AJSR AFRICAN JOURNAL OF SCIENCE AND RESEARCH

African Journal of Science and Research,2015,(4)5:18-23
ISSN: 2306-5877
Available Online: http://ajsr.rstpublishers.com/

POTENTIAL OF ANTIOXIDANT ENZYMES IN DEPICTING DROUGHT TOLERANCE IN COCOA (*THEOBROMA CACAO* L.) GENOTYPES AT YOUNG AGE.

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Received:25,Aug,2015 ***Accepted:04,Oct,2015.***

Abstract
In an effort to determine the biochemical markers for screening The obroma cacao L. for drought tolerance, changes in lipid peroxidation and activities of antioxidant enzymes were assessed in the seedlings of ten cocoa genotypes in normal and water deficit conditions. In comparison with non-stress seedlings, the catalase (CAT) and superoxide dismutase (SOD) specific activities were increased respectively by more than 88% and 82.9% in the leaves of stressed seedlings. However, peroxidase (POX) and polyphenol oxidase (PPO) specific activities were upgraded by only 9.84% and 4.7% in the leaves of stressed seedlings. The comparison of cocoa genotypes under deficit water stress conditions revealed that the genotypes, VTLCH-4, VTLCP-26 and VTLCP-22 shown effective antioxidative potential against the effects of oxidative stress. The observed data showed that status of antioxidant enzymes could provide a meaningful tool for depicting drought tolerant nature of a cocoa genotype.

Keywords: *Antioxidant enzymes, Lipid peroxidation, Cocoa, Drought, Theobroma cacao L.*

INTRODUCTION

Abiotic stresses such as drought causes an imbalance of oxidative metabolism, changes the components of the mitochondrial membrane and limits the transport of electrons through the respiratory chain of cytochromes (Juszczuk *et al*. 2001), therefore, there will be an accumulation of free radicals of oxygen (Wang *et al*. 2009). Oxidative stress results from the generation of reactive oxygen species (ROS), such as superoxide ion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) detrimental to the survival of plants in a stress environment (Luna *et al*. 2004). In response to this toxicity due to excessive accumulation of AOS (active oxygen species), plants at the cellular level establish an effective antioxidative system, which contains the enzymes of superoxide dismutase (SOD, EC1.15.1.1), peroxidase (POX, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) (Foyer and Noctor 2000; Aroca *et al.* 2003). Superoxide dismutase (SOD) enzymes are metalloenzymes, which are the first defense forms formed by catalyzing the dismutation of O_2^- radicals to H_2O and O_2. Martinez *et al.* (2001) studied *Curtilobum solanum* and *Solanum tuberosum* which showed tolerance to water stress due to over production of SOD in chloroplasts. The principal enzymes for the detoxification of hydrogen peroxide (H_2O_2) are catalase, located in the peroxisomes and peroxidases in the chloroplast, the cytosol and the apoplast (Asada 1992). The complex protein catalase and peroxidases act together to thwart the H_2O_2. Catalase detoxifies most of H_2O_2 produced by photorespiration, while peroxidases neutralize H_2O_2 molecules not destroyed by catalase.

Polyphenoloxidase (PPO) is a plastid enzyme generally catalyzes the oxidation of phenolic compounds to quinones using molecular oxygen as an electron acceptor (Sommer *et al*. 2004). These enzymes catalyze both types of oxidative reactions. The hydroxylation of monophenols to o-diphenols, and the oxidation of o-diphenols to o-quinones. According to Rivero *et al*. (2001), the activities of POX and PPO increasing among tomato plants and watermelon in response to cold and heat. The polyphenolic structure enables detoxification of toxic oxygen species.There are many reports in the literature that underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increasing resistant to environmental stress (Bowler *et al.* 1992; Bor *et al.* 2003). All of these mechanisms involved in oxidative defense system against the water shortage and drought were mentioned in many plant species. However, in cocoa, antioxidative responses to water deficit stress remain little known or unknown.This study aims to determine the effect of deficit hydric stress upon the anti-oxidative system of cocoa genotypes by studying the specific activity of the POX, PPO, SOD and CAT to assess the existence of genotypic variability. Meanwhile, the level of malondialdehyde content was measured.

