

Leaf antioxidant activity in contrasting *Brachiaria* genotypes under waterlogging conditions

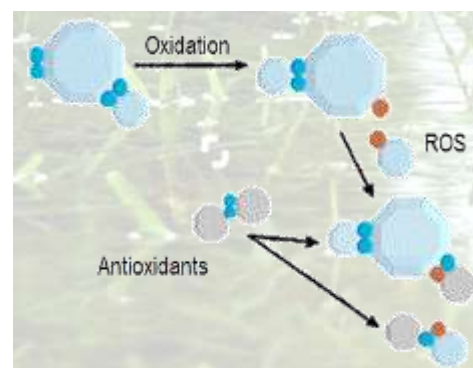
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KEYWORDS: Antioxidant capacity, Superoxide dismutase (SOD), Catalase (CAT), thiobarbituric acid assay (TBA), forages, waterlogging, *Brachiaria*, stress conditions.

ABSTRACT: *Brachiaria* grasses face, due principally to climate change, to extreme conditions like drought and waterlogging, which severely limit their productivity and adoption. Therefore is of particular interest its study with aim to develop future breeding programs. Phenotypic differences have been reported in their tolerance to waterlogging. However, there are a few published information regarding the biochemical responses to waterlogging. In this study was evaluated the antioxidant activity of two contrasting genotypes (*B. humidicola* -679- tolerant and *B. hybrid Mulato II* -36087- sensitive) under waterlogged soil conditions. The assays realized were defined in basis of two factor: Antioxidant system (enzymatic-Catalase- and non-enzymatic-DPPH assay-) and Oxidative damage (protein content-Bradford assay- and lipid peroxidation-TBARS assay-). We conclude that the outstanding adaptation of *B. humidicola* to waterlogged soils to long-term (15 days) is related to their non-enzymatic antioxidant system and is probably related with increase of anthocyanin as waterlogging adaptation. This study is an important step (an initial contribution) in elucidating the biochemical response behind physiological response to waterlogging conditions in tropical forages, which could support the development of breeding programs targeted to select waterlogged adapted plants.



INTRODUCTION

Pasture in Tropical America face, due principally to climate change, to extreme conditions like drought and waterlogging^{1,2}, which limit their productivity conducting to a deficient production of meat and milk³. In Colombia and in most tropical countries the staple food of bovine animals are grasses. The grasses constitute the basic and most economical diet feeding of ruminants and provide organic matter to the soil, which helps in their preservation.

Approximately 91.9% of agricultural land in Colombia was sown with pastures and meadows, where the 73.3% is permanent cultivated, this large percentage, highlights the great importance of research and development of pastures⁴. Waterlogging is characterized by oxygen deficiency in soils, this lack impedes the root respiration, therefore adversely influences the changing physiology, reducing plant growth and inducing senescence⁵.

Waterlogging conditions can create an imbalance between the production of reactive oxygen species (ROS) and the ability of a biological system to rapidly decode the reactive intermediates or repair the resulting damage, this is the called oxidative stress. As a consequence of increased ROS in tissues, some plants can counterattack the deleterious effect of ROS increasing in the antioxidant defense system (enzymatic and non-enzymatic)⁶ which is considered as a short-term response^{7,8,9}.

Brachiaria is widely used as pasture for livestock in Latin America. Its rapid adoption in agricultural regions across the region was probably due to their wide range of agronomic adaptation, the nutritional quality and high productivity¹⁰. Phenotypic differences have been reported between *Brachiaria* cultivars in terms of waterlogging adaptation¹¹.

Comparative physiological studies have classified *B. Mulato II* as sensitive and *B. humidicola* as tolerant to waterlogged soils. *B. humidicola* has less reduction of growth, gas exchange and chlorophyll under waterlogging conditions than other species of *Brachiaria* (eg *B. decumbens*, *B. brizantha* and *B. hybrid Mulato*)^{12,13,14,15,16}; however, there are not published information about the biochemical responses of *Brachiaria* spp under waterlogging conditions.

