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LUIS FLÁVIO MENEZES ROCHA JÚNIOR

SPATIO-TEMPORAL REDOX CHANGES IN RICE LEAVES EXPOSED TO HIGH LIGHT

FORTALEZA

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Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira.

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Aos meus pais, Jucivanda Rosa da Silva e Luis Flávio Menezes Rocha.

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RESUMO

Apesar do crescimento e desenvolvimento das plantas serem processos sistêmicos e espaço-temporalmente regulados, os mecanismos bioquímicos que envolvem o metabolismo redox ainda não são bem documentados. Neste trabalho, avaliamos a função da idade e posição da folha na resposta redox à luz intensa, com o objetivo de estabelecer uma relação entre as mudanças espaço-temporais e o metabolismo redox. O arroz (*Oryza sativa*) foi cultivado em casa de vegetação por 45 dias. Em seguida, as plantas foram transferidas para uma câmara de crescimento, onde foram divididas em dois grupos: baixa luminosidade - LL (300 μmol fótons m^{-2} s^{-1} PPFD) e alta luminosidade - HL (1.800 μmol fótons m^{-2} s^{-1} PPFD) por 9 horas. Após esse período, as folhas do dossel foram separadas em 3 partes: folhas inferiores (ou senescentes, L6 e L7), folhas intermediárias (ou totalmente expandidas, L8 e L9) e folhas superiores (ou últimas totalmente expandidas e folhas em expansão, L10 e L11). O conteúdo de clorofila dessas folhas foi medido periodicamente. Os conteúdos de peroxidação lipídica e extravasamento de eletrólitos foram semelhantes e fortemente aumentados nas folhas inferiores, independentemente da luz, em comparação com as folhas superiores e intermediárias. Em contraste, esses indicadores de estresse, juntamente com o teor de H_2O_2, foram intensamente aumentados nas folhas superiores expostas à HL. O estado redox do ascorbato foi fortemente diminuído da parte inferior para a parte superior e ligeiramente dependente da intensidade da luz. Surpreendemente, as atividades de CAT e APX foram fortemente estimuladas por HL nas folhas inferiores e médias, mas não no topo, enquanto a atividade de SOD não apresentou variação em resposta à luz ou parte da planta. Embora a porção superior tenha apresentado uma alta atividade de CAT, essa não foi estimulada pela luz, enquanto a atividade de APX nessas folhas foi menor do que nas folhas inferiores. O conteúdo de proteínas solúveis foi muito menor nas folhas senescentes e, portanto, as atividades enzimáticas dependerão da base escolhida: “mg de proteína” ou “g de massa fresca”. Aqui, escolhemos expressar por “g de massa fresca” porque é uma base mais estável. De acordo com nossos dados, as respostas redox exibidas por folhas ao longo do dossel de arroz expostas à luz contrastante são dependentes do estágio fisiológico da folha e/ou de uma contextualização espaço-temporal.

Palavras-chave: Oryza sativa; Antioxidante; Resposta sistêmica.

ABSTRACT

Despite plant growth and development are systemic and spatio-temporal highly regulated processes, the biochemical mechanisms involving redox metabolism are lacking. In this work we evaluated the role of leaf age and position in redox response to high light, aiming to establish a relationship between spatio-temporal changes and redox metabolism. Rice (*Oryza sativa*) hydroponically cultivated in greenhouse for 45 days. Afterwards, plants were transferred to a growth chamber, where they were divided in two groups: low light - LL (300 μmol photons m^{-2} s^{-1} PPFD) and high light – HL (1,800 μmol photons m^{-2} s^{-1} PPFD) for 9 hours. After this, the canopy leaves were separated in 3 parts: bottom or senescing leaves (L6 and L7), middle or fully expanded leaves (L8 and L9) and top or last fully expanded and expanding leaf (L10 and L11). Periodic measurements of chlorophyll content were taken in all leaves. Lipid peroxidation and electrolyte leakage were similarly and strongly increased in bottom leaves, independently of light, in comparison to top and middle leaves. In contrast, these stress indicators and H_2O_2 content were intensely increased in HL only in top leaves. Ascorbate redox state was strongly decreased from bottom to top parts and slightly dependent of light intensity. Interestingly, CAT and APX activities were strongly stimulated by HL in bottom and middle leaves but not in top, whereas SOD activity didn't present any variation in response to light or plant part. Top portion displayed high but not-HL stimulated CAT activity whereas APX activity in these leaves were lower than bottom leaves. Soluble protein were much lower in bottom and, thus enzyme activities will depend on the chosen basis: mg of protein or g of fresh weight. Here, we choose FM because it is a more stable basis. According to our data, redox responses displayed by leaves along rice canopy exposed to contrasting light are dependent on the leaf physiological stage or of a spatio-temporal contextualization.

Keywords: Oryza sativa; Antioxidant; Systemic response.

LIST OF FIGURES

LIST OF TABLES

SUMMARY

1 INTRODUCTION

Nowadays, one of the biggest difficulties in studying plant metabolism is that the most used techniques are reductionists and only allows us to analyze the event at one steady-state point in time (MUNNÉ-BOSCH, 2022). Considering plants as open complex systems, it is expected that they will present different responses to environmental conditions, such as high or low light, depending on the spatio-temporal scale (LIMA NETO et al., 2021). That is, the different phenological stages presented by the entire plant can exhibit different dynamics of response to the same stimulus.

Space and time effect in the plant response to different environmental conditions can be easily observed by leaves age and position. Leaves undergo basically 3 stages during their life span: (1) expansion, in which leaf act as a sink of nutrients and receive higher amounts of light due to its position at the canopy; (2) fully expanded, presenting higher photosynthetic activity; (3) senescence, which is generally under high oxidative stress because of the organelles dismantling and also receive lower amount of light due to the mature and younger leaves shade (LIM; NAM, 2007; MUNNÉ-BOSCH; ALEGRE, 2004). Once senescing leaves present a high oxidative stress, these leaves exhibit a decrease in photosynthesis rates and an increase in the content of reactive oxygen species (ROS, DHINDSA; PLUMB-DHINDSA; THORPE, 1981).

The formation of ROS is a result from several metabolic reactions that occur in different compartments of plant cells. When in high concentrations, ROS are extremely harmful to cells, triggering a state of oxidative stress. High concentrations of ROS can lead to protein oxidation, lipid peroxidation, damage to nucleic acids and activation of programmed cell death (SHARMA et al., 2012). To deal with these highly reactive molecules, plants have developed enzymatic and non-enzymatic antioxidant mechanisms for the ROS scavenging (NOCTOR; FOYER, 1998).

In this context, we have postulated the hypothesis that rice leaves present different redox responses to high light depending on their age and position. In accordance, our results showed a higher oxidative stress in senescent leaves, although it was not affected by the light, whereas younger presented a lower index, but high light sensitive, of oxidative stress.

GENERAL OBJECTIVE

- Determine how space and time affect the redox response to high light in rice leaves.

SPECIFIC OBJECTIVES

1. To determine the chlorophyll content in different rice leaves during the time.

2. To quantify the stress oxidation through electrolyte leakage, membrane permeability and H_2O_2 quantification in different rice leaves exposed to light stress.

3. To quantify the activity of enzymatic antioxidants (SOD, CAT, APX) and the redox state of ascorbate (ASC) in different rice leaves exposed to light stress.

2 LITERATURE REVIEW

2.1 Importance of redox metabolism for plant physiology

The oxidative stress can be characterized as a disruption of the balance between oxidant and antioxidant molecules in plant metabolism, which can be triggered by some biotic and abiotic stresses such as high light, pathogen infections, UV radiation, high and low temperatures, drought and salt stress, heavy metals, atmospheric pollutants, and physical and mechanical wounding (KERCHEV; VAN BREUSEGEM, 2021; SREENIVASULU et al., 2007; FAROOQ et al., 2008). These stress stimulators enhance the production of reactive oxygen species (ROS) in different subcellular compartments (PINHEIRO, 2004). ROS are powerful oxidant strength and can react with living cell components producing severe damage to proteins, lipids and nucleic acids (KARUPPANAPANDIAN et al., 2011). However, at low or moderate amounts, these compounds can also act as signaling molecules, regulating various physiological and developmental processes and pathogen defense in plants (FOYER; NOCTOR, 2005).