MATERIAL AND METHODS

Plant material

Ten cocoa genotypes (VTLCP-22, VTLCP-11, VTLCP-24, VTLCP-25, VTLCP-26, VTLCP-29, VTLCP-27, VTLCP-28, VTLCH-4 and VTLCH-3) were used in this study. Seeds of hybrids were sown in polybags with a thickness of 5/100[ièm], 20 cm diameter and 35 cm height. Each polybag was filled with 12 kg soil with density of 1.2 g/cm^3. After 60 days in nursery, sorting of plants was done for choosing homogeneous plants.

Experimental condition

The study was carried out at the Regional Station of ICAR- Central Plantation Crops Research Institute (CPCRI), Vittal, Karnataka, India. The relative humidity, average temperature and evapotranspiration during the study period are presented in Fig. 1. The relative humidity ranged between 74.2 and 93.3%, temperature between 25.3 and 30°C and low evaporation indicate favorable conditions for cocoa farming.

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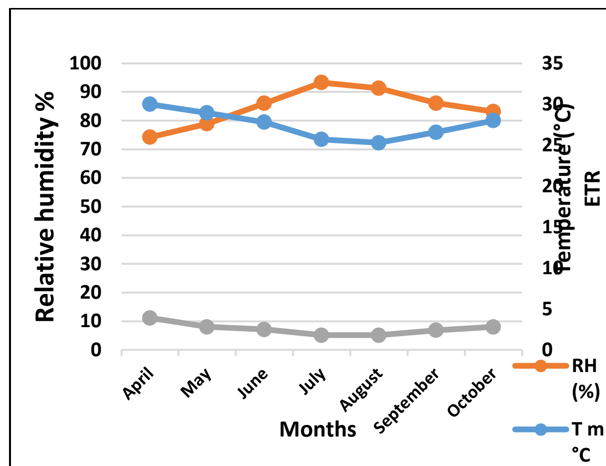


Fig(1)Change in monthly average of relative humidity (RH%) and temperature (T°C) at glass house and evapotranspiration ($ETR^{mmday-1}$) during experiment.

Experimental design

The experiment in pots was laid out in a completely randomized design. Ten cocoa genotypes were used and two levels of watering treatment were practiced with three replicates. A control (100% of Field Capacity) group with daily watering (no stress), where the pot water content was kept wet corresponding to maximal soil field capacity (FC) and a stressed group (20%FC), in which soil moisture was kept at twenty percent with a pot received twenty percent of water added to control pots.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 0.5 g leaf fresh weight according to Heath and Packer (1968). MDA is a product of lipid peroxidation by Trichloro Acetic acid (TCA) reaction. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecified turbidity) by using extinction coefficient 155 $mMcm^{-1}$.

Enzymes assays

Enzyme extract: Cocoa leaf enzyme was extracted by homogenization of 2.5 g leaf tissue in pre chilled motor and pestle with 20 ml 0.1 M sodium phosphate buffer, 0.2 ml β-mercaptoethnol and 1g insoluble polyvinylpolypyrrolidone (PVPP). The supernatant after double centrifugation was collected, then measured by the ammonium sulphate concentration conversion table. After 1 hour, supernatant was centrifuged at 4°C for 30 min. at 12,000 rpm and pellets were re-suspended in 3 ml extraction buffer. The dialyzed enzyme extract was transferred to microtube and used for the assay of scavenging enzymes *viz*., Super Oxide Dismutase (SOD), Catalase (CAT), Peroxidase (POX) and Polyphenol Oxidase (PPO).

Super oxide dismutase (SOD, EC 1.15.1.1) specific activity was assayed by following the method of Beauchamp and Fridovich (1971). 1.6 ml potassium phosphate buffer 1.6 ml, 0.1 ml of solution sodium carbonate (1.5 M) solution 0.1 ml, NBT solution (0.3 ml), methionine solution (0.3 ml), EDTA disodium salt solution (0.3 ml) and 0.1 ml of enzymes extract were used. The enzyme reaction mixture OD was taken at 560 nm by using UV visible spectrophotometer (Shimadzu UV160A, Japan). The specific activity was calculated following this method (100-(OD S/OD LC)* 100)/50= x, where, x/mg protein in enzyme extract= specific activity in units, 1 unit is defined as the 50% reduction in the blue color formed by NBT/ 30minutes/ mg protein.