The main objective of the present paper was to identify biochemical traits that are related to waterlogging adaptation in *Brachiaria* grasses. Therefore, changes in antioxidant defense system were measured in contrasting genotypes under 15 days of waterlogging conditions.

The assays realized were defined in basis of two factor: Antioxidant system (enzymatic and non-enzymatic) and Oxidative damage (protein content and lipid peroxidation). The total non-enzymatic antioxidant capacity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and the enzymatic antioxidant activity was tested by measuring Catalase (CAT) activity. Membrane lipid peroxidation was evaluated through thiobarbituric acid assay (TBARS) and protein content by Bradford method. These assays were made using methods previously reported in literature and widely used; some of these were modified as result of previous adaptation work to particular conditions of sample.

It is of important interest the *Brachiaria* waterlogging adaptation study with aim to develop future breeding programs as a key element on climate change mitigation.

MATERIAL AND METHODS

Reagents and equipment. All reagents used were obtained from sigma Aldrich, merck and BioRad; All reagents were of the highest available grade. Deionized water (milli-Q) was used. Microplate reader UQuant from BIO-TEK Instruments was used for all assays.

.Plant Material and Growth Conditions. Methodology used in this study was similar to the one described by Cardoso et al¹⁷. Vegetative propagules of *B. humidicola* cvv. Tully (waterlogging tolerant) and *B. hybrid* cv. Mulato II (waterlogging sensitive) were planted in plastic cylinders (80 cm height and 7.5 cm diameter) filled with 5 kg of a mix of an Oxisol soil. Plastic containers were inserted into PVC pipes with similar dimensions. Plantlets were grown in optimum conditions during 21 days before to start the treatment.

Experiment was performed in a completely randomized design with factorial structure: two genotypes, two treatments (waterlogging and well-drained) and six replications. Waterlogging treatment was imposed during 15 days

by sealing the bottom of the cylinder and applying water up to maintain a water lamina 3 cm above soil surface. In the well-drained treatment, soil moisture was kept close to field capacity.

After 15 days of treatment aboveground biomass was removed, leaves were freeze dried and stored at -80 °C for further biochemical analysis. An initial sampling (before starting treatment) was performed to 4 plants in order to know the basal levels of compounds from each species.

Determination of Total Non-Enzymatic Antioxidant Capacity. To determinate total antioxidant capacity by DPPH method we used the methodology proposed by Du¹⁸. Briefly, 50 mg of dry tissue was weighed and homogenized with a solution of acetone:water (70:30) during 30 minutes. After doing that, the solution was centrifuged at 6000 rpm during 10 min. Then, the supernatants obtained were mixed with a solution of DPPH for 30 minutes. Antioxidant capacity was determined at 517 nm¹⁹. Trolox, (antioxidant) a vitamin E analogue was used as standard in a 0.8-4 mM linear range.

Determination of Lipid Peroxidation by TBARS Assay. Due to high concentrations of anthocyanins²⁰, which absorb at the same wavelength (532 nm) employed to determine malondialdehyde (MDA), a methodology correcting this interference was used²¹.

100 mg of freeze dried sample was homogenized in 1,5 ml of ethanol 80%. Then was centrifuged at 10 °C 11000 rpm 30 minutes. 0,5 ml of the supernatant was added to a test tube with 2 ml of 0,5% TBA, 20% TCA, 0,01% BHT; the sample blank was made it adding 0,5 ml of the supernatant in 2 ml of 20% TCA, 0,01% BHT without TBA, which reacts with MDA thereby obtaining just anthocyanins absorbance at 532 nm. Samples and samples blanks were heated at 95 °C for 30 min, cooled and centrifuged at 6000rpm for 10 min.

