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MAIANY ALVES PATRIOTA

RELATIONSHIP BETWEEN CATALASE AND GLYCOLATE OXIDASE ACTIVITIES IN RESPONSE TO CONTRASTING PHOTORESPIRATORY CONDITIONS INDUCED BY DIFFERENT LIGHT INTENSITIES

FORTALEZA
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Dissertation presented to the Graduate Program in Biochemistry and Molecular Biology of the Department of Biochemistry and Molecular Biology of the Federal University of Ceará, as a partial requirement for obtaining the title of Master in Biochemistry. Area of concentration : Plant biochemistry.

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BANCA EXAMINADORA

Prof. Dr. Joaquim Albenisio Gomes da Silveira (Orientador)
Universidade Federal do Ceará (UFC).

Prof. Dr. Danilo de Menezes Daloso
Universidade Federal do Ceará (UFC).

Prof. Dra. Ana Luiza Sobral Paiva
Universidade Federal do Ceará (UFC).

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~Abri a janela e o **coração, o sol** inundou meu quarto,
e o **amor** inundou minha **alma ~**
Paulo Coelho

ABSTRACT

Glycolate oxidase (GO) and catalase (CAT) are two crucial enzymes involved in photorespiratory metabolism in higher plants. Although GO is the only plant enzyme capable of eliminating the glycolate accumulated by the oxygenase activity of Rubisco, its reaction has the side effect of producing hydrogen peroxide (H_2O_2) in peroxisomes that must be quickly removed by CAT activity. In fact, the co-evolutionary need for a functioning of these enzymes has been considered in the literature and recent studies point to a possible interaction between these proteins forming a large enzyme complex. Despite this interaction, the physiological significance of crosstalk between GO and CAT is still poorly understood, especially under contrasting photorespiration conditions. Thus, the present study aimed to investigate the possible functional regulation between GO and CAT in rice plants exposed to different photorespiratory levels, induced by light variations. For this, in a first experiment, rice seeds (Oryza sativa japonica cv. Nipponbare) were cultivated in a greenhouse for 40 days. Photorespiration and photosynthesis were characterized by *in vivo* measurements using an infrared gas analyzer(IRGA). The activity of GO and CAT enzymes, the content of substances reactive to tiubarbituric acid (TBARES), the accumulation of H_2O_2 and redox levels of ascorbate and glutathione were also determined. The results showed a decrease in photosynthesis and an increase in photorespiration with increasing light. The activity of GO and CAT increased under photorespiratory condition and were positively correlated to each other. In addition to the cross-talk of the enzymes among themselves, both are strongly related to the H_2O_2 pool, as well as to membrane damage and lipid peroxidation caused by it, even as respond in an negative-correlated manner to the ASC-GSH cycle .

Keywords: *Oryza sativa*; photorespiration; environmental variation; photosynthesis.

RESUMO

A glicolato oxidase (GO) e a catalase (CAT) são duas enzimas cruciais envolvidas no metabolismo fotorrespiratório em plantas superiores. Embora a GO seja a única enzima vegetal capaz de eliminar o glicolato acumulado pela atividade oxigenase da Rubisco, sua reação tem o efeito colateral de produzir peroxido de hidrogênio (H_2O_2) nos peroxissomos que devem ser rapidamente removidos pela atividade da CAT. De fato, a necessidade co-evolutiva do funcionamento dessas enzimas tem sido considerada na literatura e estudos recentes apontam para uma possível interação entre essas proteínas formando um grande complexo enzimático. Apesar dessa interação, o significado fisiológico do crosstalk entre GO e CAT ainda é pouco compreendido, especialmente sob condições de fotorrespiração contrastantes. Como objetivo, o presente estudo teve como objetivo investigar a possível regulação funcional entre GO e CAT em plantas de arroz expostas a diferentes níveis fotorrespiratórios, induzidas por variações de luz. Para isso, em um primeiro experimento, sementes de arroz (*Oryza sativa* japonica cv. Nipponbare) foram cultivadas em casa de vegetação por 40 dias. A fotorrespiração e a fotossíntese foram caracterizadas por medidas in vivo usando um analisador de gases. infravermelho (IRGA). Além disso, foram determinados a atividade das enzimas GO e CAT, teor de substâncias reativas ao ácido tiubarbitúrico (TBARES), acúmulo de H_2O_2 e níveis redox de ascorbato e glutationa. Os resultados mostraram uma diminuição na fotossíntese e um aumento na fotorrespiração com o aumento da luz. Observou-se também que GO e CAT respondem positivamente às variações fotorrespiratórias induzidas pela luz, além de se correlacionarem positivamente, influenciando diretamente na atividade uma da outra. Além do cross-talk das enzimas entre si, ambas estão fortemente relacionadas ao pool de H_2O_2, bem como ao dano de membrana e peroxidação lipídica por ele causado, ainda que respondam de forma negativamente correlacionada ao ciclo ASC-GSH.

Palavras-chave: *Oryza sativa*; fotorrespiração; variação ambiental; fotossíntese.

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SUMMARY

1 INTRODUCTION

Plant organisms daily perform vital functions in nature, acting, for example, in the nutrients cycling, being able to transform atmospheric CO_2 into organic carbon through Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) enzyme (EISENHUT et al, 2016). However, for specific plant types, such as C3 plants, their photosynthetic capacity is limited by several environmental factors, which can increase the oxygenation activity over the carboxylation in the Rubisco enzyme. This leads to decomposition of the ribulose bisphosphate, releasing 2-phosphoglycerate (2-PG) molecules, which are toxic to plant metabolism (MAIER et al, 2021). Due to its toxicity, the 2-PG is removed by a photorespiration cycle, which is capable to recover three out of every four carbons that would otherwise be lost (FERNIE & BAUWE, 2020).

Among all the processes occurring in plants, photorespiration is the second largest mass flow process, behind only photosynthesis (SHIM et al, 2020). During its steps to remove 2-PG, another type of toxic molecule is generated: the hydrogen peroxide (H_2O_2). This compound has harmful potential to cells when is at large concentrations (CUI et al, 2016). However, under low concentrations, it has a redox signaling role due to its stability and permeability across membranes (RIBEIRO et al, 2017), being also able to regulate several physiological processes such as growth and defense under environmental stresses (CHI et al, 2016).

Photorespiration is a common process and necessary for the survival of plants under unfavorable conditions (BAUWE, et al, 2010). However, it turns out to be the main route of H_2O_2 production through the oxidation activity of glycolate through the glycolate oxidase (GO) enzyme in peroxisomes (ZHANG et al, 2016). GO enzymes are able to metabolize the irreversible oxidation of O_2, using glycolate as a substrate to produce glyoxylate and, consequently, release H_2O_2. Commonly, the H_2O_2 is quickly consumed by the catalase enzyme and does not accumulate in the cell (HAGEMANN & BAUWE, 2016).

According to TYUTEREVA et al (2017), catalases have the ability to remove about 25% of all peroxisomal H_2O_2, as well as the ability to act in autophagic and programmed cell death regulations, representing the most abundant enzyme in peroxisomes (SU et al, 2018). In addition, CAT has a low affinity for the substrate and works to remove excess, where it exhibits a high activity rate, having been the first documented antioxidant enzyme (KAUSHAL et al, 2018) and which appears in all eukaryotic and prokaryotic organisms (AHMAD,2014). Beyond the catalase, other enzymes can act in the H_2O_2 scavenger, such as glutathione peroxidases

(GPX) or ascorbate peroxidases (APX), which have a greater affinity for the peroxide and act in its fine regulation (TEIXEIRA et al, 2006; RIBEIRO et al, 2017).

Some studies have been done focusing on a CAT and GO a isolated way, but no one are focusing in the relationship between both, just Zhang et al (2016) was study they enzymes in the same time, suggested the possibility of an enzyme complex formation between the enzymes, which could possibly serve as a strategy to increase H_2O_2 removal efficiency. So, considering the scarce information about these enzymes interacition, the objective of this work was investigates the possible functional regulation between GO and CAT enzymes in rice plants to expose them to different light levels to induce contrasting levels of photorespiration. The understanding of how the increase in light intensity (which increase the photorespiration level) influences the GO activity and, consequently, the CAT activity to H_2O_2 removal will be explored in this work.

CHAPTER 1
~– LITERATURE REVIEW ~

2.1 PLANTS METABOLISM AND PHOTORESPIRATORY PATHWAYS

Plants are distributed in all global regions and use the sun as a source of energy to grow up and reproduce. The uptake of this energy takes place through processes of photosynthetic activity, carried out by the enzyme Ribulose-1-5-biphosphate carboxylase oxygenase or Rubisco (EISENHUT et al, 2016). Evolutionarily, exist three different types of plants with different strategies to CO_2 concentrating mechanism: C3 metabolism, C4 metabolism and Crassulacean Acid Metabolism – CAM. The C3 metabolic pathway is predominant among plant species, corresponding to 95% of all of them (FOYER et al, 2009), and can thus be found in different environments (JONES, 2014).

In C3 plants, Rubisco fixes the organic carbon present in the chloroplasts, forming 3-phosphoglyceric acid (PGA). This compound is converted to glyceraldehyde-3-phosphate, which enters the CB cycle and generates phosphate sugar molecules consuming two ATP molecules and two NADPH molecules (ZHU et al, 2010). However, sometimes, occurs a decrease in the CO_2 concentration near the Rubisco enzyme. When this happens, it is favored the oxygen molecules use by the oxygenase catalytic site, generating two-carbon molecules. This one cannot enter the CB cycle and has an inhibitory potential for several enzymes (REINHOLDT et al, 2019), leading to the loss of one carbon each cycle. In some species, the CO_2 can be recovered in up to 30% through rearrangement of chloroplasts close to the cell periphery (BUSH et al 2013).

Due to the harmful potential of 2-PG, this molecule is exported from the chloroplast to the peroxisomes by a plastidic carrier glycolate-glycerate (PLGG1) and a sodium bile acid simulant (BASS6) (PICK et al, 2013; WALKER et al, 2016; SOUTH et al, 2017). In peroxisomes, the photorespiration (Pr) pathway consumes this compound (Figure 1), being considered a wasteful pathway by some researchers (GUPTA, 2016) which may represent a loss of 20% of net photosynthesis (CEGELSKI & SCHAEFER, 2006). However, Pr still developing a biological role linked to the expression of nitrogen metabolism (BUSCH et al., 2017), amino acid synthesis (WINGLER et al, 2000) and redox regulation by minimizing the ROS production, dissipating reducing equivalents (VOSS et al, 2013).