Catalase (Cat, EC 1.11.1.6) activities were assayed by following the method of Kar and Mishra (1976). The cuvette contained the enzyme extract (0.5 ml), hydrogenperoxide (H_2O_2) (1 ml), distilled water (1 ml) and phosphate buffer (2 ml). Reaction was started by adding enzyme extract and sample was analyzed in UV visible spectrophotometer (Shimadzu UV160A, Japan) at 240 nm. Absorbance was recorded at every 60 seconds against corresponding control of each sample. The specific activity was calculated with the formula ((Ax3)/0.5) = specific activity/ min/ mg protein in enzyme extract, where, A= specific activity.

Peroxidase (POX, EC 1.11 .1.7) activity was checked by the method of Kar and Mishra (1976). Sample solution was constituted by potassium phosphate buffer (2 ml), hydrogenperoxide (H_2O_2) (1 ml) and pyrogallol (1 ml) were mixed together. To this, enzyme extract (0.1 ml) was added and kept at room temperature for 3 minutes for incubation. Reaction was stopped by adding sulphuric acid (0.5 ml). OD reading was taken at 420 nm using "UV" Visible spectrophotometer (Shimadzu UV160A, Japan). The specific activity was calculated by the formula (A x 3/0.1)/ 3 = specific activity/ min/ mg protein. Where, A is difference in absorbance of sample and the control (ODS - ODC).

Polyphenol oxidase (PPO, EC1.10.3.2) was assayed by following the method of Kar and Mishra (1976). The reaction mixture was prepared by adding potassium phosphate buffer (2 ml) and pyrogallol (1 ml) and mixed together. To this, enzyme extract (0.1 ml) was added and kept at room temperature for 3 minutes for incubation. Reaction was stopped by adding sulphuric acid (0.5 ml) and immediately OD reading was taken at 480 nm. The enzyme specific activities was calculated following the method (A x 3/0.1)/ 3 = specific activity/ min/ mg protein, where A = Difference of absorbance of sample and the control (ODS - ODC).

Statistical analysis

The data analysis was performed with SAS 9.3 software to estimate the mean differences between cocoa genotypes under stress condition for distribution of lipid peroxidation and enzymes specific activities. Data collected from each genotype were subjected to analysis of variance using the GLM procedure of SAS. LSD test were used to compare the average at the 5% threshold. In this analysis, the residual variance E1 was used to test the influence of watering regimes (wr) also, where: E1 = rep x wr.

RESULTS

Lipid peroxidation activities in leaves of cocoa genotypes

Lipid peroxidation level in leaves of cocoa genotypes were measured as content of MDA and is given in Figure 2 and 3. In figure 2, the box plot parameters showed a homogeneous distribution of the production of malondialdehyde under deficit water stress induced with a reduction of the interquartile range (min= 0.0301 nmol/ g FM; max= 0.0599 nmol/ g MF; Q3 - Q1= 0.0093 nmol/ g MF) and normal irrigation condition (min= 0.0252 nmol/ g FM; max= 0.0568 nmol/ g MF; Q3 - Q1= 0.0131 nmol/ g MF). Half of the tested genotypes obtained a greater accumulation of leaf malondialdehyde at 0.0408 nmol/ g MF (median) under water stress and higher than 0.0421 nmol/ g MF (median) in normal irrigation condition (Fig.2.)

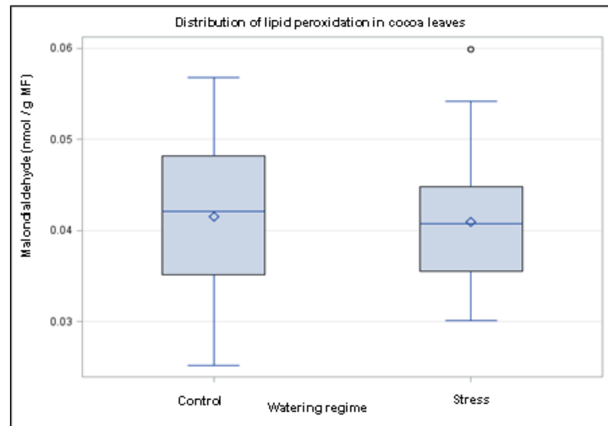
The analysis of variance revealed a high significant effect between interaction watering treatment x genotypes (p= 0.0001) and genotypes