The absorbances of samples (SPL) and samples blanks (BLK) were read at 440 nm, 532 nm and 600 nm. Malondialdehyde equivalents were calculated as follow:

$$A = (\text{Abs } 532_{\text{SPL}} - \text{Abs } 600_{\text{SPL}}) - (\text{Abs } 532_{\text{BLK}} - \text{Abs } 600_{\text{BLK}}) \quad \text{Ec.1}$$

$$B = (\text{Abs } 440_{\text{SPL}} - \text{Abs } 600_{\text{SPL}}) \cdot 0,0571 \quad \text{Ec.2}$$

$$\text{MDA equivalents (nmol.ml}^{-1}\text{)} = ((A-B)/157000)10^6 \quad \text{Ec.3}$$

In equation 1 the absorbance at 600 nm is used to correct nonspecific turbidity, in equation 2 the absorbance at 440 nm is used to correct interference generated by TBA-sugar complexes where the ratio among molecular absorbance at 532 nm and 440 nm is 0,0571 (Ec.2). The molar extinction

coefficient for MDA (157000) was used to determinate the MDA equivalents.

Determination of Protein Content and Enzymatic Activity of Catalase. Freeze dried tissues were homogenized through vortex in cold potassium phosphate buffer (50mM, pH 6,8) with EDTA (0,1mM) during 30 seconds, then it was centrifuged at 11000 rpm during 15 min at 4 °C. The supernatants obtained were used for protein assay and determination for enzymatic activity. Protein was determined according to the method of Bradford²² using gamma globulin as a standard.

Catalase activity was measured by the method of Luck²³ with some minor modifications^{24,25}. The reaction mixture contained 50 mM phosphate buffer, 5 mM H₂O₂ (pH 7.0), and a suitable aliquot of the supernatant in a final volume of 300 µl. Catalase activity was estimated by calculating the initial rate of disappearance of H₂O₂ (Fig 8) at 240 nm for 1 min.

Data analyses. Data are reported as the averages of six biologic replications (six plants for each genotype-treatment) and three technical replications for each plant in every assay. Statistical analysis was performed using the Excel Analyses of variance (ANOVA) and student's t-test were performed to identify differences between treatments and genotypes.

RESULTS

Determination of Total Antioxidant Capacity. DPPH is a free radical that is frequently used to estimate the scavenging capacity of antioxidants in many plants. This assay has been widely used in diverse studies of important crops like rice²⁶, potato²⁷ and cassava.²⁸

Antioxidants donate a radical monoatomic Hydrogen to DPPH radical, in the radical the delocalization of the spare electron over the molecule as a whole gives rise to deep violet colour, this causes the disappearance of DPPH radical and its absorption to 517 nm decreases proportionally due to its conversion to the reduced form (Fig 1), also the loss of violet color gives place to a yellow color from the picryl group still present. This strong change has been reported as an indicator of this reaction²⁹.

The free radical scavenging capacity of the *B. humidicola* and *B. Hybrid Mulato II* are shown in Fig 2.

The antioxidant capacity of *B. humidicola* (679) was not different than genotype *B. hybrid Mulato II* (36087) during initial (data no shown) and drainage treatments, but we found significant difference in waterlogging treatment.

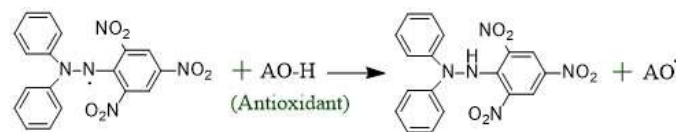


Figure 1. Reduction of DPPH radical by antioxidants

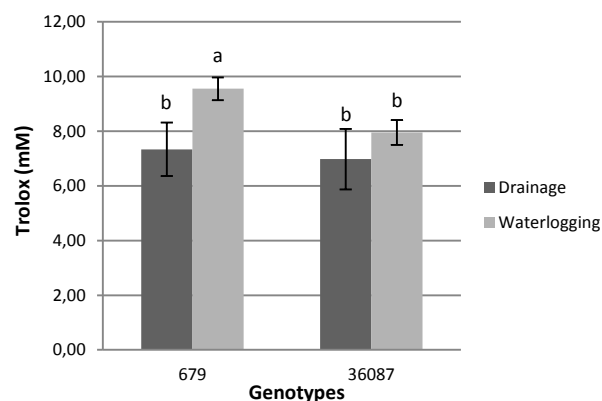


Figure 2. Total antioxidant capacity in two contrasting genotypes of *Brachiaria* under 15 days of continuous waterlogging and drainage. Columns with different letters above the bars indicate significant differences according to the student's test (n=6; P≤0,05)