ROS molecules include superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$), which are mainly produced in chloroplasts, mitochondria and peroxisomes (Table 1, SCANDALIOS, 2005). It has been estimated that 1-2% of O_2 consumed by plants is converted to ROS (BLOKHINA et al., 2003). The main production of superoxide and singlet oxygen occurs at the photosystem I and photosystem II in chloroplasts, respectively (ASADA, 2006; CORPAS; GUPTA; PALMA, 2015). Photoreduction of oxygen by the excess photochemical energy can lead to the formation of $^1O_2$. Singlet oxygen is a highly reactive molecule, being able to transfer its excitation energy to or bind to biomolecules (MITTLER et al., 2004; RAO; REDDY, 2008). The superoxide radical is produced through the reduction of O_2 by a single electron and it's considered as a moderately reactive ROS. O_2^- can be spontaneously dismuted into H_2O_2, but they are also converted into H_2O_2 by the activity of superoxide dismutase (SOD) (MITTLER et al., 2004). In addition, H_2O_2 is also produced in peroxisomes by the glycolate oxidase activity during photorespiration (FOYER; FLETCHER, 2001). Hydrogen peroxide is a moderately reactive ROS and is able to cross biomembranes, which allows it to diffuse the damage and also act as a signaling in the stress response (HALLIWELL; GUTTERIDGE, 2015; MØLLER; JENSEN; HANSSON, 2007). It has been demonstrated that H_2O_2 can also contribute to the production

of $OH^{\cdot}$ through Fenton (Equations 1 and 2) and Haber-Weiss (Equation 3) reactions. $OH^{\cdot}$ is considered the most reactive ROS, acting as one of initiation radicals for lipid peroxidation and being able to react with almost any constituent of cells (KEHRER, 2000; VRANOVA; INZE; VAN BREUSEGEM, 2002).

Fenton reaction:

$$Fe^{3+} + O_2^{\cdot} \rightarrow Fe^{2+} + O_2 \qquad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \qquad (2)$$

Haber-Weiss reaction:

$$O_2^{\cdot -} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \qquad (3)$$

Table 1. Key reactive oxygen species (ROS) and their properties (KARUPPANAPANDIAN et al., 2011).

ROS	Half-life and mobility	Mode of action	Cellular sources	Main scavenging systems
Superoxide radical ($O_2^{\cdot -}$)	1 µs, 30 nm	Reacts with double bond-containing compounds such as iron-sulphur (Fe-S) clusters of proteins; reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$)	Formed in many photooxidation reactions (flavoprotein, redox cycling), Mehler reaction in chloroplasts, mitochondrial electron transport chains (ETCs) reactions, glyoxisomal photorespiration, peroxisomes, and plasma membrane. NADPH oxidase in membranes. Xanthine oxidase and membrane polypeptides in peroxisomes. Reactions of ozone (O_3) and $OH^{\cdot}$ in apoplastic space	Superoxide dismutases (SODs)
Hydroxyl radical ($OH^{\cdot}$)	1 ns, 1 nm	Extremely reactive with protein, lipids, DNA, and other macromolecules	Reaction of H_2O_2 with $O_2^{\cdot -}$ (Haber-Weiss reaction), reactions of H_2O_2 with Fe^{2+} (Fenton reaction). Decomposition of O_3 in apoplastic space	Flavonoids, prevention of $OH^{\cdot}$ formation by sequencing Fe
Hydrogen peroxide (H_2O_2)	1 ms, 1 µm	Oxidizes proteins; reacts with $O_2^{\cdot -}$ in a Fe-catalyzed reaction to form $OH^{\cdot}$	ETCs of mitochondria, chloroplasts, endoplasmic reticulum, and plasma membrane. Photorespiration, fatty acid β-oxidation, urate oxidase, and MnSOD in peroxisomes	Catalases, various Peroxidases, peroxiredozins, and flavonoids
Singlet oxygen ($^1O_2$)	1 µs, 30 nm	Directly oxidizes protein, polyunsaturated fatty acids, and DNA	Photoinhibition, photosystem II electron transfer reactions in chloroplasts	Carotenoids and α-tocopherols

In order to maintain the redox homeostasis and to mitigate the effects of oxidative stress, plant cells have developed wide-ranging enzymatic and non-enzymatic antioxidant systems to deal with the negative effects of ROS in plant metabolism (BONIFACIO et al., 2011). Non-enzymatic antioxidants are separated into two classes: lipid soluble membrane associated antioxidants (α-tocopherol and β-carotene) and water soluble reductants (glutathione, ascorbic acid and phenolics). These antioxidants play a key role in ROS scavenging by donating

electrons or hydrogen (ASADA, 1999). Besides that, ascorbic acid (AsA) is considered the main ROS-detoxifying compound in the aqueous phase because it can directly scavenge $OH^{\cdot}$, O_2^{-} and $^1O_2$, and can reduce H_2O_2 to H_2O through ascorbate peroxidase (APX) activity (NOCTOR; FOYER, 1998).

Enzymatic antioxidants include SOD, APX, catalase (CAT), glutathione peroxidase (GPX) and some other enzymes required for the regeneration of the active forms of the non-enzymatic antioxidants in ascorbate-glutathione cycle, like monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (APEL; HIRT, 2004; MUNNÉ-BOSCH; ALEGRE, 2004). CAT is located in peroxisomes and plays a key role in the catalytic scavenging of H_2O_2 into H_2O and O_2. SOD and CAT combined activity also combats the formation of the highly reactive hydroxyl radical (JALEEL et al., 2009). APX uses AsA as an electron donor to scavenge H_2O_2 into H_2O and monodehydroascorbate (MDHA) and has different isoforms distributed in different cellular compartments, like cytosol and chloroplasts (ASADA, 1999). MDHA can be reduced back into AsA by MDHAR activity using NAD(P)H as an electron donor or suffer spontaneous disproportionation to AsA and dehydroascorbate (DHA), a divalent oxidant of ascorbate (HOEKSTRA; GOLOVINA; BUITINK, 2001).

2.2 Spatio-temporal changes during plant development

Plants are complex open systems that are in constant interaction with an ever-changing environment, and they must be studied in its all complexity. However, the major physiologic events are analyzed in a fragmented, reductionist and simplistic way at one steady-state point in time, underestimating spatial and temporal dynamics of plant metabolism. Biotic and abiotic stress responses depend on levels of plant organization, and considering it as a homogeneous event can lead to misinterpretation of the effects of environmental stimuli on the plant as a whole (SOUZA; CARDOSO, 2003; PINHEIRO; CHAVES, 2010). According to Schneider (1998), multiscale analysis in biological systems is important because single experimental results cannot be directly transferred to larger scales and environmental conditions trigger different responses depending on the biosystem scale.

Leaf age and position is a great example of how space (position) and time (age) affect the responses to different environmental conditions on plants. During its life span, a leaf

undergoes several developmental stages. Initially, leaf acts as a sink of resources, importing carbon and nitrogen, increasing protein synthesis and cell expansion and accomplishing little net photosynthesis (the expansion stage, FIELD, 1987). When fully expanded, leaf photosynthetic capacity typically reaches its maximum and it starts to contribute to the supply of carbon. Mature leaves also present a low level of protein turnover, which remain until internal or external conditions trigger the initiation of senescence (LIM; NAM, 2007).

Leaf senescence can be understood as an integrated response of leaf cells to developmental age information and other internal and environmental signals (Figure 1). The environmental factors include high or low temperature, low light, drought, nutritional limitation, pathogen infection, shading and wounds. The internal signals include age, reproductive stage, hormonal level and high ammonium concentration (SOUSA et al., 2021; NOODÉN; LEOPOLD, 1988; THOMPSON et al., 1988; BLEECKER; PATTERSON, 1997; BUCHANAN-WOLIASTON, 1997). In this stage, leaf acts as a source of minerals and biomolecules for the whole plant level, contributing to the maintenance and formation of new organs (MUNNÉ-BOSCH; ALEGRE, 2004). Because of that, these leaves present specific metabolic changes, such as protein degradation, higher levels of ROS and lipid peroxidation, decrease in photosynthesis rates and an increase in membrane permeability (DHINDSA; PLUMB-DHINDSA; THORPE, 1981).