(p= 0.0001). However, the effect of water treatment was not statistically significant (p= 0.855) for leaf lipid peroxidation of cocoa. The Comparison of lipid peroxidation by malondialdehyde accumulation in cocoa leaves indicated significant genotypic differences. The average value of malondialdehyde accumulation which was 0.041 ± 0.008 mg/ g MF in regular water supply condition, fell to an average value of 0.040 ± 0.007 mg/ g MF (Fig. 2) in stress condition.

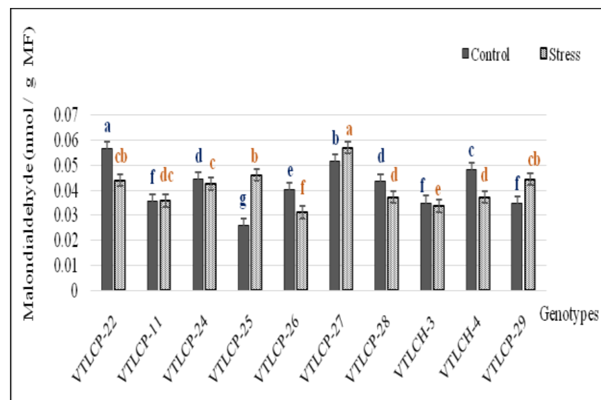
In normal irrigation condition, the genotypes VTLCP-22 (0.056 mmol/ g MF), VTLCP-27 (0.051 mmol/ g MF) and VTLCH-4 (0.048 mmol/ g MF) accumulated high levels of foliar malondialdehyde. However, the genotype VTLCP-25 (0.025 mmol/ g MF) presented a low value. Under hydric deficit stress induced, the genotypes VTLCP-27 (0.057 mmol/ g FM) VTLCP-25 (0.046 mmol/ g FM) and VTLCP-29 (0.044) accumulated a high value of leaf malondialdehyde levels than those of their respective control. However, VTLCP-26 (0.031 mmol/ g MF) and VTLCH-3 (0.033) had small leaf levels (Fig. 3).

Superoxide Dismutase (SOD) specific activities in leaves of cocoa genotypes

Figure (4) shows the distribution of SOD activity in the leaves of cocoa genotypes under hydric stress and non-stress condition. The box plot results showed a comparison of the inter quartile deviation and an increase of SOD activity under hydric deficit stress (mini= 0.3 specific activity; max= 0.718 specific activity; Q3 - Q1= 0.097 specific activity), compared to those in normal irrigation condition (min = 0.0197 specific activity; max = 0.541 specific activity; Q3 - Q1 = 0.233 specific activity).

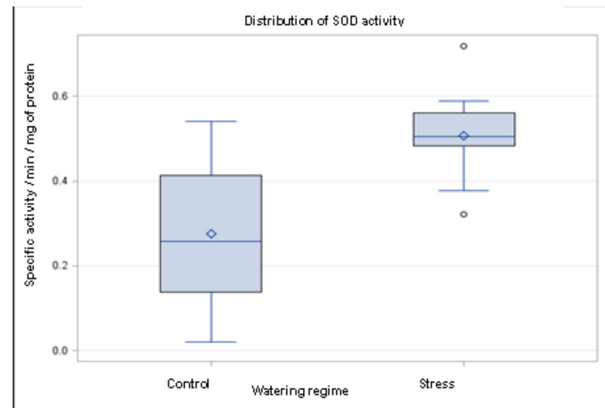


Fig(2)Effect of watering treatment on the distribution of accumulation of malondialdehyde in cocoa genotypes