Lipid Peroxidation. Waterlogging stress induces reactive oxygen species (ROS), with O₂ and H₂O₂ radicals being the most abundant in plants. Accumulation of H₂O₂ cause lipid peroxidation³⁰. The MDA is the first product produced by membrane lipid peroxidation and has been reported as an indicator of plant oxidative damage³¹. A MDA formation route is described in figure 3.

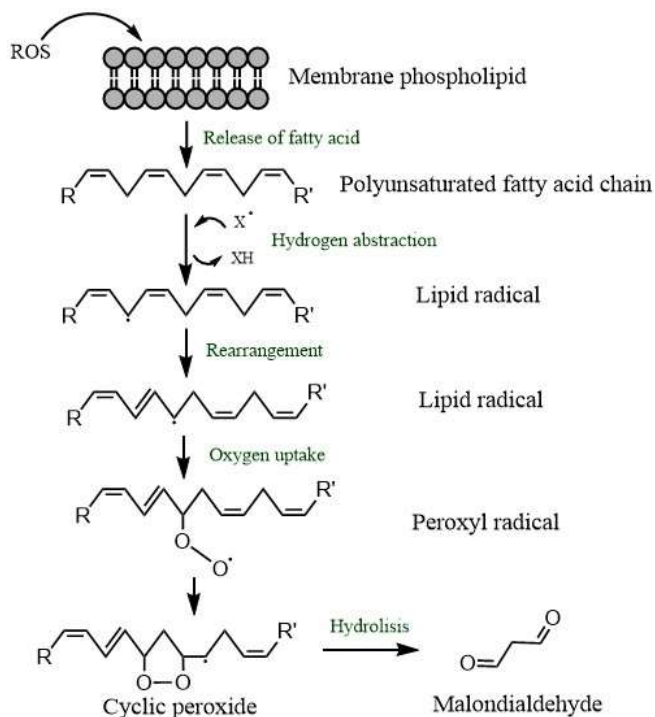


Figure 3. Reactions involved in lipid peroxidation, with MDA as final product.

MDA and other aldehydes have been identified as products of lipid peroxidation, these react with two molecules of thiobarbituric acid (Fig 4) via an acid-catalyzed nucleophilic-addition yielding a pinkish-red chromogen with an absorbance maximum at 532 nm^{32,33}. For this method we got a 90,36% of efficiency.

The Malondialdehyde content in genotypes of *Brachiaria* is presented in Figure 5.

The MDA content, in *B. humidicola* (679), was stable under initial (data no shown), drainage and waterlogging treatments. The genotype *B. hybrid Mulato II* (36087) shows a significantly increase in MDA content under waterlogging stress.