Furthermore, the photorespiration importance is indicated by the presence of a typical phenotype in mutants with defects in Pr metabolism, that only can grow in an atmosphere with high concentrations of CO_2, exhibiting impaired or inhibited growth when they are cultivated under low CO_2 environmental conditions (KUHNERT et al, 2021).

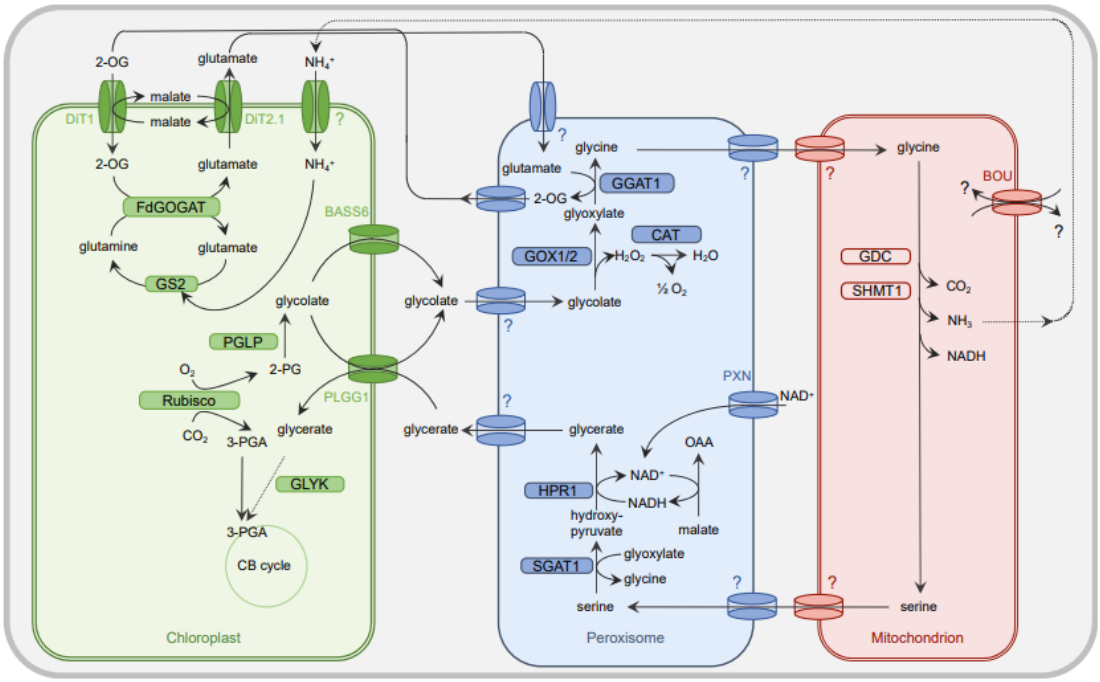

Figure 1. Simplified presentation of the plant PR metabolism interconnected with photosynthetic Calvin-Benson cycle and NH_3 assimilation in higher plants. Reproduced by Kuhnert et al, 2021.

2.2 PLANT CATALASE

2.2.1 Functional, structural and biochemical aspects of the catalase enzyme

Catalases [EC 1.11.1.6.] are the most abundant enzymes in plant peroxisomes, corresponding to 10-25% of the proteins in this organelle (TOLBERT, 1980), playing a crucial role (AKRAM et al, 2017). In animals, catalase may also be present in mitochondria and cytoplasm (PALMA et al, 2020). The author Loew (1901) was the first to report the function of CAT as H_2O_2 scavengers, while Zeile and Hellstrom (1930) were the first to observe that catalases had a prosthetic group of hematin. With advances in research, isoforms encoded by specific genes were discovered, as well as that they can convert two peroxide molecules per cycle, acting at a rate of up to 40 million molecules catalyzed per second (TEHRARI & MOOSAVI-MOVAHEDI, 2018).

Acting as antioxidant enzymes, CATs have a heme group, containing iron atoms in the center of their constitution (BIRBEN et al, 2012; KAUSHAL et al, 2018). This heme group is a key component for enzymatic activity. The structure of the enzymes is presented in a tetrameric and dumbbell form, with four identical monomeric subunits with 220-350 kDa (PALMA et al, 2020). Additively, according to Loewen et al (2002), there are three different types of catalase according to the size and structure of thets subunits (I – monofunctional

catalases; II–- catalase peroxidases; and III–- non-heme catalases), beingthee monofunctional catalase the most common. Furthermore, according to AHMAD (2014), in plant catalase, there is a specific non-polypeptide unit linked to the catalytic center of the enzyme and, in some species, the catalase monomer also contains an NADP strongly linked to each of the four subunits.

In relation to the functional and biochemical aspects of this enzyme, the catalases family has two forms of action: peroxidative (acting at low concentrations of peroxides) in which the enzyme is reduced by hydrogen donors such as ethanol, formic acid, formaldehyde, etc., producing useful products and water; and the catalytic mode (acting at high substrate concentrations), in which H_2O_2 acts as an acceptor and donor of hydrogen molecules for an extremely fast catalytic reaction (SCANDALIOS, 1994). CAT, as well as othesr functional antioxidant enzymes, is very required in plants, especially when they are under biotic and abiotic stressfull situations, in which the enzyme plays a direct role in breaking down H_2O_2 to oxygen and water (GILL & TUTEJA, 2010).

2.2.2 **Isoforms, regulation and inhibition**

In general, there are three catalase isoforms distributed in plants species, such as arabidopsis (CAT1, CAT2, and CAT3) and rice (CATA, CATB, and CATC), as well as in corn, pumpkin and tobacco (LIU et al., 2019). However, there are cases such as cotton, in which five isoforms are present (NI & TRELEASE, 1991) and are regulated by at least seven different genes (WANG W et al., 2019). In arabidopsis plants, the CAT2 and CAT3 isoforms have opposite cicardial profiles, while CAT1 is not expressivelly induced. It also occurs in pepper plants (LI & NA, 2005; PALMA et al., 2020). Furthermore, the expression of the three isoforms varies according to the development of the plant and/or plant tissue under different environmental conditions.

Regarding the isoforms expression, *AtCAT1, AtCAT2* and *AtCAT3* genes regulate the CAT codification, with each gene expressing a specific isoform of the enzyme. The first one is usually more expressed in pollen grain and seeds, being strongly induced by H_2O_2; the second is usually more expressed in photosynthetic tissues, being regulated by salt, cold and high pH; and the latter in vascular tissues and senescent leaves (SU et al., 2018). In rice, the responsible genes are *OsCATA, OsCATB,* and *OsCATC*, respectively, with *OsCATA* corresponding to *AtCAT3, OsCATB* corresponding to *AtCAT1* and *OsCATC* corresponding to *AtCAT2* (LIU et al, 2019).

The expression of catalase isoforms is commonly associated to plant tolerance against various stressful situations, such as the presence of metals in the soil (TYAGI et al, 2020).

However, when exposed to certain compounds, the plant catalases are inhibited, as in the case of NO (KRYCH-MADEJ & GEBICKA, 2017), H_2S (CORPAS et al, 2019), salicylic acid (HERNANDEZ et al 2017), azo dye, cryosidine, phenylhydrazine (ORTIZ DE MONTELLANO & KERR, 1983), ifendate DDB (WANG et al 2013) and flavonoids (KRYCH & GEBECKA, 2013) that strictly regulate its activity. In addition to the compounds mentioned, some reactions are also capable to inactivate the enzyme, such as non-enzymatic glycation (MOFIDI NAJJAR et al, 2017) and disturbances in the tryptophan residue (REGELSBERGER et al 2001; WANG et al 2013) or even physical effects, as when there is deactivation by ultrasound (POTAPOVICH, 2003).

The most diverse compounds can inhibit the expression of CAT, as well, as its activity. These compounds are commonly called inhibitors and classified into four classes (GHADERMARZI & MOOSAVI-MOVAHEDI, 1999). Class A act in the first stage of catalysis, preventing the formation of the second compound. The class B is slow and irreversible, as is the case of sulfates, in which the anionics deactivate the enzyme by electrostatic and hydrophobic bonds (MOOSAVI-MOVAHEDI et al., 1989). Class C inhibitors are most used in experiments (3-aminotriazole or 3-AT), involving the second compound in the reaction instead of ferricatalase. Finally, class D inhibitors reduce compound II and hold an NADH (SINGH et al, 2014).

2.2.3 **Catalase response to the environment**

Daily, plants are exposed to different environmental conditions, many of them stressful (SIMÕES, 2007) and capable of altering plant metabolism. The photorespiratory rate is one of the main factors that can be changed, due to environmental conditions, which end up influencing the levels of intracellular ROS (FOYER et al 2009; VOSS et al., 2013). This ends up reflecting on the content and response of both the photorespiratory compounds and in the ROS scavenger efficiency.

In rice subjected to cold, was observed an increase in catalase expression (CHENG et al, 2007), as well, in the activity levels of the same enzyme(GUO et al., 2006), improving its tolerance to this stress. Furthermore, it was observed through the studies by Rivero et al. (2009), that isopentenyl transferase overexpression tobacco plants, show an increase in peroxisomal catalase. The same can be seen in the compilation of studies accounted by Leung (2018), which bring the increase in CAT under abiotic stresses (Table 1). This behavior is considered a common regulatory response, which promotes greater plant tolerance under these environmental conditions, since such conditions can change the enzyme transcription (TYAGI

et al, 2020), regulate its activity, and consequently the redox balance, during the stressful period.

Table 1. Adapted table with some of the examples raised in the studies by Leung (2018), showing recent findings on increased catalase activity in plants under different abiotic stresses.