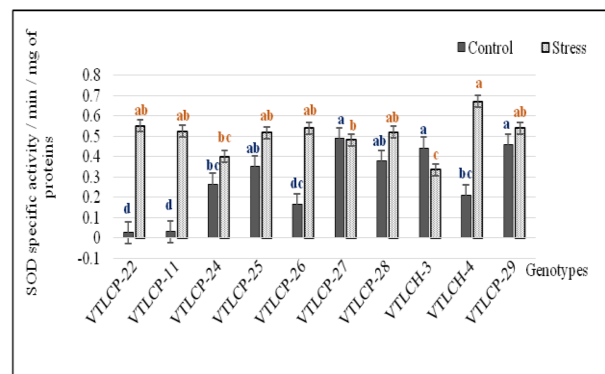


Fig(3)Accumulation of MDA in leaves of cocoa genotypes under hydric treatment. Comparisons of means were done under each watering treatment.Means followed by the same letter are statistically identical at 5% (LSD).

Half of all genotypes tested recorded higher enzyme activity 0.50 specific activity (median) under water stress condition and higher than 0.27 specific activity (median) in control treatment.The average enzyme activity that was 0.281 specific activity in normal irrigation condition, rose to 0.514 specific activity under water stress treatment (Fig. 4). The analysis of variance revealed a highly significant effect of interaction of water treatment x genotype (p = 0.0001), water treatment (p = 0.0003) and genotype (p = 0.0001). A significant deviation of superoxide dismutase activity was observed under both conditions of irrigation (Fig. 5). In regular water condition, genotypes VTLCP-28 (0.48 specific activity), VTLCP-29 (0.457 specific activity) and VTLCH-3 (0.44 specific activity) had the strongest activity of SOD. However, genotypes VTLCP-22 (0.027 specific activity) and VTLCP-11 (0.032 specific activity) had low values of SOD. Under water deficit stress condition VTLCH-4 (0.673 specific activity), VTLCP-22 (0.553 specific activity), VTLCP-26 (0.541 specific activity) and VTLCP-29 (0.541 specific activity) showed strong activity of SOD. The genotype VTLCH-3 (0.337 specific activity) obtained the lowest enzyme activity.



Fig(4)Effect of water treatment on the distribution of SOD specific activity in leaves of cocoa genotypes



Fig(5)SOD specific activity in cocoa genotypes under watering treatment

*** Comparison of means was done under each water treatment. Means followed by the same letter are statistically identical at the 5% (LSD)**

Peroxidase (POX) specific activities in leaves of cocoa genotypes

Figure 6 shows the distribution of the peroxidase activity in the leaves of cocoa genotypes under hydric stress and non-stress condition. The parameters of the box plot showed a greater dispersion of the activity of the POX under hydric stress (min= 0.282 specific

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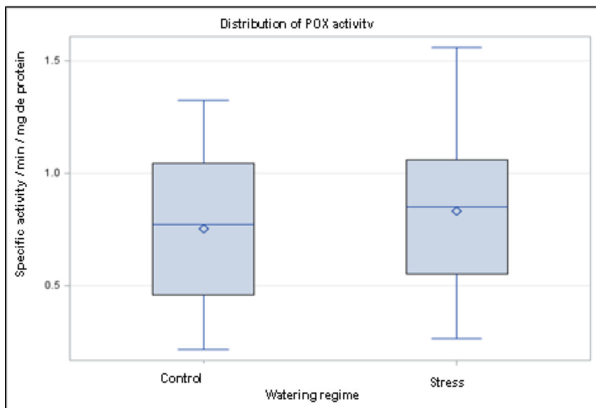
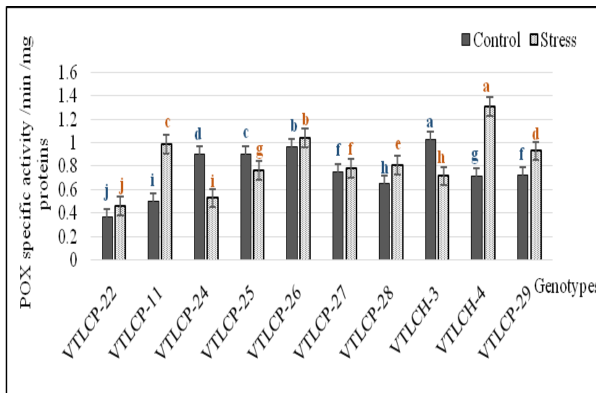
activity; max= 1.56 specific activity; Q3 - Q1= 0.51 specific activity) than non-stress condition (min= 0.214 specific activity; max= 1.324 specific activity; Q3 - Q1= 0.588 specific activity). Half of the tested genotypes were obtained the POX activity greater than 0.848 specific activity (mean) under water stress and specific activity greater than 0.772 specific activity (median) in normal irrigation condition (Fig. 6).