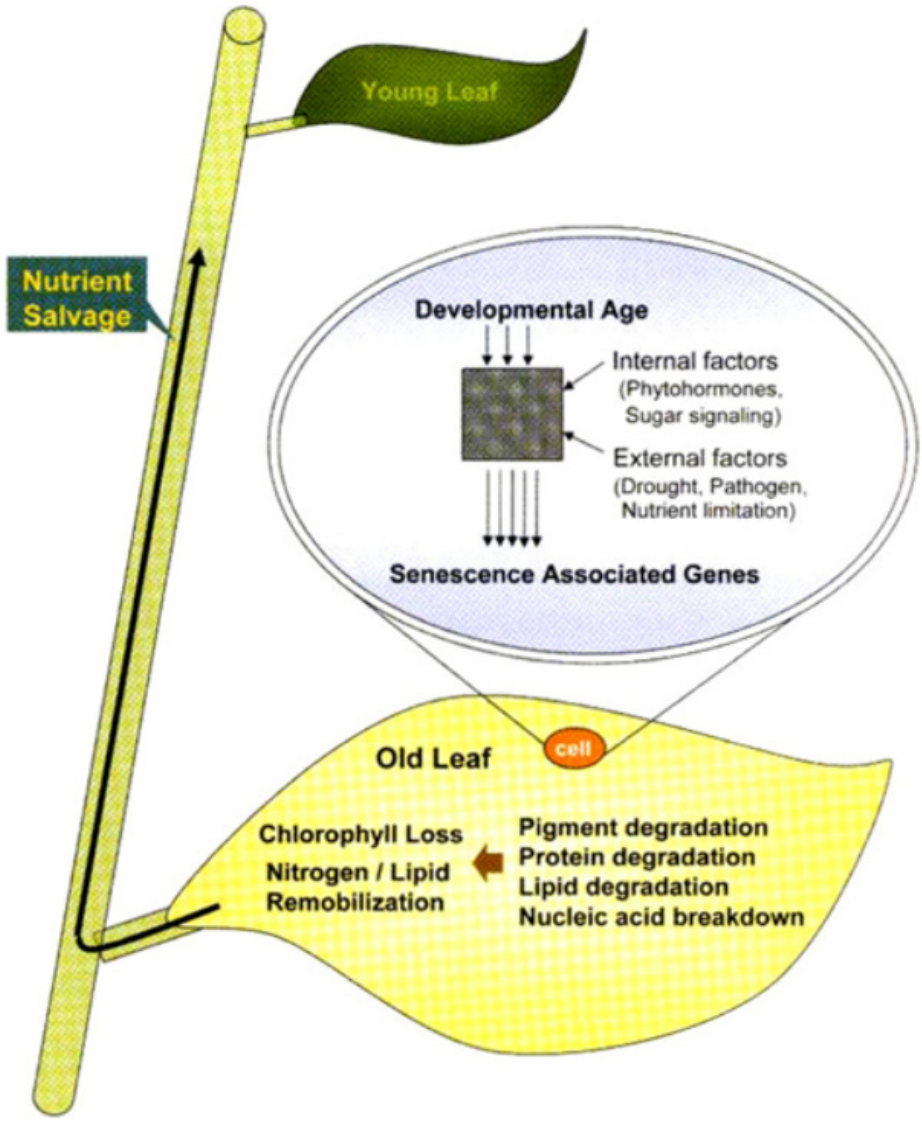

Figure 1: Leaf senescence-related processes (LIM; NAM, 2007).

Almost inevitably, as leaves age they also assume lower positions within plant canopy. Thus, these leaves receive smaller amounts of energy due to the overlapping leaf layers (HIROSE; WERGER, 1987). Besides leaf aging process, the vertical gradient of light also acts in the formation of a vertical N gradient through resorption and redeployment of N to better-lit and young leaves, maximizing canopy photosynthesis and N utilization efficiency (DROUET; BONHOMME, 1999; ANTEN; SCHIEVING; WERGER, 1995). In addition, some studies have shown the decrease of some leaf properties in parallel with decreasing light

availability, such as leaf erectness and leaf mass per area of individual leaves (KUROIWA, 1971; GUTSCHICK; WIEGEL, 1988).

2.3 Importance of light for rice growth and productivity

One of the most important environmental factors that determines the basic characteristics of rice development is light intensity. It decreases exponentially from the upper to lower layers of the plant canopy (MONSI; SAEKI, 1953) and also varies at different times of the day; for example, there is a lot of light at noon and numerous light fluctuations due to shadows from neighboring clouds or leaves moving in the wind. Plants exposed to excessive or insufficient intensities, known as light stress, affect their productivity characteristics by inhibiting physiological metabolic processes such as photosynthesis, antioxidant machinery, and their ability to fix atmospheric carbon and nitrogen (YANG et al., 2019).

When plants are exposed to low light conditions, the duration of growth and, consequently, the agronomic conditions change. Low light, for example, causes a longer period of growth and increases height and leaf area (LIU et al., 2014). Plants exposed to low light show morphological effects on height, biomass, and growth. Low light also reduces tillering, panicle and spikelet number, weight, and grain quality in rice growth (LIU et al., 2020; WANG ET AL, 2013; SEKHAR et al., 2019).

Rice production can be reduced by 30% to 50% in regions with continuous cloudy or rainy weather (VENKATESWARLU, 1977; VIJI et al., 1997; LIU et al., 2020), indicating that light lacking is a problem that affects rice production. A global study of the relationship between biomass accumulation and photosynthesis revealed that photosynthetic rate under low light is related to biomass accumulation and has a high potential for use as a target for high-yield rice breeding (QU et al., 2017).

Fluctuating light (FL) treatment inhibited rice plant growth, and the difference in plant growth rate between control and FL-treated plants became significant after 4 days of treatment. The rate of leaf area growth in plants treated with FL gradually decreased as well (Wei et al., 2020). As the climate changes, high light, high temperature (HLHT) stress may become more common and severe, affecting crop growth and yields. The phenotype of local lesions in the rice ls1 mutant is caused by DNA damage and excessive ROS accumulation caused by HLHT stress, and LS1 is required to protect genome stability and leaf structure from HLHT damage in rice (QIU et al., 2018).

Photosynthesis is also vulnerable to light-induced damage caused by reactive oxygen species production (ROS). Chlorophyll synthesis and solar conversion efficiency were higher in the genotype with pale green leaves (pgl) when exposed to high light treatment, whereas the normally pigmented control (Z802) showed the opposite trend due to the high level of photoinhibition under high light. Excessive absorption of solar energy in Z802 not only increased the generation of ROS and NPQ, but also exacerbated the effects of temperature increases, causing midday photosynthesis depression. These findings suggest that reducing the size of the light-harvesting chlorophyll antenna could improve photosynthesis and yield potential in rice (GU et al., 2017).

2.4 Rice as crucial crop for human nutrition

Rice (*Oriza sativa*) is considered as an important staple food, providing more than 20% of the calories consumed worldwide for two-third of the world's population, especially in South and East Asia, parts of Caribbean and Latin America (SHARIF, 2013). This cereal is grown in more than 100 countries with 90% of these being Asian countries (FUKAGAWA; ZISKA, 2019). In addition, the world production of paddy rice was 782.0 million tons in 2018, being surpassed only by corn and followed by wheat (FAO). Rice has over than 40,000 varieties, which differ in grain length, color, thickness, aroma and growing conditions and/or production practices, impacting quality and nutrient profile (LI et al., 2019; FUKAGAWA; ZISKA, 2019).

People generally consume polished (or milled) rice, although it contains a smaller amount and variety of nutrients due to the milling process, which consists in the removal of embryo and bran layers (ZHAO; LIN; CHEN, 2020). In general, rice has higher amounts of carbohydrates and mild quantities of proteins and lipids. It also has a higher variety of secondary metabolites, such as phenolic compounds, vitamin E (tocopherols and tocotrienols) and vitamin B (thiamin, riboflavin and niacin). Furthermore, rice present some minerals, mostly including K, Na and Mg, among macroelements, and Cu, Fe, Zn and Mn, among microelements (SHAR et al., 2003;). In some regions of South Asia, rice contribution to per capita dietary of energy and protein is more than 50%, while this value is 17-27% for dietary fat (KENNEDY; BURLINGAME; NGUYEN, 2002).

Although rice production has increased substantially in recent years, it is still insufficient to supply the increasing global demand (SASAKI; BURR, 2000). Global rice consumption has been higher than the production since 2000, and this deficit has been solved by drawing on bumper stocks (NGUYEN, 2002). The annual shortage of rice is estimated to increase from 400,000 to 800,000 tons in 2016 and 2030, respectively (THIRZE, 2016).