PLANT	TRATAMENT	REFERENCE
Cucumis sativus	Heat, polyethylene glycol (osmotic stress), cold and NaCl (salt stress)	Zhou et al. (2017)
Oryza sativa L.	Simulated acid rain at pH 4	Ju et al. (2017)
Brassica rapa L.	Cadmium stress (leaf spray with 50 mg L^{-1} $CdCl_2$)	Zong et al. (2017)
Transgenic tobacco (overexpressing a Cu/Zn-SOD gene)	Drought, freezing, and oxidative stress	Zhang et al. (2017)
Trifolium arvense	Drought	Ma et al. (2017)
Nerium oleander	NaCl (salt stress)	Kumar et al. (2017)
Ipomoea batatas [L.] Lam. (Sweet potato)	Potassium Deficiency	Liu et al. (2017)
Citrus sinensis [L.]	Water Deficit	Oliveira et al. (2017)
Transgenic Orange Sweet Potato (overexpressing a zeta-carotene desaturase gene)	200 mM NaCl	Li et al. (2017ª)
Tolerant rice genotype	60 mM NaCl	Li et al. (2017b)

2.3 PLANTS GLYCOLATE OXIDASE

2.3.1 Functional, structural and biochemical aspects of the GO enzyme

Glycolate oxidase (GO) [EC 1.1.3.1] is the first enzyme acting in the photorespiration process. This enzyme mediates glycolate oxidation to glyoxylate and H_2O_2 (ROSALES et al., 2012; BORELLA et al., 2017) in peroxisomes (HAVIR , 1983). Additively, GO may play a key role in gene-to-gene resistance in tobacco and arabidopsis (ZHANG et al, 2012). It is also reported that this biomolecule participates in the conversions of glyoxylate to oxalate/L-lactate to pyruvate (KENTEN & MANN 1952; CLAGETT et al., 1969; ZELITCH & OCHOA, 1973). In cyanobacteria and green algae, the enzyme that catalyzes these reactions is the glycolate dehydrogenase (GD) (HOFFMAN, 2015).

GO is classified as a flavoprotein, holds tetrameric format in C3 plants, or octameric format in C4 plants (POPOV et al, 2003; EPRINTSEV et al, 2009). Each GO subunit has approximately 43 kDa (MULLER F., 1992) in spinach plants (FRIGERIO NA, & HARBURY HA, 1958), peas (KERR MW & GROVES D, 1975), pumpkin and cucurbits, as well as 37-44kDa in soybean leaves and corn mesophylls respectively (EPRINTSEV et al, 2009). Furthermore, in enzyme crystallography, the existence of 369 amino acid residues and two types of structures has been determined: One obtained using butane as precipitant, in which the

tetragonal have unit cell dimensions of a = b = 148.1 Å and c = d=135.1 Å; and in the other crystalline form, obtained with ammonium sulfate as a precipitating agent, cell dimensions of a = b = 145.5 Å and c = d = 103.5 Å (MULLER F.,1992). Complementing these data, it has been reported that the amino acid residue Lis230, contained in this enzyme, confers a positive charge resultant on the amino group at position 1 and the neighboring carbon, thus maximizing the electrical efficiency of the fifth nitrogen atom in the chain (MACHEROUX et al., 1993). This electrostatic aspect stabilizes the anions of the flavin semiquinone molecules, 8-mercapto-flavin, and influences the formation of N-sulfide complexes (MACHEROUX et al., 1993).1993).

2.3.2 **Isoforms, regulation and GO inhibition**

It has been shown in *Arabidopsis thaliana* and rice that GO isoforms are encoded by a family of conserved genes. In rice are reported four genes, *OsGO1*, *OsGO3*, *OsGO4*, and *OsGO5* (ZHANG et al 2012; ZHANG et al, 2017) and three into Arabidopsis, *AtGO1*, *AtGO2*, and *AtGO3*. Arabidopsis isoforms GO1 and GO2 are related to photorespiration (so that they perform different functions during the period of oxidative stress (KERCHEV et al., 2016)) and GO3 is related with lactate metabolism (ENGQVIST et al., 2015). In addition, the first two isoforms have a combined expression, being 300 times greater than the last one (SCHMID et al, 2005; WINTER et al, 2015; al, 2007; DELLERO et al, 2016). In some studies, regulation of photorespiratory genes, such as GO, through signaling compounds and putative post-translational modifications has been proposed. This findings showed the GO activity inhibition in peas being proven through S-nitrosylation (JOSSIER et al, 2019), as well, by aldehyde bisulfite, glyoxylate bisulfite and α-hydroxysulphonates (MULLER, 1992), being a common practice in certain experiments.

2.3.3 **Glycolate oxidase response to the environment**

According to the literature, the physiological functions of GO are often considered to have a link with various responses induced by environmental stresses (CUI et al., 2016). An example for this is the gene for “enzymatic resistance” in melon, identified by Taler et al., (2004). They suggested that increased peroxisomal serine/glyoxylate aminotransferase (SGAT) expression correlated with increased GO activity play a role in resistance to *Psilocybe cubensis* by increased production of H_2O_2. It is also suggested that transcripts are increased in plants transgenics under water deficit conditions and GO levels in pea peroxisomes, that also grow on exposure to cadmium (McCARTHY et al, 2001). Despite this, plants with overexpression of GO tend to increase the H_2O_2 production, being a risk to the cellular constituents that can be

damaged. However, they present high photosynthesis, even at high temperatures or light (CUI et al, 2016), while suppression causes glyoxylate accumulation and photosynthetic inhibition (LU et al, 2014), as well, a reduced GO activity leading to the display of photorespiratory phenotypes. In this type of phenotype, plants show reduced growth and light green leaves when grown in the air under natural light conditions (DELLERO et al, 2016). In addition to drastically reduced electron transport rates in high light (ZELITCH et al., 2008).

2.4 GLYCOLATE OXIDASE X CATALASE INTERACTION

As previously stated in this study, the generation of H_2O_2 in peroxisomes is mainly given by the oxidation of glycolate catalyzed by GO (BORELLA et al, 2017) and then, in the same organelle, CAT is the main scavengers of this peroxide (MHAMDI et al., 2012). In this way, both enzymes work by proposing a modulation in the redox balance within the organelle. As a result, several studies have explored the functioning of these enzymes both in plants with CAT deficiency and in plants with overexpression of GO (SEWELAM et al., 2014). Additionally, due to the exposure of plants to the environment, the modulation of GO ends up occurring quite similarly to what happens with CAT, and exposure to salt, for example, increases the expression of both enzymes, providing better tolerance to salinity in *Puccinella tenuiflora* plants (YU et al, 2011).

Complementing these data, Zhang et al (2016) proposed that GO and CAT could work interacting in vitro and in vivo, with a possible dynamic association-dissociation between them in response to environmental stimuli. In this interaction, which may serve as a physiological mechanism for modulating the levels of H_2O_2 in C3 plants, this association by proximity is a possible evolved mechanism to enhance the efficiency of H_2O_2 elimination, since CATs have a low affinity for it. Unfortunately, few studies have been carried out to analyze the existence and formation of a possible complex with these enzymes, when they are requested. Consequently, there are no studies exploring the interdependence and correlation between them, as well, analyzing the gene modulation caused by the light variation.

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CHAPTER 2

~ RELATIONSHIP BETWEEN ACTIVITIES OF CATALASE AND GLYCOLATE OXIDASE IN RESPONSE TO CONTRASTING PHOTORESPIRATORY CONDITIONS INDUCED BY LIGHT INTENSITIES ~

3.1 RELATIONSHIP BETWEEN ACTIVITIES OF CATALASE AND GLYCOLATE OXIDASE IN RESPONSE TO CONTRASTING PHOTORESPIRATORY CONDITIONS INDUCED BY LIGHT INTENSITIES

ABSTRACT- Photorespiration is a common and necessary process for the survival of plants under certain environmental conditions. This pathway is capable of producing H_2O_2 through the activity of oxygen oxidation through the enzyme glycolate oxidase (GO) in peroxisomes, using glycolate as a substrate. Commonly, H_2O_2 is quickly consumed by the catalase enzyme and does not accumulate in the cell. Even with the existence of several studies on photorespiration as a whole, little is known about the interaction of enzymes within the process, especially when it comes to the physiological interaction between GO and CAT, especially under contrasting photorespiration conditions. Here, we aimed to investigate the possible functional regulation between the aforementioned enzymes in rice plants exposed to different photorespiratory conditions, induced by light variations. For this, in a first experiment, rice seeds (Oryza sativa japonica cv. Nipponbare) were cultivated in a greenhouse for 40 days, then submitted to *in vivo* analysis with an infrared gas analyzer (IRGA) and biochemical analysis with the samples collected. The results showed a decrease in photosynthesis and an increase in photorespiration with increasing light. It was also observed that GO and CAT respond positively to light-induced photorespiratory variations, in addition to being positively correlated, directly influencing each other's activity. In addition to the cross-talk of the enzymes with each other, both are strongly related to the H_2O_2 pool, as well as to the membrane damage and lipid peroxidation caused by it, although they respond in an negative-correlated way to the ASC-GSH cycle.

Keywords: *Oryza sativa*, photorespiration, environmental variation, photosynthesis

3.2 INTRODUTION

Sunlight is the primary source of energy on the planet, which is essential to various metabolic processes, especially for plants (PEREIRA et al., 2015). After collecting light energy, plants carry out the fixation of organic carbon, which can be through the metabolism C3, C4, and CAM (BASSAM, 2013). The C3 photosynthetic metabolic pathway is the dominant one among plant species. Which are distributed across the globe and comprise mostly crops, such as rice, barley, and wheat (JONES, 2014).

Under normal conditions, the C3 plants use an enzyme called ribulose-1-5-biphosphatecarboxylase-oxygenase (RUBISCO) to fix organic carbon, forming a compound called 3-phosphorus-glyceric acid (PGA), which is converted to triose phosphate (using itself ATP). The triose phosphate enters the Calvin cycle and generates phosphate sugar molecules, in which, for each cycle, necessary to use two molecules of ATP and 2 NADPH to carry out the conversion of CO_2 (ZHU et al., 2010). However, when under stressful conditions, CO_2 becomes less available and oxygen ends up adhering to Rubisco's catalytic site, giving rise to a toxic compound called 2-phosphoglycolate (2-PG) (MAIER et al., 2021; KUHNERT et al., 2021), which is removed by a photorespiratory pathway, which sometimes ends up being confused with the Rubisco oxygenation reaction, but is an interrelated, independent process with distinct stoichiometry (BUSH et al.,2020).

Photorespiratory results by loss of carbon and energy, reducing 23% of photosynthetic assimilation in a C3 plant (SAGE et al., 2012; SOUSA et al.,2018). Because of this, photorespiration has often been considered waste. However, reducing or removing photorespiration, could increase the complication of food production (WALKER et al., 2016; BUSH et al.,2020). Since plants deficient in photorespiratory enzymes or pathway inhibition, cannot survive and reproduce in environmental air, only when placed under conditions of high CO_2 (DELLERO et al.,2016; SOUSA et al.,2018).