Analysis of variance with two criteria for classification revealed a highly significant interaction effect of the water treatment x genotypes (p= 0.0001) and genotype (p= 0.0001). However, a statistical homogeneity was observed for the effect of water treatment (p= 0.494).

The specific activity of the POX has undergone statistically significant variations according to water treatment and also according to genotypes in each treatment (Fig. 7). In normal irrigation condition, the genotypes VTLCH-3 (1.03 specific activity), VTLCP-26 (0.96 specific activity) and VTLCP-25 (0.90 specific activity) indicated strongest foliar POX activities, unlike genotypes VTLCP-22 (0.365 specific activity) and VTLCP-11 (0.5 specific activity). Similarly, under water deficit stress the genotypes VTLCH-4 (1.309 specific activity), VTLCP-26 (1.041 specific activity) and VTLCP-11 (0.98 specific activity) exhibited high enzymatic activity. The low activity was observed in the genotype VTLCP-22 (0.459 specific activity).



Fig(6)Effect of water treatment on the distribution of POX specific activity in leaves of cocoa genotypes



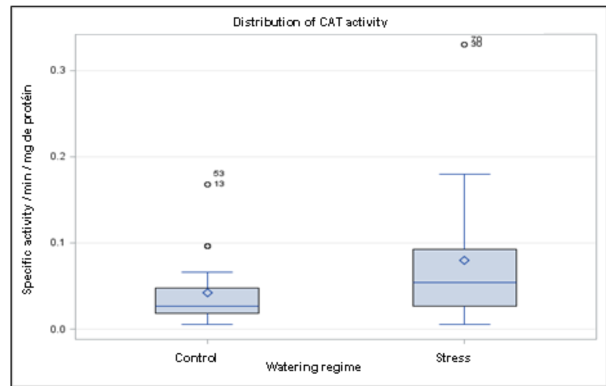
Fig(7)POX specific activity in cocoa genotypes under watering treatment

*** Comparison of means was done under each water treatment. Means followed by the same letter are statistically identical at the 5% (LSD)**

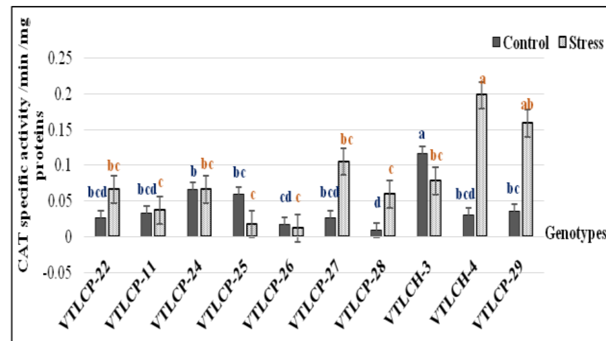
Catalase (CAT) specific activities in leaves of cocoa genotypes

Figure 8 shows the distribution of catalase activity in the leaves of cocoa genotypes under water stress and normal irrigation. The box plot parameters showed a greater dispersion of CAT activity under water stress condition (mini= 0.006 specific activity; max= 0.33 specific activity; Q3 - Q1= 0.066 specific activity), than normal irrigation condition with a reduced interquartile range (mini= 0.006 specific activity; max= 0.168 specific activity; Q3 - Q1= 0.03 specific activity). More than half of the tested genotypes scored above the CAT at 0.0546 specific activity (median) under water stress and higher 0.027 specific activity (median) for condition normal irrigation (Fig. 8).