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3 MATERIAL AND METHODS

3.1 Growth and treatment conditions

Rice seedlings (Oryza sativa L. cv. Niponbare) 7-days-old, which were germinated in germitest® paper and then transferred to 2,7 L pots filled with Hoagland-Arnon's nutritive solution (HOAGLAND; ARNON, 1950). The nutritive solution was changed weekly. They were grown at greenhouse under natural conditions (day/night mean temperature of 30/26° C, mean relative humidity of 79% and photoperiod of 12 h). Non-destructive measurements of chlorophyll (Chl) content were taken periodically using a portable SPAD 502 Chlorophyll Meter (Minolta Co., Ltd.) to observe the senescence progress. After this period, plants were moved to a growth chamber, two group of plants were individually exposed to 300 μmol photons m^{-2} s^{-1} PPFD (low light treatment, or LL) and 1800 μmol photons m^{-2} s^{-1} PPFD (high light treatment, or HL) for 9 h, with a mean temperature of 25° C and a mean relative humidity of 64%. For the harvest, rice canopy were separated in 3 groups: bottom, or senescing leaves (leaves 6 and 7), middle, or expanded leaves (leaves 8 and 9) and top, or last fully expanded and expanding leaves (leaves 10 and 11), which were collected separately in the presence of liquid N2 and stored at -80° C for posterior analysis.

3.2 Electrolyte leakage and lipid peroxidation

Membrane damage or cellular viability was measured by the electrolyte leakage, as in Blum and Ebercon (1980). Ten leaves segments of 5 cm were placed in test tubes containing 10 ml of deionized water. The flasks were kept in a shaking for 12 h and then the medium electrical conductivity was measured (C1). After this, the segments were boiled at 95 °C for 1 h and cooled at 25 °C, and the electrical conductivity was measured again (C2). The membrane damage (MD) was estimated by MD=(C1/C2)x100. The lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS), as previously described by Cakmak and Horst (1991). TBARS concentration was calculated using its absorption coefficient (155 mM cm^{-1}) and the results were expressed as nmol TBA-MDA g^{-1} FW.

3.3 Hydrogen peroxide content

For the extraction, fresh leaves were grounded with 100mM phosphate buffer pH 7,5 in the presence of liquid N_2 and then centrifuged at 12,000 x *g* for 30 min at 4 °C. The H_2O_2 content was determined by the resofurin formation (ZHOU et al., 1997), using the Amplex-red™ kit. For the assay, 100 μL of the resulting supernatant was added in a 500 μL microtube containing a reaction buffer (1358 μL of 100 mM potassium phosphate buffer pH 7,5 + 14 μL of 0,2 M Amplex Red + 28 μL of 0,2 U horseradish peroxidase) and was incubated at 25 °C for 30 min, then it was read in a spectrophotometer at 560 nm and the H_2O_2 concentration was estimated by a standard curve.

3.4 Enzymatic activity assays

The total soluble protein extract was obtained by grounding fresh leaves with 100mM phosphate buffer pH 7,0 containing 1mM EDTA and 1 mM L-Ascorbic acid in the presence of liquid N_2 and then centrifuged at 14,000 x *g* for 30 min at 4 °C. The supernatant was used for all enzymatic assays. The APX activity was measured following ascorbate oxidation by the decrease in absorbance (NAKANO; ASADA, 1981). For the assay, 100 μL of the supernatant was added in a 5 mL tube containing a reaction buffer (2,5 mL of 50 mM potassium phosphate buffer pH 7 + 200 μL of 6,75 mM L-Ascorbic acid + 200 μL of 30 mM H_2O_2) at 25 °C, then the absorbance at 290 nm was followed for 240 s. The activity of APX was expressed as μmol AsA mg^{-1} protein min^{-1}. CAT activity was measured following the H_2O_2 consumption (HAVIR; MCHALE, 1987). The reaction was performed at 30 °C by adding 50 μL of the supernatant in 2,95 mL of 50 mM potassium phosphate buffer (pH 7) containing 20 mM H_2O_2 and the absorbance at 240 nm was followed for 240 s. The CAT activity was estimated according to the molar extinction coefficient of H_2O_2 (36 M cm^{-1}) and was expressed as μmol H_2O_2 mg^{-1} protein min^{-1}. SOD activity was measured by the inhibition of blue formazan production (BEAUCHAMP; FRIDOVICH, 1971). For the assay, 30 μL of the supernatant was added in 2,97 mL of reaction buffer (50 mM sodium phosphate buffer pH 7,8 + 13 mM methionine + 75 μM nitrotetrazolium blue chloride + 0,1 mM EDTA + 2 μM riboflavin), then it was placed in a chamber with a 30 W fluorescent lamp for 5 min and the absorbance was read at 540 nm. The activity was expressed as U SOD mg^{-1} protein min^{-1}.

3.5 Ascorbate redox state

For the extraction, fresh leaves were grounded with liquid N_2 and 6% TCA, and then samples were centrifuged at 15,000 x *g* for 15 min at 4 °C. Total ascorbate (AsA+DHA) and reduced ascorbate (AsA) content were determined according to Kampfenkel et al. (1995). For the reduced ascorbate assay, 50 μL of the supernatant was added in a 5 mL tube containing the reaction medium (150 μL of 200 mM potassium phosphate buffer pH 7,4 + 50 μL of deionized water + 250 μL of 10% TCA + 200 μL of 45% H_3PO_4 + 200 μL of 4% bipyridine + 100 μL of 6% $FeCl_3$), then it was placed in a water bath at 42 °C for 40 min. Total ascorbate assay was performed by adding 50 μL of the supernatant in a 5 mL tube containing 50 μL of 10 mM dithiothreitol and 100 μL of 200 mM potassium phosphate buffer pH 7,4, then it was moved to the water bath at 42 °C for 15 min. After this, it was added 50 μL of 0,5% N-ethylmaleimide, 250 μL of 10% TCA, 200 μL of 45% H_3PO_4, 200 μL of 4% bipyridine and 100 μL of 6% $FeCl_3$ in the same tube. It was also maintained in a water bath at 42 °C for 40 min. Both absorbance was read at 525 nm, and the oxidized ascorbate (DHA) was obtained by the difference between reduced and total ascorbate. The ascorbate redox state (%) was calculated as (AsA/AsA+DHA)x100.

3.6 Experimental design and statistical analysis

The experiments were arranged in a completely randomized design, with two different light intensities and four replicates per treatment, each one represented by an individual pot containing two plants. Data were analyzed by ANOVA and averages were compared by Tukey's test at the $P \leq 0.05$ significance.

4 RESULTS

Rice plants were grown at greenhouse under natural conditions for 5 weeks (Figure S1). Non-destructive measurements of chlorophyll content were taken periodically in order to observe the leaf aging process through the chlorophyll degradation in senescent leaves. As expected, older leaves presented lower amounts of chlorophyll, which gradually decreased over time, representing the senescence progress (Figure S2).

The measurement of stress indicators were taken to evaluate the effect of age and position in the oxidative response to HL. Although not affected by the light, senescing leaves presented higher levels of electrolyte leakage (Figure S3) and lipid peroxidation (Figure S4) when compared to fully expanded and young ones. Light stress was observed only in top leaves, which were increased by the HL. Similarly, only top leaves showed a strongly increase in H_2O_2 content when exposed to HL, whereas middle leaves showed higher levels of H_2O_2 when compared to bottom leaves in both treatments and top leaves in LL (Figure S5). In addition, leaves at the middle and top position presented an increase of 63% and 69%, respectively, in total soluble proteins when compared to bottom leaves (Figure S6). However, it was only sensitive to light in middle leaves, which showed an increase of 23%.

Although the redox state of ascorbate did not undergo significant changes in the middle part after HL exposure, it was decreased by 7% and 23% in leaves at the bottom and top parts, respectively (Figure S7). There was also observed a gradually increase in total ascorbate content from bottom to the top. The activities of CAT (Figure S8) and APX (Figure S9) were highly stimulated by HL in bottom and middle leaves, whereas the same response was not noticed in young leaves. Top portion displayed higher CAT activity whereas APX activity in that canopy leaves were lower than bottom leaves. SOD activity did not show significant changes between treatments or among parts (Figure S10). Interestingly, when CAT (Figure S11), APX (Figure S12) and SOD (Figure 13) activities are expressed by “mg of soluble protein” they show different patterns, which might be due to the strong difference between senescent leaves soluble proteins content and the others. Here, we chose express by “g of fresh weight” because it reflects better plant cells and tissues and is a more stable basis.