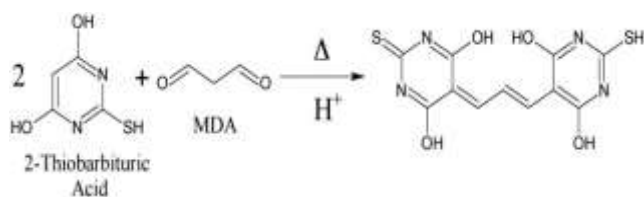


Figure 4. Reaction between TCA and MDA chromogen pinkish-red

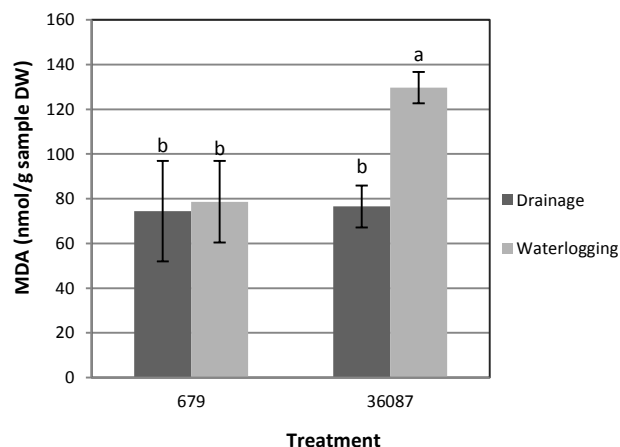


Figure 5. Content of oxidative products expressed as MDA equivalents in *B. humidicola* and *B. hybrid Mulato II* in treatments Drainage and Waterlogging. Different letters above the bars indicate significantly different means between treatments or genotypes (n=6; $P \leq 0,05$)

Determination of Protein Content and Enzymatic Activity of Catalase. Bradford protein assay has been widely used as an indicator of protein concentration^{34,35} it is based on the interaction promoted by Coomassie Brilliant Blue G-250 (Bradford reagent) (Fig 6), this dye under acidic conditions is predominantly red cationic form, when this binds (primarily to basic and aromatic amino acid residues³⁶) to protein it is converted to a stable unprotonated blue form causing a shift in the absorption maximum of the dye from 465 to 595 nm and the increase of absorption at this wavelength is directly proportional to protein concentration³⁷.

The protein concentration of genotypes *B. hybrid Mulato II* and *B. humidicola*, in treatments drainage and waterlogging are shown in Figure 7. For the two *Brachiaria*'s genotype the protein concentration does not show significant difference among drainage treatment and waterlogging treatment.

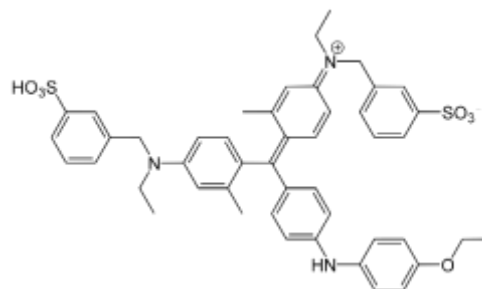


Figure 6. Chemical structure of Coomassie Brilliant Blue G-250,

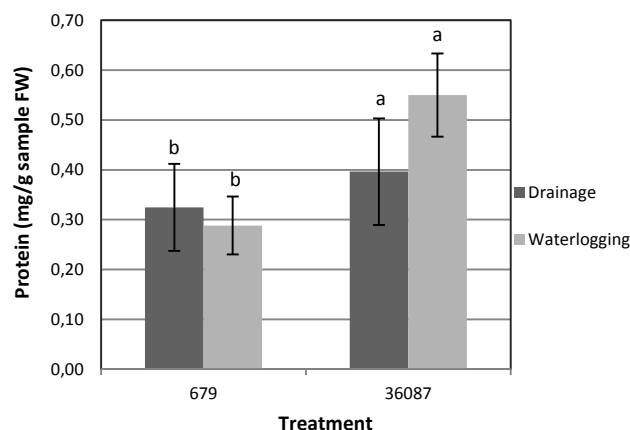


Figure 7. Protein concentration in genotypes *B. humicola* and *B. hybrid Mulato II*. Different letters above the bars indicate significantly different means between treatments or genotypes (n=6; $P \leq 0,05$)

Catalase is involved in regulation of H_2O_2 intracellular levels. Catalase is a homotetramer with four Heme prosthetic group where the Iron nucleus acts as cofactor interacting with peroxide hydrogen and converts in O_2 and H_2O by redox reaction.²⁵ This reaction is strongly associated with oxidative stress response mediated by waterlogging and allow characterize the response to this type of stress in plants^{38,39}. At 240 nm the absorbance of H_2O_2 decrease (Fig 8) proportionally with Catalase Content (Fig 9). For this method we got a 91,13% of efficiency.

The enzymatic activity of Catalase of genotypes *B. hybrid Mulato II* and *B. humicola*, in treatments drainage and waterlogging are shown in Figure 10.

