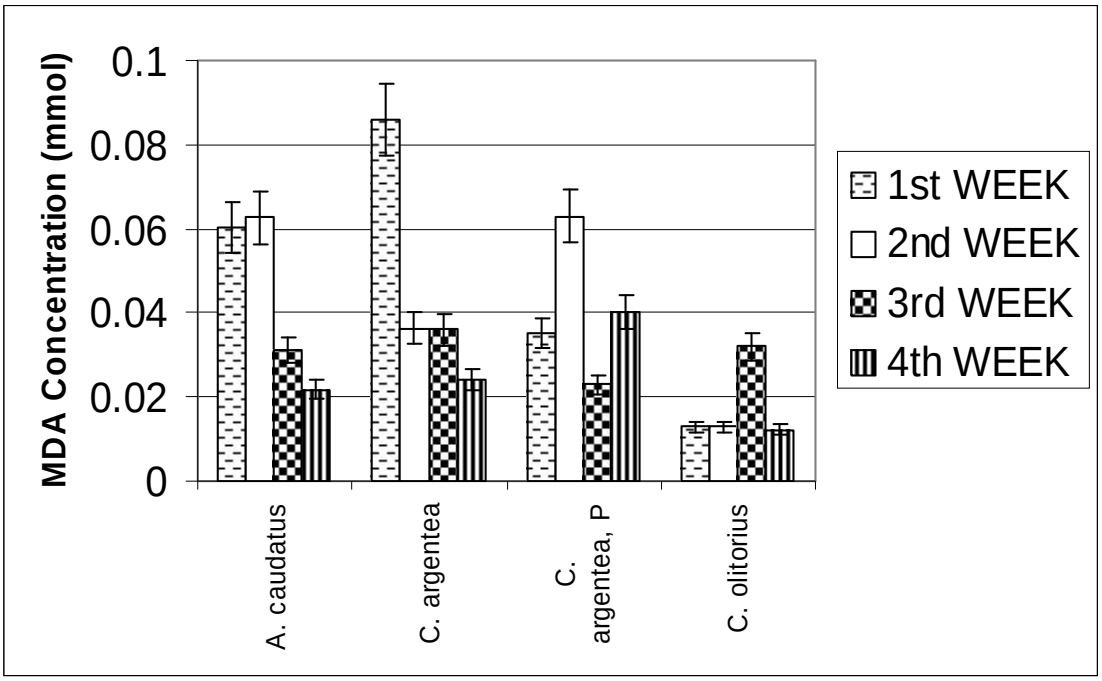

Figure 2: MDA levels in vegetables during four weeks of growth

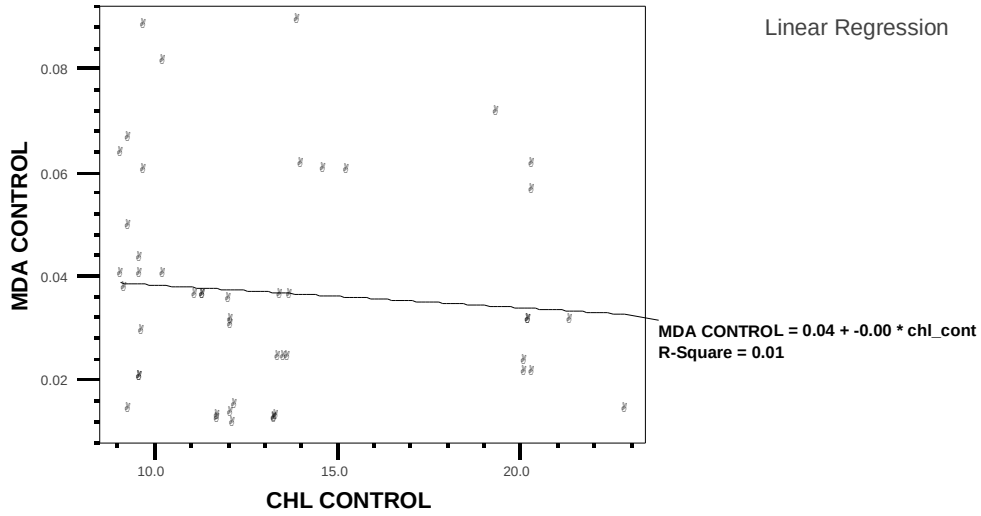

Figure 3: Correlation between the levels of MDA and chlorophyll formation among different vegetables over a period of four weeks