5 DISCUSSION

Since many studies have shown the importance of considering space and time in biological processes analyzes for a better understanding of biosystems, in this work we have postulated the hypothesis that rice leaf age and position play an important role in redox response to light (MUNNÉ-BOSCH, 2022;). It was observed a gradual increase in chlorophyll content during leaf expansion; meanwhile it was decreased over time in older leaves. It is well known that expanding leaves construct their photosynthetic apparatus gradually, so the levels of chlorophyll increase steadily during leaf expansion (JIANG et al., 2005, LEPEDUS, 2011). Chloroplasts are one of the first organelles affected by the onset of senescence, leading to the breakdown of photosynthetic apparatus, chlorophyll degradation and a decrease in photosynthesis (WINGLE et al., 2006; ABREU; MUNNÉ-BOSCH, 2009). However, the degradation of this photosynthetic pigment during senescence does not occur in all species, as demonstrated by Krupinska et al. (2012) in a barley variety, which exhibited an increase in the chlorophyll *a/b* ratio at a late phase of senescence.

Senescing leaves present changes in cell structure, metabolism and gene expression (NOODÉN; LEOPOLD, 1988). Since senescing leaves act as a source of nutrients to young tissues and reproductive organs, these leaves naturally present high level of protein and RNA degradation, lipid peroxidation and membrane permeability (BUCHANAN-WOLLASTON, 1997). These properties were confirmed by the higher levels of electrolyte leakage and lipid peroxidation and the lower amount of soluble proteins exhibited by bottom leaves. The non-sensibility of these stress indicators to HL in older leaves might be due to their position within canopy, where these leaves receive lower amounts of energy because of the mature and younger leaves shade (HIROSE; WERGER, 1987). Light-related and age-related distinction of leaf traits is important for better understanding plant canopies and improve the optimization of canopy models. Interestingly, bottom part did not present higher content of H_2O_2, contrasting with the higher amount of H_2O_2 in tobacco senescent leaves observed by Ohe et al. (2005). Supporting our results, Liu et al. (2019) also observed an increase in soluble protein content in mature leaves of wheat exposed to long-term HL. They attributed this to a potential difference between leaf senescence induced by HL and the natural senescence.

Top portion displayed low but HL-stimulated lipid peroxidation and higher amounts of soluble protein. In accordance, Lepedus et al. (2019) also found higher protein amounts and

lower lipid peroxidation in emerging leaves of *Acer platanoide* L. when compared to the mature ones. In addition, other studies have shown higher amounts of lipid peroxidation in younger leaves (JUVANY; MÜLLER; MUNNÉ-BOSCH, 2012), thus suggesting that emerging leaves suffer oxidative stress depending on species and/or growth conditions. Indeed, young leaves are more sensitive to many environmental insults since their photosynthetic apparatus still in construction, being more susceptible to photoinibition, and their location at the top part of the canopy, where they receive more direct energy than mature and senescent leaves (JIANG et al., 2005; MAAYAN et al., 2008). This higher susceptibility to oxidative stress presented by these leaves can be observed through the increase of electrolyte leakage, lipid peroxidation and H_2O_2 content in response to HL.

The results of electrolyte leakage, lipid peroxidation and H_2O_2 are strongly correlated with ascorbate redox state. The relevant decrease in ascorbate redox state presented by younger leaves shows that, although these leaves are still expanding, they already have antioxidant mechanisms to deal with the increase in H_2O_2 content induced by HL. Correlating to our results, Ohe et al. (2005) also has shown a gradual increase in total ascorbate content from older to younger leaves. This pattern might be related to the role of ascorbate in cell division, cell expansion and in modulating plant senescence (SMIRNOFF; WHEELER, 2000). Lower levels of total ascorbate in older leaves have been reported for various species, such as *Betula alba*, *Corylus avellana*, *Alnus glutinosa*, *Pistacia lentiscus* and *Pisum sativum* (GARCÍA-PLAZAOLA; HERNÁNDEZ; BECERRIL, 2003; MUNNE-BOSCH; PENUELAS, 2003; PALMA et al., 2006). Unfortunately, SOD, CAT and APX activities were not correlated to the other results presented here. Since rice has a lot of isoforms of these enzymes distributed in various organelles, such as chloroplasts, mitochondria and cytosol (SOUSA et al., 2019; LEE; KIM; LEE, 2001), it is possible that individual analysis of the activity of each isoform will lead us to a better understanding of its role in each cell compartment (PINTO-MARIJUAN; MUNNE-BOSCH, 2014).

6 CONCLUSION

Our results suggest that space and time thus affect the redox response to high light in rice leaves. Although it is still necessary to perform finer measurements of antioxidant enzymatic activities to better understand their role along canopy, it was observed that older leaves naturally present higher oxidative stress, which is not affected by the high light, whereas young leaves presented a lower but high light-stimulated oxidative stress.

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ANEXO A – RESULTS AND SUPPLEMENTARY FIGURES

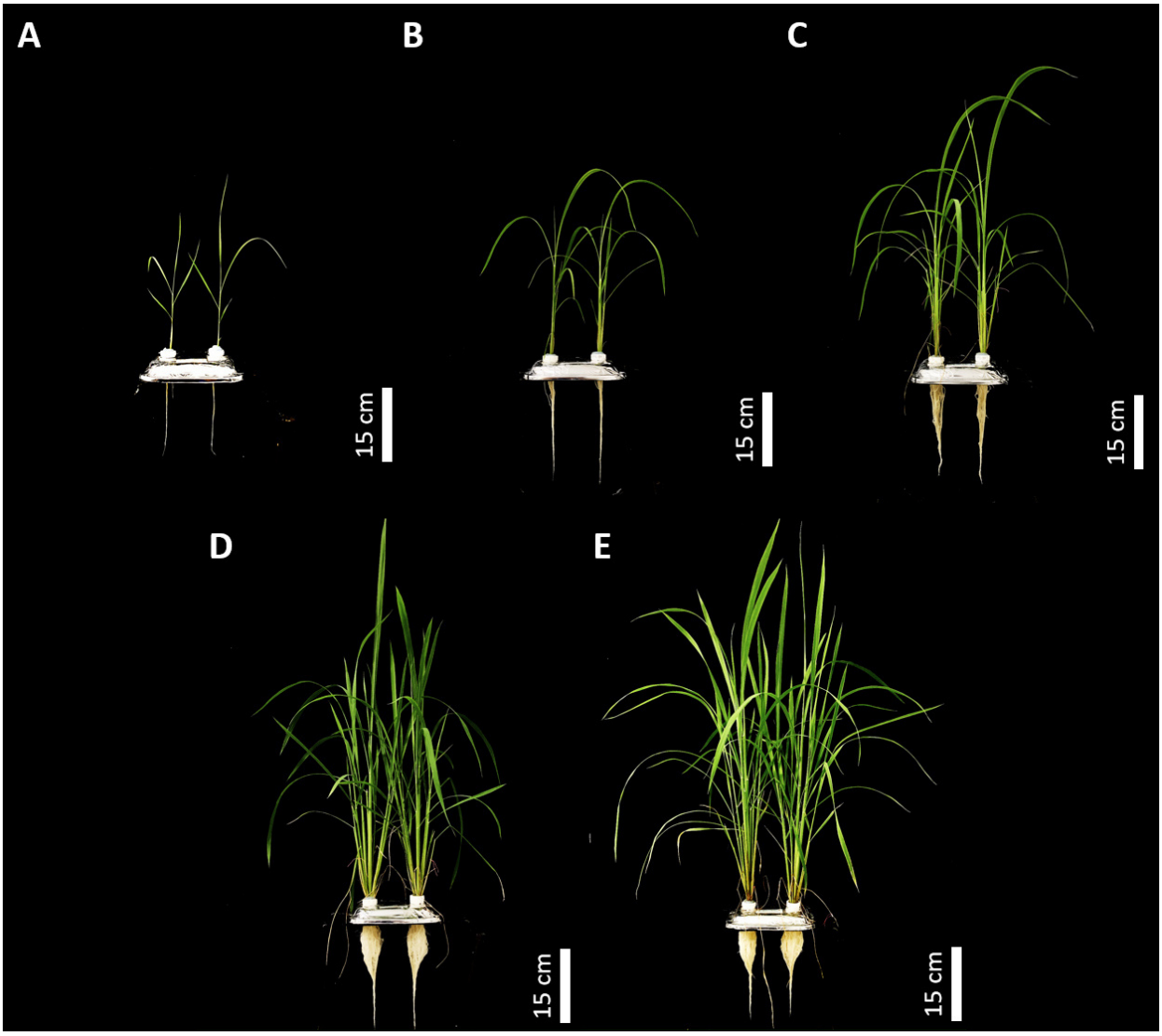

Figure S1: Rice plant development after 1 (A), 2 (B), 3 (C), 4 (D) and 5 weeks (E).