The implemented waterlogging treatment did not alter significantly the total CAT activity in *B. humicola* genotype, however *B. hybrid Mulato II* increased significantly in CAT activity in waterlogging treatment with regard to drainage treatment.

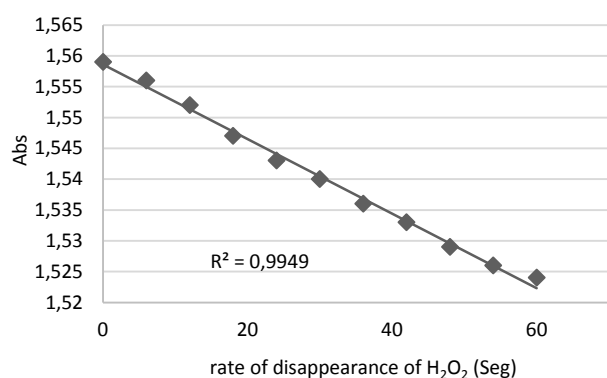


Figure 8. H_2O_2 rate of disappearance for 1 min in presence of Catalase.

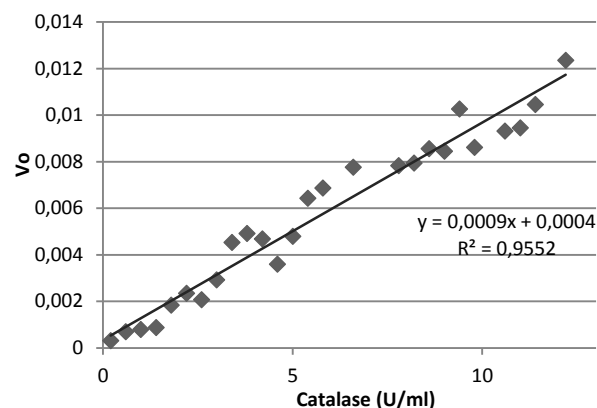


Figure 9. Initial rate proportionally linear with Catalase concentration.

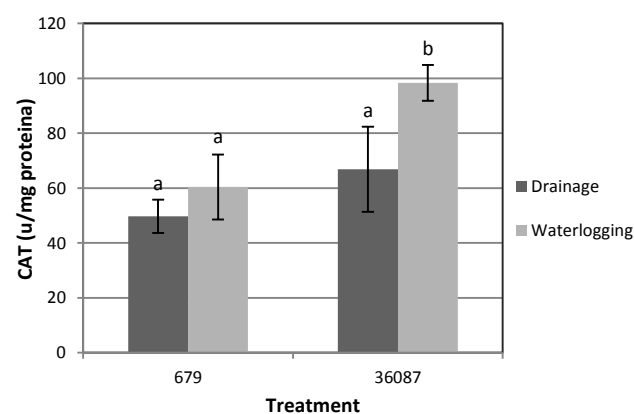


Figure 10. Enzymatic activity of Catalase in genotypes *B. humicola* and *B. hybrid Mulato II*. Different letters above the bars indicate significantly different means between treatments or genotypes (n=8; $P \leq 0,05$)

DISCUSSION

According to reports of biochemistry and physiology response associated with waterlogging stress^{9,40}, had been expected in tolerant genotype (*B. humicola*) a greater increases in total non-enzymatic antioxidant capacity than sensitive genotype (*B. hybrid Mulato II*). However, only it was observed this difference in non-enzymatic antioxidant capacity (ascorbate, glutathione, tocopherol, proline, etc.) by DPPH assay. In enzymatic activity of Catalase only the genotype *B. hybrid Mulato II* shows significant increment in Catalase activity between drainage and waterlogging treatments after 15 days of treatment.

Even so, genotype *B. humicola* didn't present significantly loss of protein between treatments. Additionally, it was also evidenced lower damage due to lipid peroxidation

than sensitive genotype (*B. hybrid Mulato II*). This is consistent with previous physiological studies⁴⁰.