When 2-PG is produced, due to your toxicity, it is quickly converted to glycolate and sent to peroxisomes. In peroxisome, the glycolate is detoxified by Glycolate oxidase (GO, EC 1.1.3.15), the key enzyme for this process. This enzyme catalyzes glycolate oxidation to generate glyoxylate and H_2O_2 (LI et al., 2021). Additively, may play a key role in gene-to-gene resistance in Nicotine and Arabidopsis (ZHANG et al., 2012). GO is classified as a flavoprotein, holds tetrameric format in C3 plants, or octameric format in C4 plants (POPOV et al., 2003; EPRINTSEV et al., 2009). Each GO subunit has approximately 43 kDa (MULLER F., 1992) in spinach plants (FRIGERIO NA, & HARBURY HA, 1958), peas (KERR MW & GROVES D, 1975), pumpkin and cucurbits, as well as 37-44kDa in soybean leaves and corn mesophylls respectively (EPRINTSEV et al., 2009).

According to the literature, the physiological functions of GO are often, considered to have a link with various responses induced by environmental stresses (CUI et al., 2016). An example for this is the genes for "enzymatic resistance" in melon, identified by Taler et al., (2004), who suggested that increased peroxisomal serine/glyoxylate aminotransferase (SGAT) expression correlated with increased GO activity, to play a role in resistance to *Psilocybe cubensis* by increased production of H_2O_2, as well, transcripts that are increased in plants transgenics (McCARTHY et al, 2001).

H_2O_2 is a reactive oxygen species (ROS) capable of causing damage to cells (CUI et al., 2016), because of this, photorespiratory H_2O_2 is eliminated, mainly by peroxisomal catalase (CAT, EC 1.11.1.6) that it can remove until 25% of all H_2O_2 (TYUTEREVA et al., 2017). Acting as antioxidant enzymes, CATs have a heme group, content iron atoms at the center of their constitution (BIRBEN et al., 2012; KAUSHAL et al., 2018) and this heme group is the key component for enzymatic activity. The structure of the enzymes is presented in a tetrameric and dumbbell form, with four identical monomeric subunits with 220-350 kDa (PALMA et al., 2020).

GO and CAT usually acts together to regulate the intracellular photorespiratory levels of H_2O_2 in plants (LI et al.,2021) and the redox homeostasis. Some studies report that plants with catalase deficiency and transgenic plants with overproduction of plastid glycolate oxidase provide a genetic system to disrupt H_2O_2 levels (SEWELAM et al., 2014). However, little emphasis has been given to the CAT-GO relationship in plants, Thus, no studies are exploring the interdependence and correlation between these two enzymes (GO and CAT), as well as a description of the modulation that occurs when plants are submitted to different photorespiratory levels. Based on this, the present investigation investigates the possible functional regulation between GO and CAT enzymes in rice plants to expose them to different light levels to induce contrasting levels of photorespiration.

3.3 MATERIAL AND METHODS

Growth and treatment conditions

Rice plants (*Oryza sativa* L. cv. Nipponbare) were germinated in germitest paper and transferred to 3L pots containing Hoagland-Arnon's nutritive solution (Hoagland & Arnon., 1950) in a greenhouse under natural conditions (See supplementary figure 1) for 40 days. After, the plants are changer for a growth chamber, when were acclimated one day (day/night means temperature 28/26 ºC, mean relative humidity of 70%, photoperiod of 12h and 300 μmol $m^{-2}s^{-1}$ PPFD), and immediately applicated different light treatments during 7 hours (exposition to 300 (low light- LL- treatment), 800 (control treatment), 1200 or 1800 (both high light -HL- treatment) μmol $m^{-2}s^{-1}$ PPFD) with temperature of 28 ºC and relative humidity of 70%. Subsequently, were performed analysis of photosynthesis and harvest of leaf sample in the presence of liquid nitrogen followed by storage at -80ºC until the biochemistry analysis.

Gas exchange and photochemical measurements

The steady state gas exchange parameters (A–CO_2 assimilation and g_s – stomatal conductance) were measured using a portable infrared gas analyzer system (LI6400XT, LI-

COR, Lincoln, NE, USA), equipped with a leaf chamber fluorometer (LI-6400- 40, LI-COR, Lincoln, NE, USA) in plants acclimated to different light conditions (300, 800, 1200 or 1800 μmol $m^{-2}s^{-1}$ PPFD) during seven hours. The photochemical parameters were measured by the saturation pulse method (SCHREIBER et al. 1995) and the leaves were previously acclimated in the dark for 30 minutes. Induction curves were performed, which consisted of 6 minutes of light induction from 0 μmol $m^{-2}s^{-1}$ to 1,700 μmol $m^{-2}s^{-1}$ of actinic light.

Electrolyte leakage and lipid peroxidation

Membrane damage (DM) was measured by described Blum and Ebercon (1981). Ten leaves segment (5 cm) were placed in test tubes containing 10 mL of deionized water. The tubes were closed and maintained to 24h in ambient temperature, and the electrical conductivity (C1) was measured. The tubs were boiled (95º C) for 60 min and cooled to ambient temperature, and the conductivity (C2) was measured again. The DM was estimed based by DM (%) = C1/C2 x 100. The lipid peroxidation was measured based on the formation of tiobarbituric acid reactive substance (TBARS) in accordance with Cakmak and Horst (1991).

H_2O_2 quantification

Rice leaves were macerated with 100 mM phosphate pH 7.5 and centrifuged at 12,000 g for 30 min at 4ºC. They were be placed in 500 μL eppendoffs, with the composite reaction medium 100 mM potassium phosphate buffer pH 7.5; Amplex Red 0.2 M and 0.2 U peroxidase. The assay was incubated for 30 minutes at 25º C in a thermomixer, protecting from light and read in a spectrophotometer at 560 nm.

Determination of ascorbate-glutathione redox state

The ascorbate reduced and total contents (ASA and ASA-DHA) were assayed according to Kampfenkel., et al (1995), using fresh leaves. The glutathione reduced and total contents (GSH and GSH + GSSG) were assayed according as described Griffith (1980). The leaves were

macerated with liquid nitrogen and 5% TCA, and were centrifuged at 12000 g (4ºC). The supernatant was immediately used for ASC and GSH determination. The assay for reduced was based on the reduction of Fe^{3+} to Fe^{2+} by AsA, and total ascorbate was measured after complete reduction with 10mM DTT and 0.5% N-ethylmaleimide. The total glutathione was measured using a reation in the presence of 1.0 unit of glutathione reductase, 0.15mM NADPH, 100mM sodium phosphate buffer (pH 7.0), and 6Mm DTNB. The ascorbate and glutathione redox state was calculated as ASA/ (ASA+DHA) x 100 and GSH/(GSH+GSSG) x 100, both expressed as a percentage.

CAT and GO activity assay

The catalase activity was performed according to the method of Havir and McHale (1987), based on the consumption of H_2O_2 in the medium. Aliquots of 50 µL of protein extract, added to 2.95 mL of reaction buffer (potassium phosphate pH 7.0 and 50 mM, containing 20 mM H_2O_2) were read at 240 nm in a spectrophotometer for 3 minutes (intervals of 30 seconds). To calculate the enzymatic activity, the molar extinction coefficient used was 362 mM cm^{-1}. The enzymatic activity of glycolate oxidase was done according to Baker and Tolbert (1966). Being 100 µL of the extract added to 2900 µL of reaction medium containing 100 mM potassium phosphate buffer, pH 8.3, 4 mM cysteine, 7 mM glycolate, 4 mM phenylhydrazine-HCl and 0.033 mM flavin mononucleotide (FMN). The activity was measured after 3 minutes of reaction by spectrophotometry at 324 nm and the activity calculation used the molar extinction coefficient of 17 mM^{-1}.

Statistical analysis and experimental design

The experiments were organized in a completely randomized design, with three replicates per treatment, each one represented by a pot containing two plants. The data were submitted to analysis of variance (ANOVA) and the averages were compared by Tukey's test or t-test with 5% of confidence level ($P < 0.05$), as indicated in the figure captions. All statistical

analyses were conducted using Sisvar statistic program and the picture are makes in SigmaPlot 12.0 (Systat Software, San Jose, USA) and R statistic program.

3.4 RESULTS

In the present work, wild-type rice plants (WT) were grown under partially controlled environmental conditions in a greenhouse until 40 days after sow (DAS). (FIGURE S1). Subsequently, they were taken to a phytotron-type growth chamber, acclimatized, and then subjected to different light levels for seven hours. Several ways to induce the photorespiration have been previously reported, one of which is the application of high levels of light (HL). In this work, the photosynthesis (A) tended to decrease in response to HL treatment in increasing levels of intracellular carbon (Ci) when rice plants (Figure S2). At the same time that Pr was increased with the luminous increment (Table S1). It is also observed an increase in the maximum carboxylation velocity (V_{cmax}) and in the mesophilic conductance (Table S1) in HL, despite these changes, the plants did not show visual differences after treatment (figure 1).

In figure S3, still study the photosintetic performance, was observed in the HL treatment, the electron flow to oxygenation/carboxylation electron flow ratio was slightly increased from 0.2 to 0.45. As well, the photorespiration/ photosintese ration was increase too. Similar to P_r/P_n the LL induced the reduction of J_o/J_c rate. These results corroborate the previous ones, proving that energy is being diverted to photorespiration, and thus, this is being increased with HL treatments.

In the figure 2, was increase the H_2O_2 content in both ML and HL levels, and consequently in membrane damage, when compared to the control treatment (800 μmol photons $m^{-2}s^{-1}$ or 800 μE), being both reduced in LL. In figure 3, the same happens with the content of substances to thiobarbituric acid (TBARS), which increases significantly. The accumulation of TBARS occurs due to lipid peroxidation of the cell membrane, caused by the possible increase in the amount of ROS (GILL and TUTEJA, 2010), possibly the increased H_2O_2.

As shown in Figure 4, compared to the control, the ML and HL treatments significantly reduced the AsA and GSH contents, and the AsA/DHA and GSH/GSSG ratios in rice leaves. An inverse behavior was observed when the plants were submitted to LL. With the inversion of the reduced and oxidized content of both ascorbate and glutathione, it is possible to observe the reduction of the AsA/DHA redox status from 86.5% to 71.6%; and from 75.8% to 31.7% in GSH/GSSG, when plants are exposed to 1800 μE of light, compared to the control.

From the evaluation of enzyme activity assays, CAT activity (figure 5a) was reduced approximately 22% in low light conditions, while an increase of almost 30% was recorded in HL. The GO activity (figure 5b) showed similar activity with a 50% reduction under the LL condition and 70% under the HL condition. These enzymes have shown a similar behavior in response to light-induced photorespiratory enhancement, with a positive linear correlation close to 1 between them being observed (figure 7).