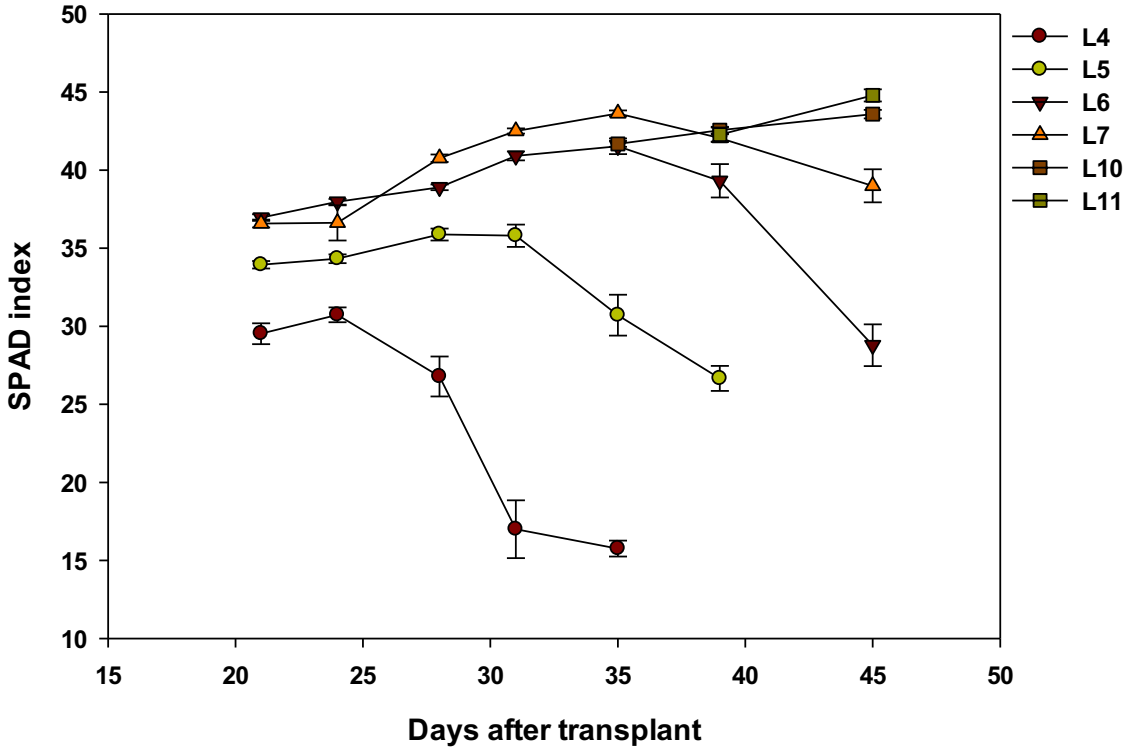

Figure S2: Chlorophyll content expressed by SPAD-502 readings of leaf 4 (L4), 5, 6, 7, 10 and 11 (L11) during the time. Represented values indicate the average of four independent replicates (±SE).

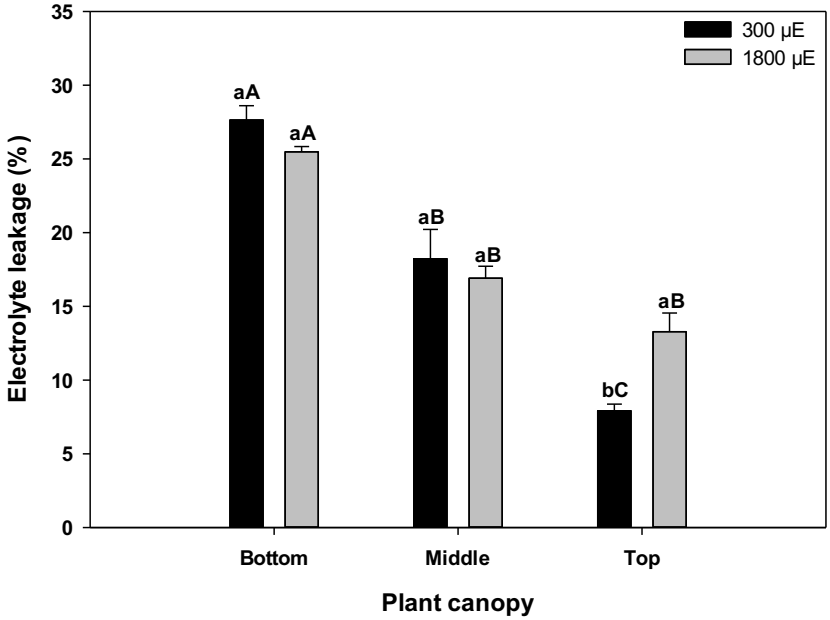

Figure S3: Electrolyte leakage of leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

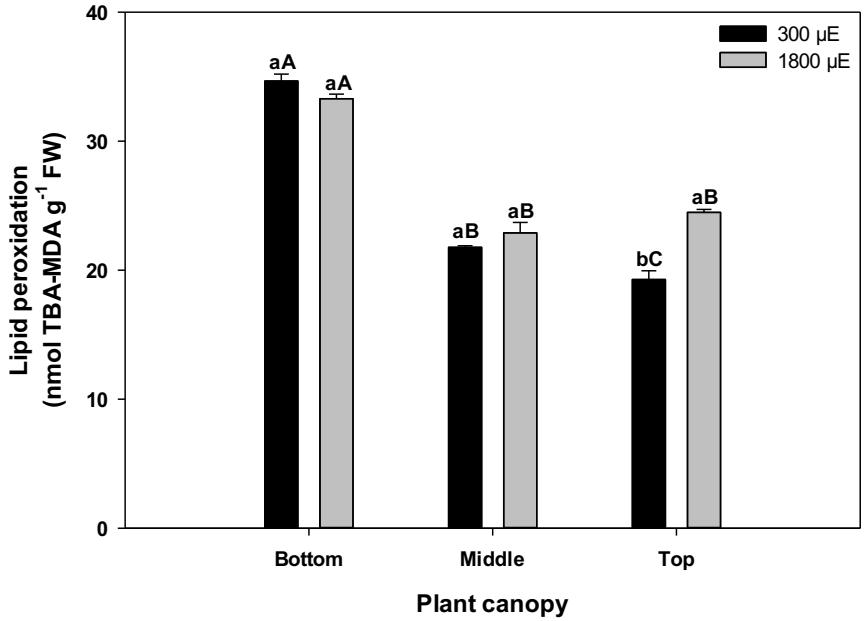

Figure S4: Lipid peroxidation of leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

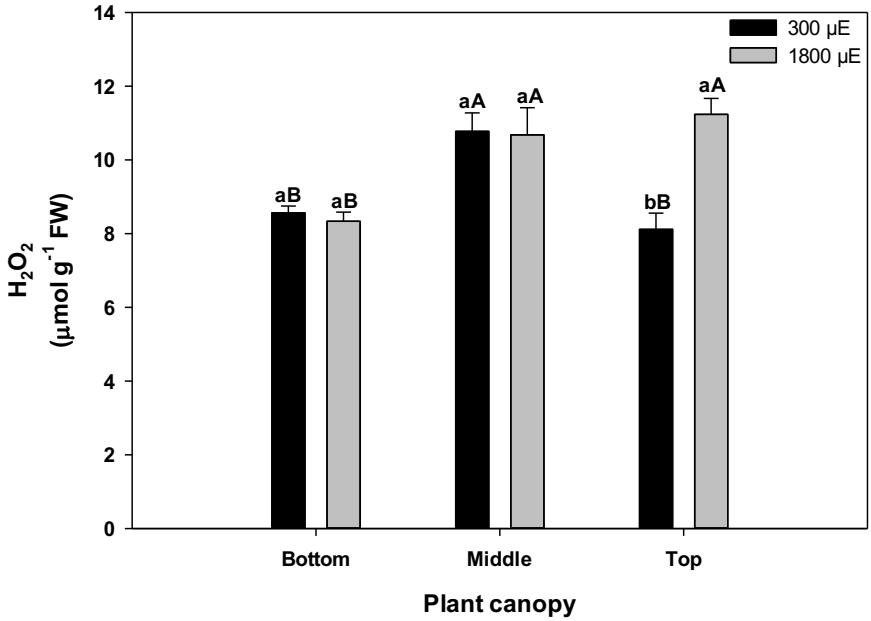

Figure S5: Hydrogen peroxide content in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