The analysis of variance revealed a highly significant effect of interaction between water treatment x genotypes (p = 0.0001) and genotype (p= 0.0003), unlike, the effect of water treatment which was homogeneous (p= 0.77). Our results revealed differences between water treatments. The overall average of the specific activity of the control group (0.042 ± 0.038 specific activity) increased to a value of (0.079 ± 0.07 specific activity) under water stress or over activation of catalase over 47% compared to unstressed control (Fig. 8). In normal irrigation condition, the genotypes VTLCH-3 (0.17 specific activity), VTLCP-24 (0.066 specific activity) and VTLCP-25 (0.06 specific activity) showed high activities of CAT in cocoa leaves, unlike genotypes VTLCP-28 (0.009 specific activity) and VTLCP-26 (0.18 specific activity). Similarly, under water stress, the genotypes VTLCH-4 (0.198 specific activity) followed by genotypes VTLCP-29 (0.159 specific activity), VTLCP-27 (0.105 specific activity) and VTLCP-22 (0.066 specific activity) had strong activities of catalase (Fig. 9).



Fig(8)Effect of water treatment on the distribution of CAT specific activity in leaves of cocoa genotypes



Fig(9)CAT specific activity in cocoa genotypes under watering treatment

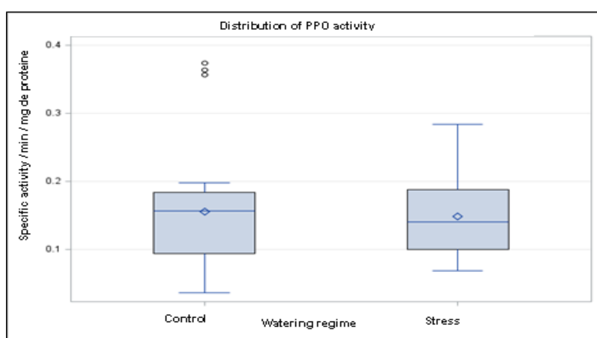
*** Comparison of means was done under each water treatment.**

Means followed by the same letter are statistically identical at the 5% (LSD)

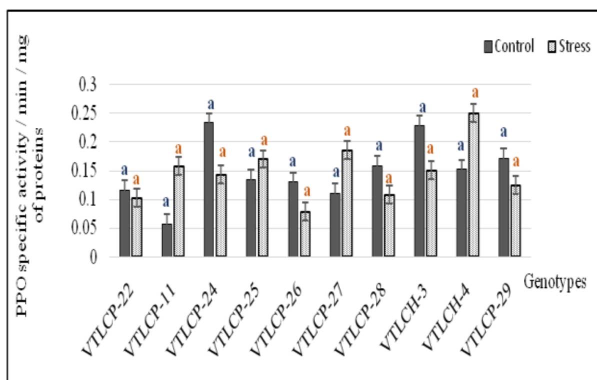
Polyphenol oxidase (PPO) specific activities in leaves of cocoa genotypes

Figure 10 shows the distribution of the activity of polyphenol oxidase in the leaves of cocoa genotypes under water stress and normal irrigation conditions. The parameters of the box plot showed a strong dispersion of the PPO activity in normal irrigation condition (min= 0.036 specific activity; max= 0.374 specific activity; Q3 - Q1= 0.09 specific activity), than in deficit induced water stress condition (min= 0.068 specific activity; max= 0.284 specific activity; Q3 - Q1= 0.088 specific activity). More than half of the tested genotypes presented a PPO specific activity greater than 0.14 specific activity (median) under water stress and higher than 0.156 specific activity (median) under normal irrigation condition.

The analysis of variance revealed homogeneous effect of interaction water treatment x genotypes (p= 0.053), genotype (p= 0.145), and treatment (p= 0.8756) (Fig. 10). The comparison of averages showed no significant statistical difference between the mean of activity of polyphenol oxidase in cocoa genotypes under water stress condition (0.147 ± 0.05 specific activity) and non-stress (0.154 ± 0.083 specific activity). The statistical analysis also showed no significant differences between the genotypes of cocoa trees in each irrigation regime. Nevertheless, genotypes VTLCH-4, VTLCP-27, VTLCP-25 and VTLCP-11 got high PPO activities under deficit stress as compared to their respective control in normal irrigation conditions. Among other genotypes of cocoa, enzymatic activities PPO decreased under water stress deficit relative to their non-stressed condition (Fig. 11).