A principal competent analysis (PCA), identified light intensity as the main contributing variable (81.7%) (figure 6). The total variance explained by Dim1 and Dim2 is 90.9%, which means that 9.1% of the variation in the analyzed data is explained by possible environmental variation or parameters that were not measured. However, for this experiment, such variations or parameters are less important, as they represent a low percentage of interference. The parameters of GO and CAT activity, as well as the accumulation of H_2O_2, DM and TBARS were shown to be correlated with each other, however, AsA, GSH and total ascorbate were shown to be anti-correlated to the other parameters mentioned above. When linear correlated parameter by parameter with enzyme activity, these effects can be better seen, being H_2O_2, DM and TBARS linear positive correlated (figure 8) while AsA and GSH are negative correlated or anticorrelated with the enzyme activities (figure 9).

3.5 DISCUSSION

Photorespiration is generally stimulated by an increase in light level (BROW and MORGAN, 1980; GERBAUD and ANDRE, 1980; VINES et al, 1982; HAUPT-HERTING et al, 2001; CUI et al, 2016). High light induces overproduction of glycolate and if not removed, it can be oxidized to glyoxylate by a PSI-dependent system (MURAI and KATOH, 1975; GOYAL, 2002; CUI et al, 2016). Excess glyoxylate is known to inhibit Pn (LU et al, 2014), so a higher level of GO is natural (figure 5b), to avoid glyoxylate toxicity. On the other hand, the increase in enzyme activity induces an increase in H_2O_2 (figure 2a) can be responsible for about 70% of its accumulation (ZHANG et al., 2017). Increasing levels of peroxide intensify DM (figure 2b) through lipid peroxidation (figure 3) and could also, according to Suzuki et al (2011), induce activation of programmed cell death (PCD), if not controlled by the enzyme CAT, which justifies the increase in its activity (figure 5a). Xiao-ping et al (2016) attribute this reduction in CAT enzymatic activity in LL to the fact that it is necessary to maintain a certain level of hydrogen peroxide (enzyme-substrate), since the same is necessary for signaling pathways. The high levels of activity can be explained by the high levels of H_2O_2 produced in high light by the photorespiratory enzyme GO, which has its activity intensified with the photorespiratory increment induced by light.

Beyond to H_2O_2 levels and lipid peroxidation, can also examined the activity of catalase and glycolate oxidase enzymes (figure 5). Were observed in the activities of both enzymes. A strong correlation was observed between the enzymes (figure 7), in which we obtained an R^2 of 0.9879. By observing the positive linear behavior and the high correlation index, can affirm that the catalase depends on the glycolate oxidase, thus having its activity modulated by it.

Regarding the increase in V_{cmax} induced by HL (table S1), this demonstrates the acceleration of the P_n and/or the CB cycle, which means a faster utilization of ATP and NADPH produced, thus exerting a protective role for the photosystems, avoiding a status excess electron

redox (YIE et al, 2015), because this photosynthesis cannot be reduced, thus supporting the diversion of energy directed to Pr. V_{cmax} is a parameter given as a function of the slope angle of the P_n curve, obtained by the method of Farquhar et al (1980), through the derivation dP_n/dCi. V_{cmax} represents the rate of regeneration of ribulose 1,5-bisphosphate carboxylase oxidase (RuBP or rubisco) during carboxylation within the plant leaf, which is of great importance since the decrease in photosynthetic rates in the plant can occur by limitation of rubisco regeneration (YAMORI et al, 2006). As for J, it has a strong relationship with V_{cmax}, so that when light limits photosynthesis, there is a high investment in J to V_{cmax} to maximize photosynthetic rates (WALKER, et al 2014). Concerning gm, significant intraspecific variation has been reported in several species, including wheat (BARBOUR et al, 2016).

The results of AsA/DHA (figure 4a), expose the functioning as a sensor of the status of the plant, being able to say if at the level of physiological stress (MIRET and MÜLLER, 2017). As well as the disulfide-thiol conversion is likely important in redox signal transduction (SHAO et al. 2008). Thus, it is known that plants with low AsA biosynthesis are more sensitive to environmental stressors (GALLIE, 2013), thus, the AsA/DHA reduction is a sign that it is stressed. Regarding the high content of GSH and the proportion of GSH/GSSG in plants (figure 4b), the same justification is valid, and the aforementioned changes may lead to the maintenance of an appropriate redox environment and the reduction of oxidative stress possibly caused by high light levels.

A principal competent analysis (PCA), identified light intensity as the main contributing variable (81.7%) (figure 6). Since with increasing light intensity photorespiration is stimulated, resulting in greater activity of the GO enzyme, which ends up by producing more H_2O_2 and this, when accumulated, causes an increase in lipid peroxidation and membrane damage, in addition to stimulating an increase in catalase activity as previously mentioned. Because this, all these parameters are on the same side of the graph. The negative correlation of AsA and

GSH with the activity of the enzymes (figure 9), mainly with CAT, should probably happen due to the functioning of the water-water cycle in which they are then inserted and that act as a parallel non-enzymatic antioxidant pathway for H_2O_2 removal, as well as , when the pathway is active the less the enzyme needs to work to remove peroxide and the opposite is also valid.

4 CONCLUSION

GO and CAT respond positively to light-induced photorespiratory variations, as well as being positively correlated, having a direct influence on each other's activity. In addition to the cross-talk of the enzymes among themselves, both are strongly related to the H_2O_2 pool, as well as to membrane damage and lipid peroxidation caused by it, even as respond in an negative-correlated manner to the ASC-GSH cycle.

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ANEXO A –RESULTS FIGURES AND TABLES

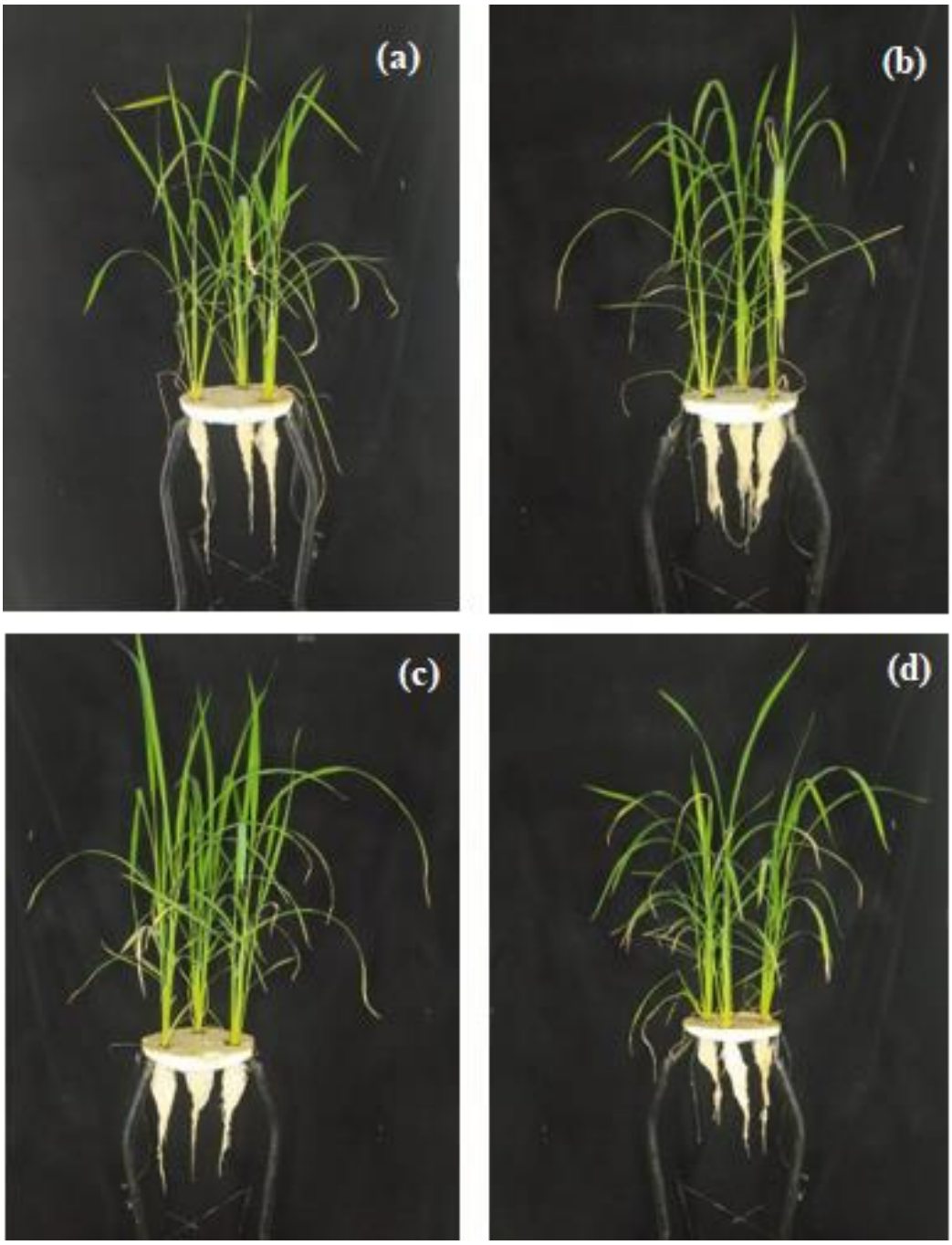

Figure 1. Visual aspects of rice plants exposed for seven hours to 300 (a), 800 (b), 1200 (c) and 1800 μE (d) of light intensities.