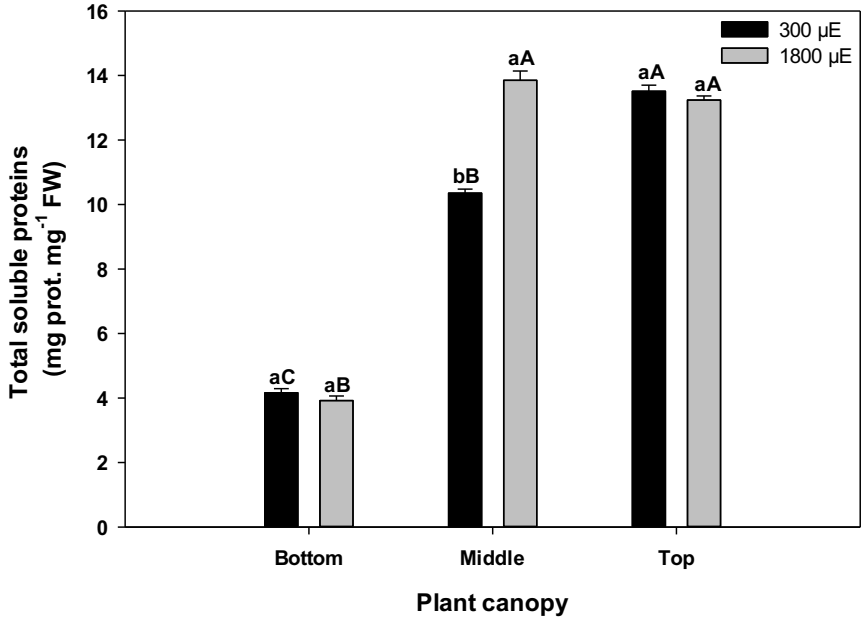

Figure S6: Total soluble proteins of leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

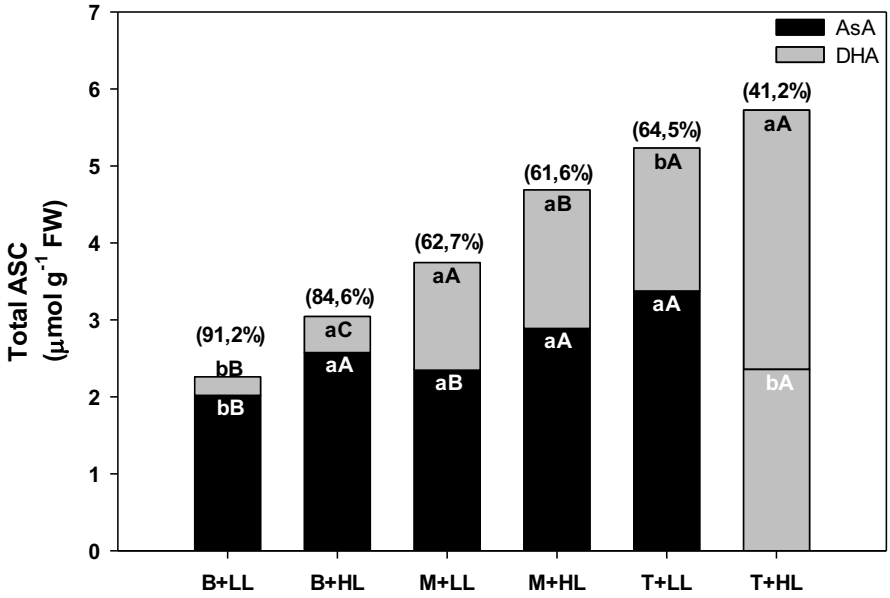

Figure S7: Redox state of ascorbate (%) and reduced ascorbate (AsA), oxidized ascorbate (DHA) and total ascorbate (AsA+DHA) in leaves at different positions on rice canopy: bottom –B– (senescing leaves), middle –M– (expanded leaves) and top –T– (last fully expanded and expanding leaves), which were exposed to 300 (low light –LL–) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light –HL–). Different lowercase letters represent significant differences of AsA and DHA between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences of AsA and DHA among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

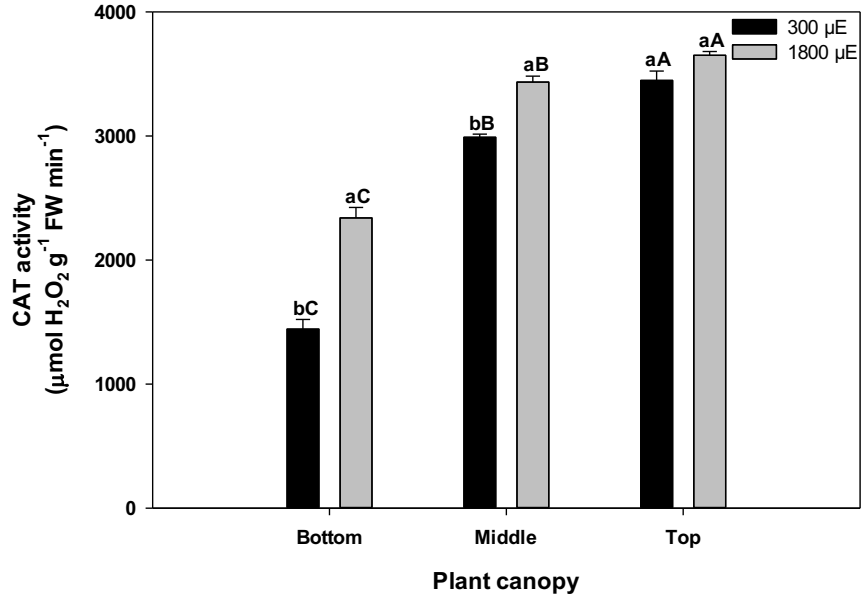

Figure S8: Catalase (CAT) activity in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

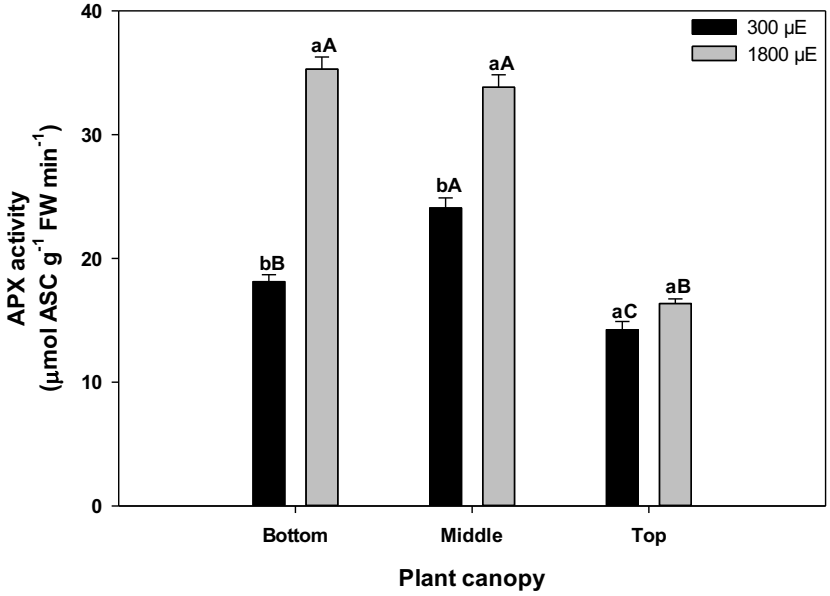

Figure S9: Ascorbate peroxidase (APX) activity in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