Fig(10)Effect of water treatment on the distribution of PPO specific activity in leaves of cocoa genotypes



Fig(11)PPO specific activity in cocoa genotypes under watering treatment

*** Comparison of means was done under each water treatment.**

Means followed by the same letter are statistically identical at the 5% (LSD)

DISCUSSION

The response of cocoa genotypes to oxidative stress showed wide variability to the induced water deficit stress. It is well established that the plants subjected to water deficit are known to evolve reactive oxygen species (ROS) and the accumulation of free radicals related to damage of the membranes, lipid peroxidation, protein denaturation and DNA (Kaminska-Rozek and Pukacki 2004). But different mechanisms set up by the plants used to annihilate the accumulation of ROS. Our investigation was limited to the response of lipid peroxidation and some enzymes involved in the detoxification mechanism. The results showed genotypic variability in MDA accumulation in leaves and the enzymatic response of cocoa genotypes in young age.

The SOD activity increased in response to the induced water deficit. A similar response under conditions of water deficit has been reported in other plant species by several other authors (Iturbe-Ormaetxe *et al*. 1998; Bueno *et al.* 1998). In this study, cocoa genotypes showed a high activity of SOD. Indeed, increased SOD activity may reflect an increase in production of O_2^-, as shown in the role of SOD on the dismutation of O_2^- and protection of the photosynthetic apparatus (Jiang and Huang 2001). However, a decrease in SOD activity was noted particularly in the VTLCH-3 genotype. This reduction may be related to an excess of active forms of oxygen such as superoxide radical as indicated by Muckenschnabel *et al.* (2001), and or the reduced synthesis or increased degradation of the SOD enzyme. Peroxidases are located in the chloroplast, the cytosol and the apoplast, POX has a high affinity for it detoxifies and decomposes H_2O_2. Our results revealed a significant increase in the activity of the POX in cocoa genotypes under water deficit stress. The same response was reported under drought conditions for wheat by Zhang and Kirkham (1994). But in our genotypes tested, high variability was observed with the activity of POX. Several authors have shown a positive link between the abiotic stress tolerance and activity of peroxidases. However, our results confirm those of Molina *et al*. (2002); Appel and Hirt (2004) and Shao *et al*. (2007), which recalled the crucial role of peroxidases in the protection of plant cells. Similar to the activity of POX, water stress induced a significant increase in the activity of catalase, whose role at the cellular level is to break down and detoxify H_2O_2 to H_2O and O_2.

The results of this study are consistent with those of Luna *et al.* (2004) and Bueno *et al*. (1998) who found an increase in CAT activity in tobacco and wheat genotypes under stress. Low activity of CAT was observed in some genotypes, in case of VTLCP-26 and VTLCH-3 compared to their unstressed controls, which could be attributed to photo-inactivation of CAT deficit under water stress (Zhang and Kirkham 1994). Unlike SOD, CAT and POX, water stress induced no effect on the activity of polyphenol oxidase in cocoa genotypes. This suggests a low level of involvement of the PPO in oxidative degradation processes and cellular detoxification against ROS in cocoa. Our results are contrary to those reported by Rivero *et al.* (2001) and Ashraf *et al.* (1994).

Our results showed that application of water deficit stress induces lipid peroxidation resulting in large accumulation of MDA in the leaves of some genotypes as reported by several studies (Dhanda *et al*. 2004; Bohnert *et al*. 2006). It is interesting to note that some of our genotypes showed low levels of accumulation of MDA under water stress. As indicated by Dhanda *et al.* (2004) and Shao *et al.* (2007), the low content MDA shows great anti-oxidative capacity, reflecting a high tolerance to water deficit stress.

CONCLUSION

The results of our work revealed that there exists wide variability for antioxidant activity of leaf tissue of cocoa genotypes to the induced water deficit. The variability of responses of enzyme complexes of oxidative metabolism and the production of malondialdehyde under water stress shows a defensive mechanism of cocoa genotypes to protect against the ROX. Significant increases in the activities of antioxidant enzymes SOD, CAT and POX were observed under water stress deficit. However, PPO activity did not significantly vary. The greater activity of SOD, POX and CAT accompanied with lower leaf level MDA, is an important indicator of abiotic stress tolerance. It was concluded that the variability observed in antioxidant activity could be a promising way for the early selection of cocoa genotypes tolerant to water deficit stress.

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