The high antioxidant capacity of tolerant genotype *B. humidicola* probably is related with the high concentration of anthocyanin^{41,42,43,44}, these molecules are included in the antioxidants metabolites that were measured in DPPH assay^{45,46}. This could explain the increase in non-enzymatic antioxidant capacity and also the fact that enzymatic antioxidant activity was relatively unaffected after 15 days of waterlogging treatment.

The *B. humidicola* protrudes from other species of *Brachiaria* for its high content of anthocyanins in response to stress²⁰, figure 10 shows foliar color changes in *B. Humidicola* and *B. hybrid Mulato II* during drainage and waterlogging treatments, the red color pigments in Fig 11 –b– (*B. Humidicola* during waterlogging treatment) evidences the presence of anthocyanins in high concentration. Anthocyanins act like photo protective pigments inhibiting the oxygen radical's formation, the phenolic structure of anthocyanins conveys marked antioxidant activity in model systems via donation of electrons or hydrogen atoms from hydroxyl moieties to free radicals⁴⁷, and the absence of significant concentration of ROS doesn't allow the activation of enzymatic activity, (Catalase in this specific case). This activity only increase significantly when the balance between ROS and scavenging molecules is broken.⁸ Also, a lower concentration of free radicals implies a lower damage by lipid peroxidation, as was observed (Fig 5).

Accordingly, the high concentration of anthocyanins, in *B. humidicola*, has an oxygen radical scavenging effect and therefore inhibits lipid peroxidation^{47,48}. The increase of this metabolite could be enough to keep the ROS and scavenging molecules balance making unnecessary the increase of catalase activity to long term (after 15 day of waterlogging treatment). In addition, there is not reported secondary effects of high concentration of anthocyanin in bovine metabolism, in fact is reported potential health benefits of plant-derived anthocyanin-rich food⁴⁹.

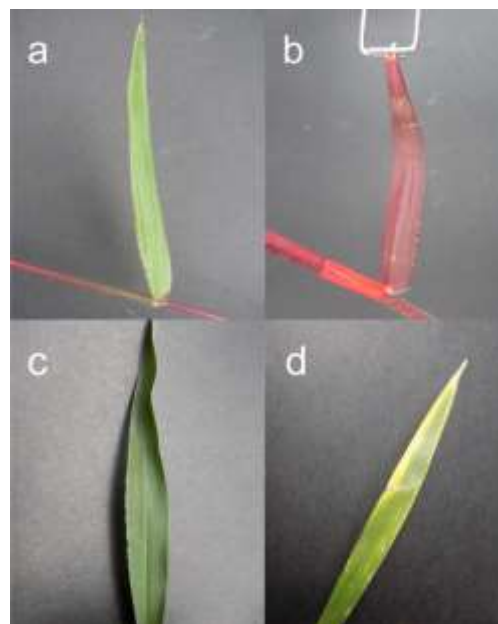


Figure 11. Foliar changes in *B. humidicola* (a) Drainage and (b) Waterlogging treatments; *B. hibryd Mulato II* (c) Drainage and (d) Waterlogging treatments.

CONCLUSIONS

The total non-enzymatic antioxidant capacity was significantly increased in *B. humidicola* after 15 days of continuous waterlogging conditions. TBA analysis showed that *B. humidicola* suffered less lipid peroxidation than *B. hybrid Mulato II* under waterlogging conditions. However, the amounts of CAT activity were higher in hybrid Mulato II in contrast with *B. humidicola* after 15 days of waterlogging treatment.

The high antioxidant capacity of tolerant genotype *B. humidicola*, after 15 days of waterlogging treatment, resides in non-enzymatic antioxidant capacity and is probably related with increase of anthocyanin as waterlogging adaptation.

This study is a first step in elucidating the biochemical response behind physiological response to waterlogging conditions on tropical forages; In addition, the analytical techniques used and modified could support the development of breeding programs targeted to select waterlogging adapted plants.

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ABBREVIATIONS

MDA, malondialdehyde; TBA, thiobarbituric acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CAT, Catalase; SOD, Super-oxide dismutase; TEAC, Trolox equivalent antioxidant capacity; ROS, reactive oxygen species.

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