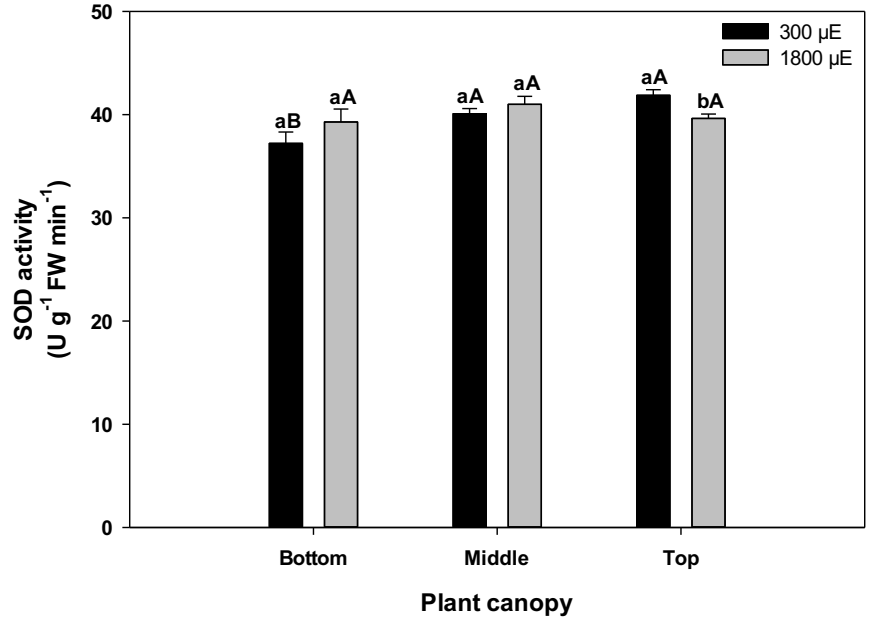

Figure S10: Superoxide dismutase (SOD) activity in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

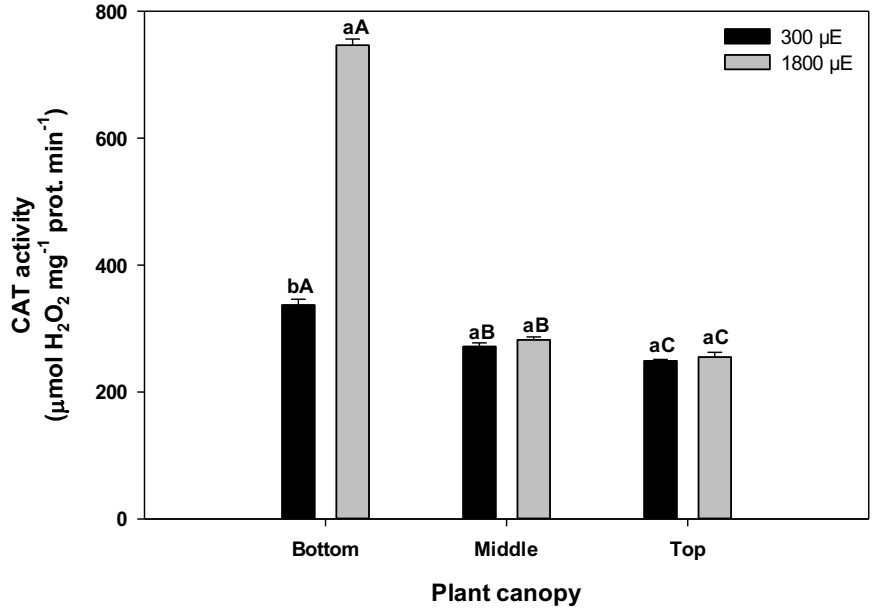

Figure S11: Catalase (CAT) activity in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 μmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 μE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

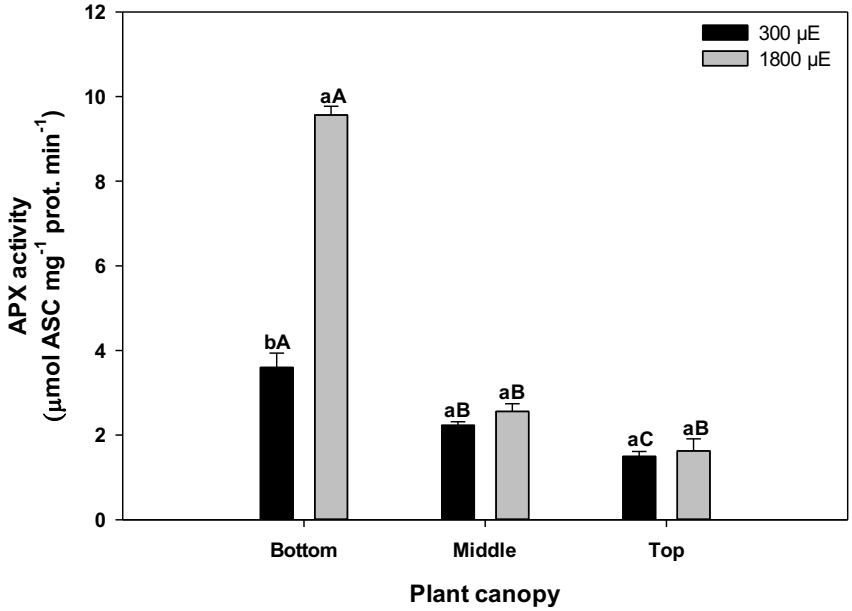

Figure S12: Ascorbate peroxidase (APX) activity in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 μmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 μE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

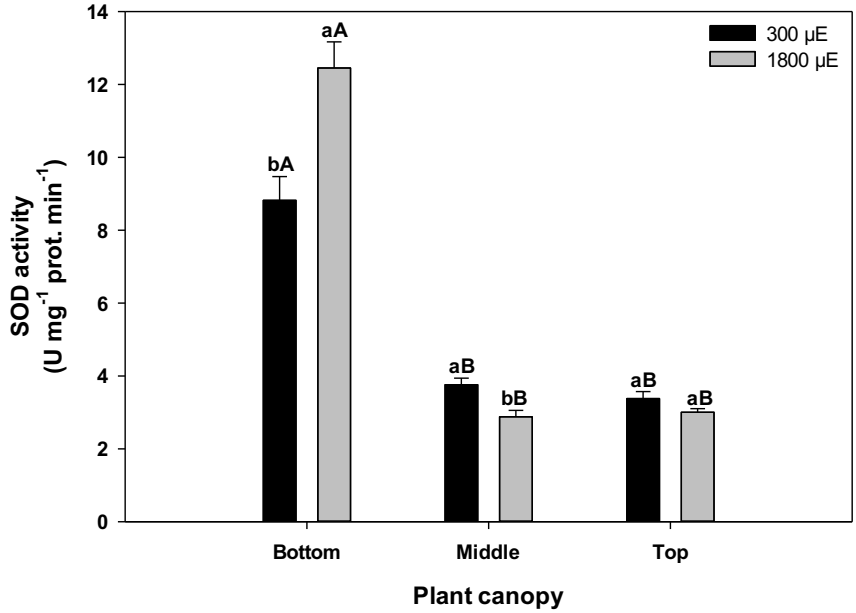

Figure S13: Superoxide dismutase (SOD) activity in leaves at different positions on rice canopy: bottom (senescing leaves), middle (expanded leaves) and top (last fully expanded and expanding leaves), which were exposed to 300 (low light) and 1800 µmol photons m^{-2} s^{-1} PPFD (high light). Different lowercase letters represent significant differences between treatments (300 and 1800 µE) within the same parts of the plant (bottom, middle and top), and different uppercase letters represents significant differences among the parts in the same treatment, at a confidence level of 0.05. Represented values indicate the average of four independent replicates (±SE) and were compared by Turkey's test.

ANEXO B – SUBSTRACT SUBMISSION

XVIII BRAZILIAN CONGRESS OF PLANT PHYSIOLOGY

SUBMISSION RESULT

The submission titled "Redox changes along rice canopy reveal spatio-temporal regulation in response to light" was **APPROVED**

- **Title:** Redox changes along rice canopy reveal spatio-temporal regulation in response to light
- **Number:** 516488
- **Submission date:** 16/07/2022
- **Submission type:** Abstract
- **Topic Area:** Plant Physiology
- **Authors:** Luis Flávio Menezes Rocha Júnior,Raysa Mayara De Jesus Sousa,Rikaely Torres de Sousa,Vicente Thiago Candido Barros Alencar,Joaquim Albenisio Gomes da Silveira

Regards,
Scientific committee
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