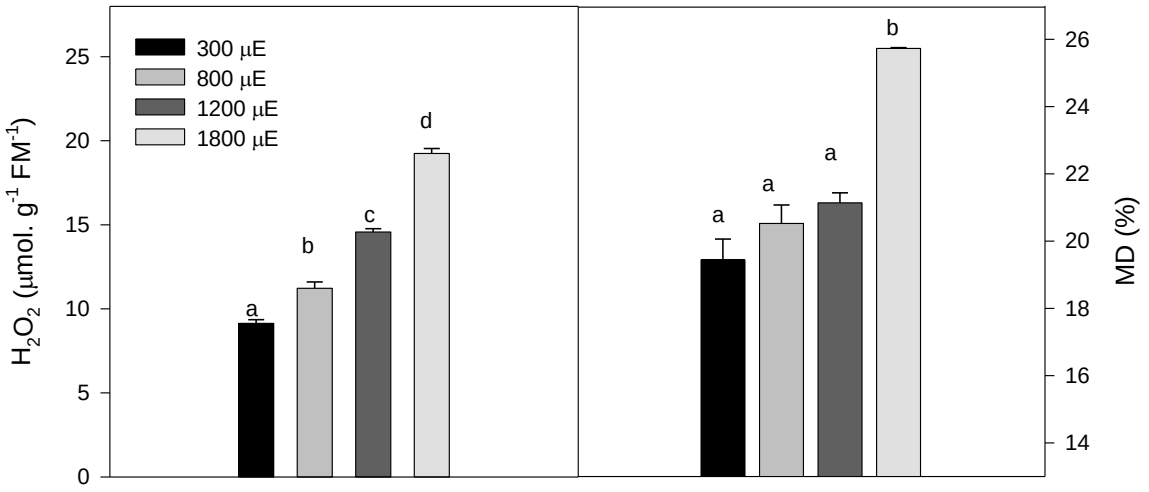

Figure 2. Hydrogen peroxide content (a) and membrane damage (MD) through electrolyte leakage (b), in rice plants leaves exposed for seven hours to different light levels (300, 800, 1200 and 1800 μE). The value represents an average of four replicates.

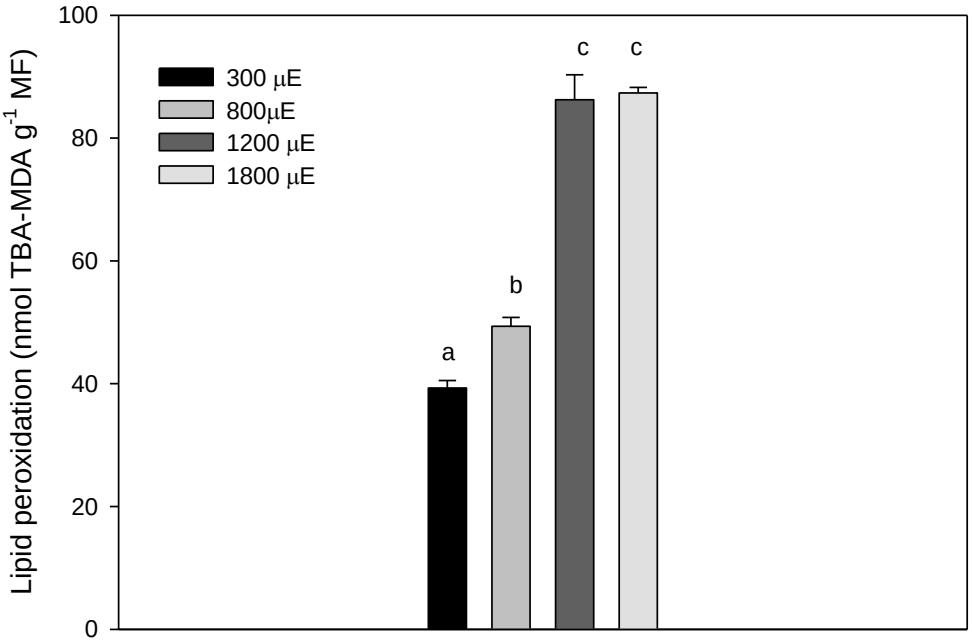

Figure 3. Lipid peroxidation determined through the content of thiobarbituric acid reactive substance (TBARs), in rice plants leaves exposed to seven hours to different light levels (300, 800, 1200 and 1800 μE). The value represents an average of four replicates.

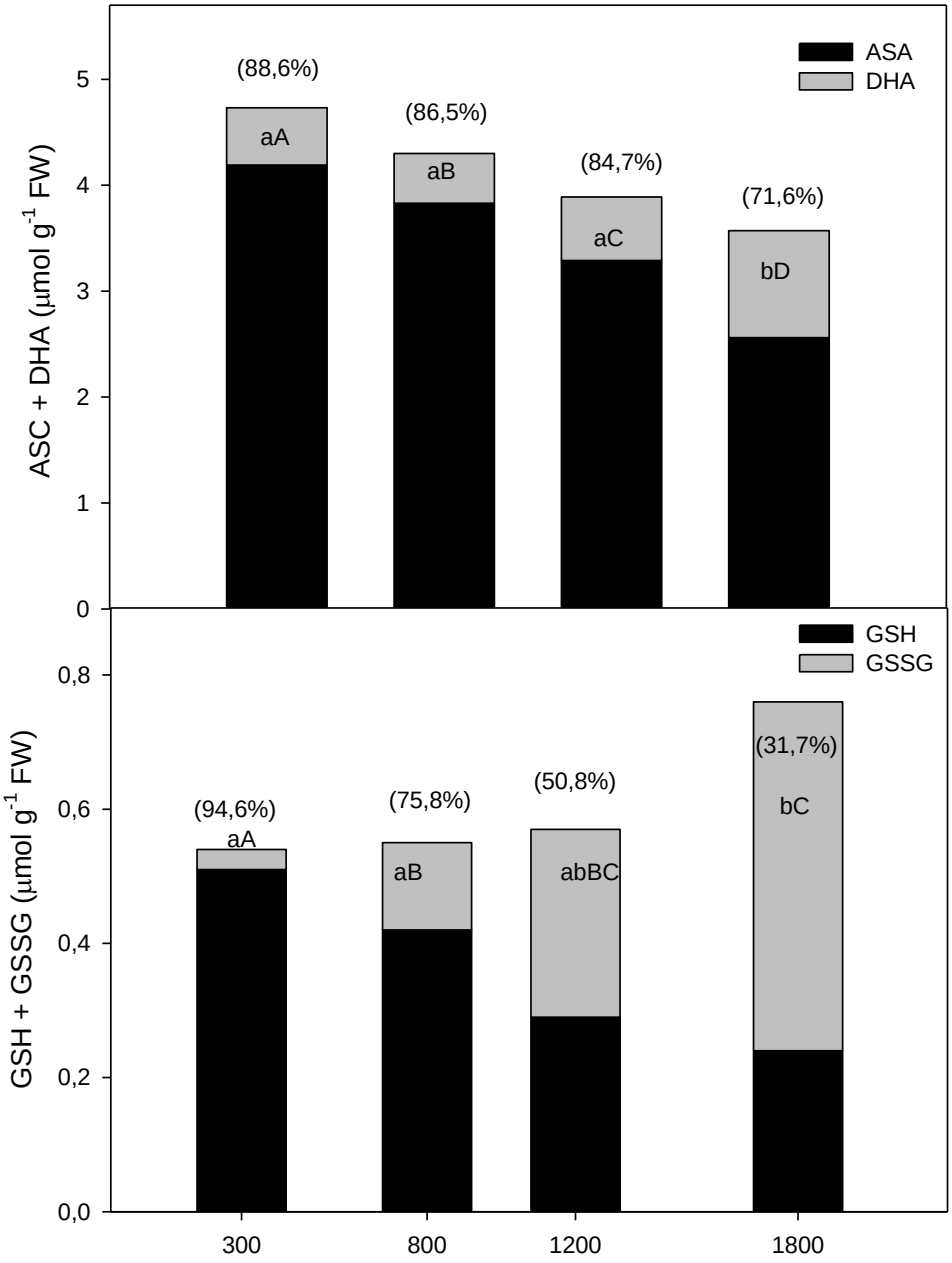

Figure 4. Total ascorbate content, ASA + DHA (μmol g^{-1} MF) of (a); total glutathione content (μmol g^{-1} MF) (GSH +GSSG) (b), and redox balance in of rice plants leaves exposed to seven hours to different light levels (300, 800, 1200 and 1800 μE). The value represents an average of four replicates. Lower case letters represent the oxidized state and capital letters represent the reduced state of the compound.

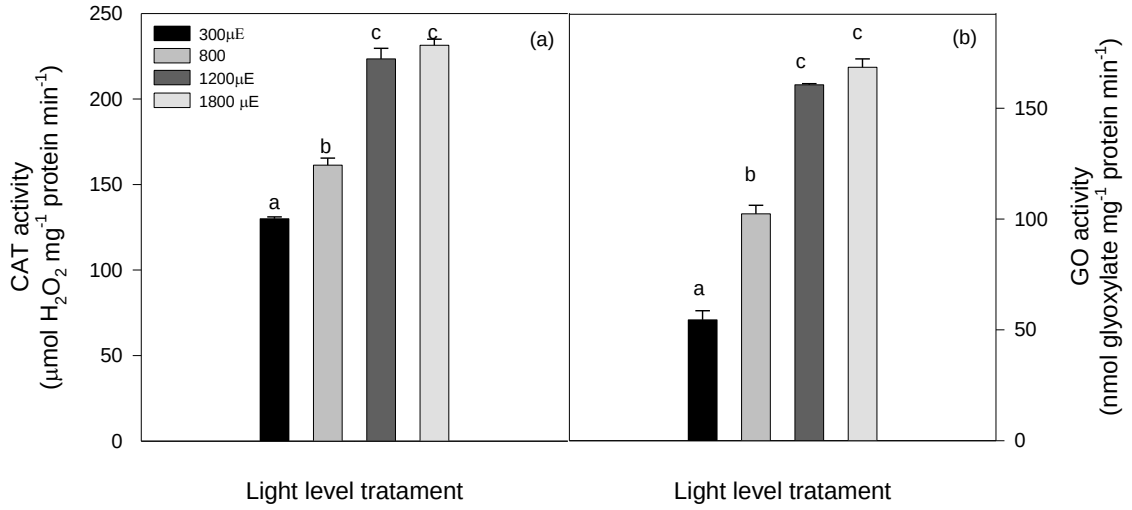

Figure 5. Catalase activity (µmol H_2O_2 mg^{-1} protein. Min^{-1}) (a) and GO activity (nmol glyoxylate mg^{-1} protein. Min^{-1}) (b) in rice plants leaves exposed to seven hours to different light levels (300, 800, 1200 and 1800 µE). The value represents an average of four replicates.

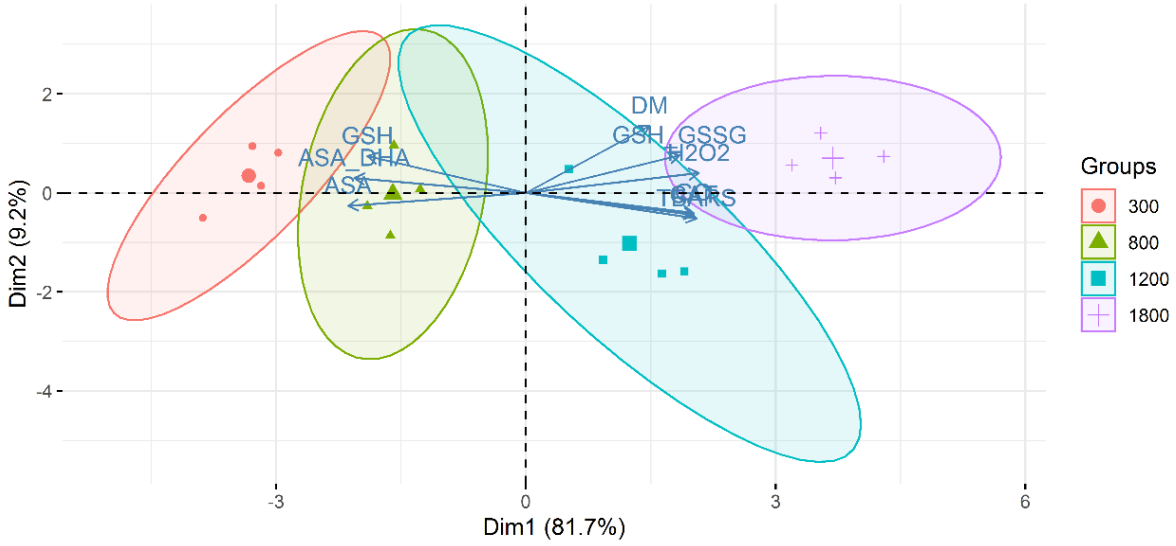

Figure 6. Principal component analysis (PCA) of normalized data rice plants leaves exposed to seven hours to different light levels (300, 800, 1200 and 1800 µE). Dots represent low light, triangles represent control light, crosses/squares represent high light.

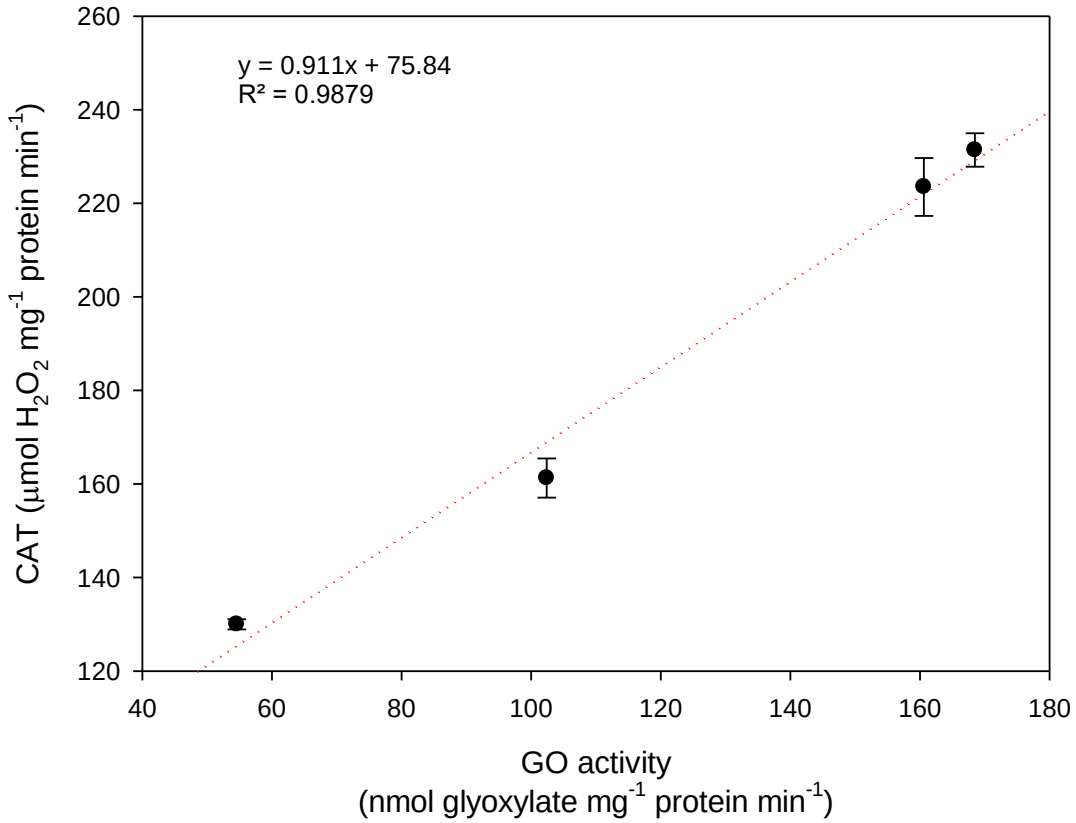

Figure 7. Catalase and GO correlation in rice plants leaves exposed to seven hours to different light levels. The value represents an average of four replicates.

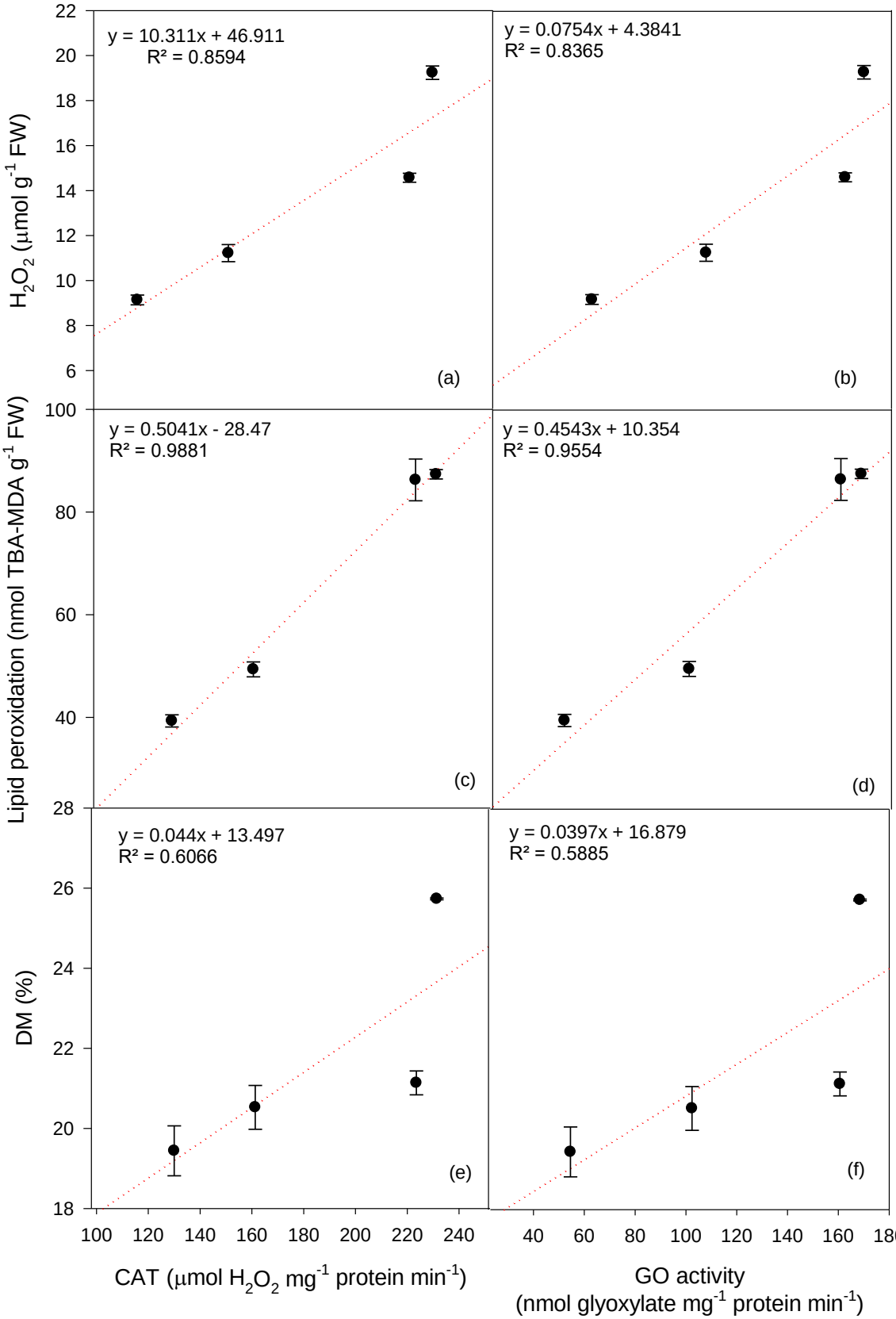

Figure 8. Correlation between the H_2O_2 content (a) and (b); the lipid peroxidation (c) and (d); the DM © and (f); with the catalase and GO activities in rice plants leaves exposed to seven hours to different light levels. The value represents an average of four replicates.

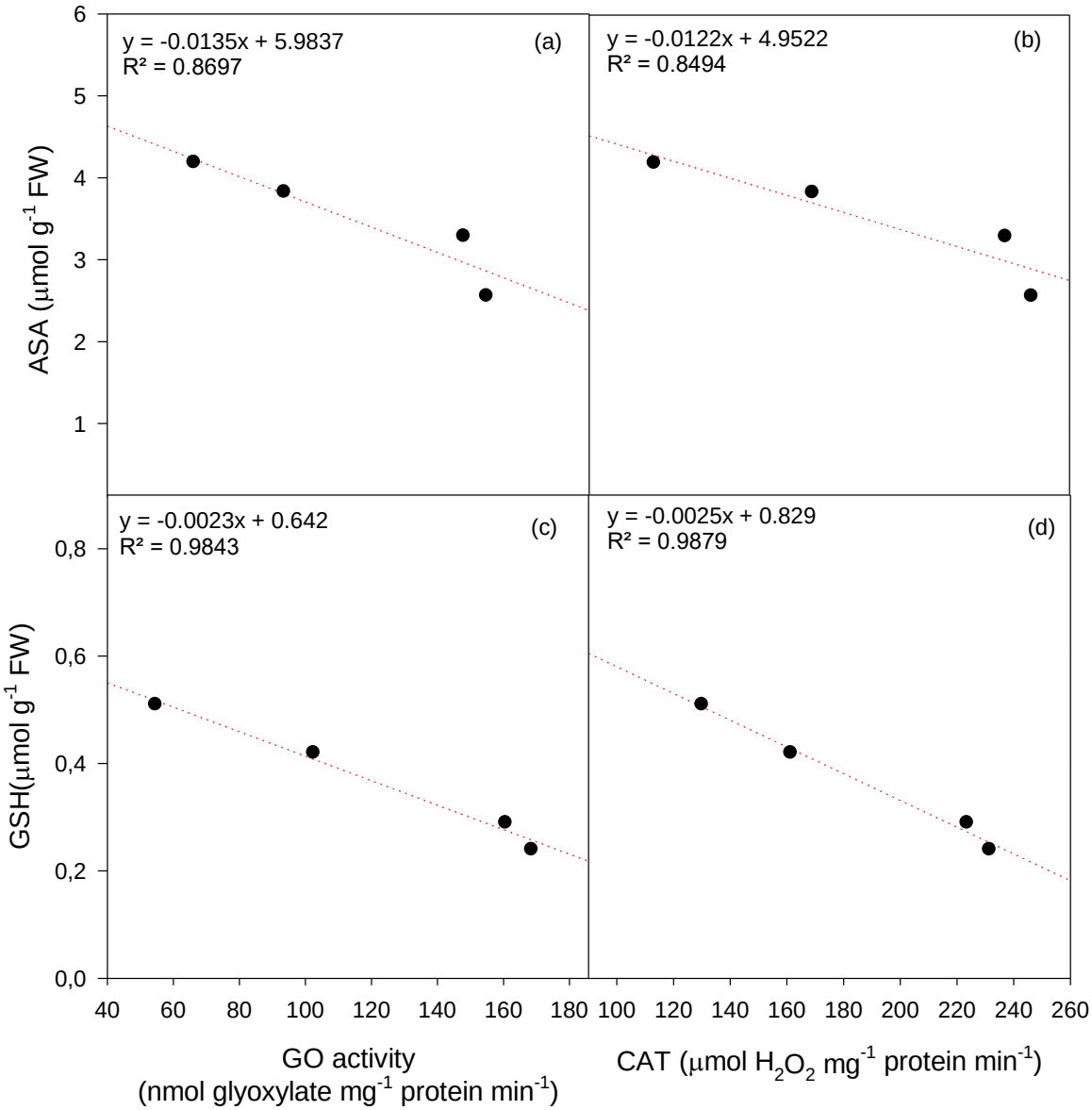

Figure 9. Correlation between the ASA (a) and (b); the GSH content (c) and (d); with the GO and Catalase activities in rice plants leave exposed to seven hours to different light levels. The value represents an average of four replicates.

ANEXO B - SUPLEMENTAR MATERIAL

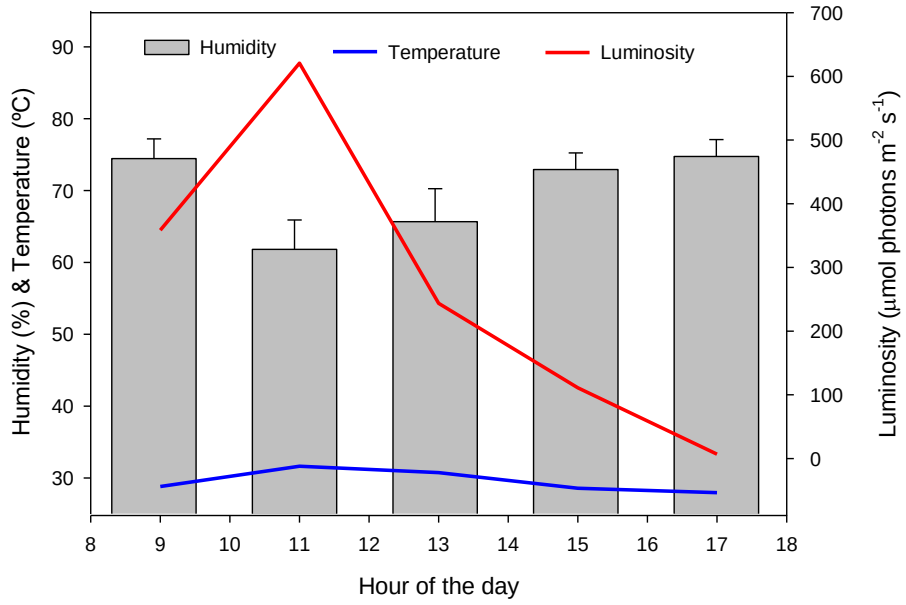

Figure S1. Greenhouse climatic parameters for rice growth conditions: relative humidity (%), temperature (º C) and luminosity (µmol photons m^{-2} s^{-1}).

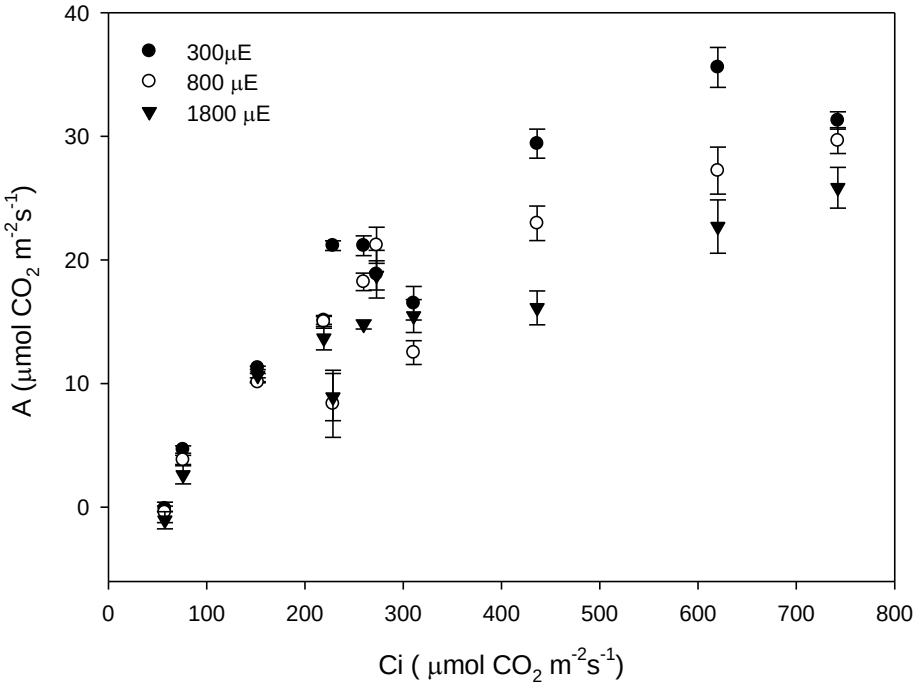

Figure S2. Net photosynthesis (A) in response to internal CO_2 (Ci) increment in rice plants leave exposed to seven hours to different light levels (300, 800, 1200 e 1800 µE). Points represent the averages and vertical bars the standard deviations (n=3).

Table S1. Maximum carboxylation velocity (Vc_{max}), Maximum electron flow (J), Dark respiration (R_d), Effective rate of electronic usage (J_{max}/V_{max}), mesophilic conductance (g_m), photorespiration (Pr) and CO_2 compensation point (Γ) in rice plants leave exposed to seven hours to different light levels (300, 800 and1800 μE).

Parameter	PPFD (μmol m^{-2} s^{-1})		
	300 μE	800 μE	1800 μE
Vc_{max}(μmolCO_2 $m^{-2}s^{-1}$)	81,40 ± 4,22[a]	104,90 ± 11,07b	143,83±7,95c
J_{max} (μmol e^- $m^{-2}s^{-1}$)	171,47 ± 6,71[a]	155,77 ± 1,83b	123,64 ± 1,57c
R_d (μmolCO_2 $m^{-2}s^{-1}$)	-0,31 ± 0,87[a]	1,77 ± 0,14b	1,05 ± 0,91b
J_{max}/V_{max}	2,11 ± 0,07[a]	2,07 ± 0,06[a]	0,81 ± 0,01b
g_m (mol CO_2 $m^{-2}s^{-1}$)	0,09 ± 0,00a	0,11 ± 0,01[a]	0,19 ± 0,00b
Pr (μmol CO_2 $m^{-2}s^{-1}$)	7.11 ± 0.21[a]	9.24 ± 0.013b	11.57 ± 0.01c
Γ	50.76 ± 0,00a	50.8 ± 0,00a	50.76 ± 0,01[a]

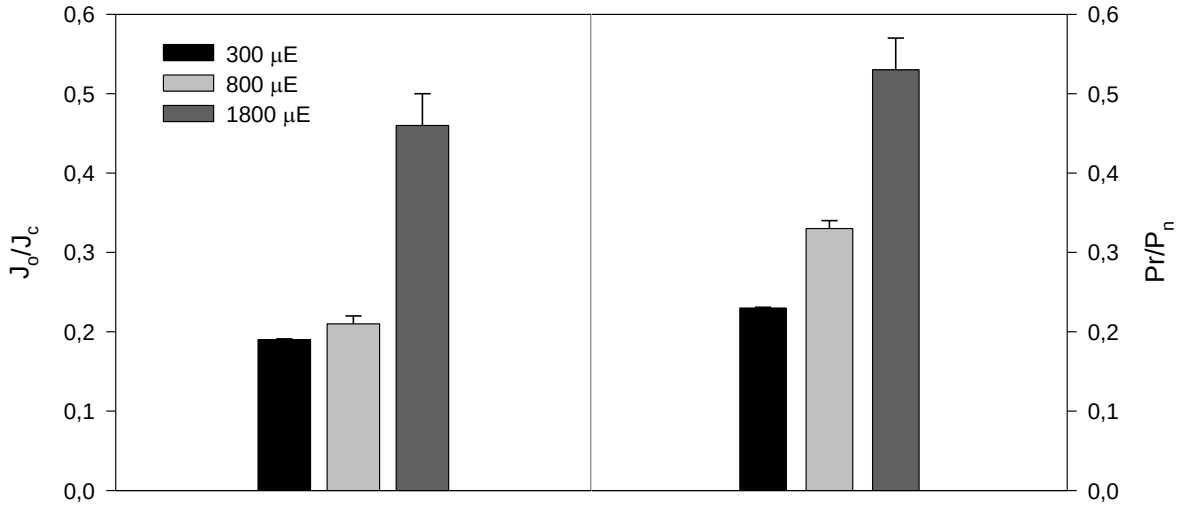

Figure S3. Electron flow devoted to oxygenation (Jo) / electron flow devoted to carboxylation (Jc) ratio (A) and photorespiration/ net photosynthesis ratio (B) of rice plants under effects of light intensities (300, 800 and1800 μE) during sever hours of exposition. Points represent averages and vertical bars the standard deviations (n=3).