Influence of Solar Radiation on the Production of Secondary Metabolites in Three Rice *(Oryza sativa)* Cultivars

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Abstract. Rice (*Oryza sativa* L.) is one of the most produced and consumed cereals worldwide and has its importance highlighted mainly in developing countries, where it plays a strategic economic and social role. Due to the importance of rice in the diet, its composition and nutritional characteristics are directly related to the health of the population. In the rice production systems, some climatic factors are determinants for the good performance of the crop, inducing the biosynthesis of primary and secondary metabolites. The present study determined the metabolic profiles through UV-visible spectrophotometry of leaf samples of three rice cultivars (Marques – white, Ônix – black, and Rubi – red pericarp) throughout the rice's vegetative stages in two experimental times, from September to December 2015 and from January to April 2016. Solar radiation was recorded along the experimental period. To the organosolvent extracts of leaf samples, UV-vis spectrophotometric techniques were applied and the quantitative results of certain metabolites, e.g., chlorophylls, carotenoids, phenolics, flavonoids, and sugars, as well the antioxidant activity, which were analyzed by chemometrics tools. The results showed that biochemical parameters carotenoids, chlorophylls and sugars are more affected by the intensity of the radiation do que as variáveis phenolics, flavonoids and these alterations may be detected through statistical analysis of biochemical concentrations and UV-vis spectra.

Keywords: Rice · Spectroscopy · Metabolic profiles · Statistical models · UV-vis spectrophotometry

1 Introduction

Rice (*Oryza sativa* L.) is one of the most produced and consumed cereals in the world, being socially and economically important mostly in developing countries [1]. Due to

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the importance of rice in the diet, its composition and nutritional characteristics are directly related to the health of the population. This cereal is capable of supplying 20% of energy and 15% of the daily need of an adult's protein, as well as containing vitamins, lipids, minerals, phosphorus, calcium, and iron [2].

In Brazil, where the annual consumption is on average 25 kg/inhabitant [3], the southern region accounts for most of the national rice production [4]. In the state of Santa Catarina, the guarantee of the economic viability of the pre-germinated rice crop results from relevant technologies developed by public research and rural extension efforts, notably based on the actions of the Agricultural Research and Extension Company of Santa Catarina (EPAGRI). Two new cultivars of rice were introduced by EPAGRI, with peculiar characteristics that, besides the nutritional attributes of the traditional grains (white), are characterized by the accumulation in the pericarp of pigments of great nutraceutical importance [5, 6]. Thus, the cultivars Rubi (red pericarp) and Ônix (black pericarp) are considered special due to the coloring of the grains, attributed to the presence of compounds beneficial to health [7, 8]. In the production, climatic factors, isolated or in association, are determinants for the good performance of the rice culture [9] and production of primary and secondary metabolites. In this sense, two important factors are the temperature and the average insolation over the growth stages of the plants [10].

The present study determined the metabolic profiles of leaf samples of three rice varieties developed by EPAGRI along the vegetative stage in two periods: (*i*) September to December 2015 (spring – summer, southern Brazil) and (*ii*) January to April 2016 (summer – autumn). Insolation, i.e., the amount of solar energy/cm^2/min reaching the leaf surface, has been daily measured over the experimental period and was further correlated with the metabolic profiles through chemometrics tools. The biochemical and climatic datasets were further related aiming to build statistical models to better understand the regulatory effect of the solar radiation on the *O. sativa* secondary metabolism. For that, spectrophotometric techniques were adopted, since the UV-vis spectrophotometry allows the rapid and low cost acquisition of qualitative and quantitative data from the plant metabolism whose contents can be altered in response to external stimuli. To the biochemical dataset, bioinformatics tools developed by our research group were applied, using multivariate statistical techniques as further described.

2 Materials and Methods

2.1 Biological Material

In a greenhouse at EPAGRI, Itajaí Experimental Station (26°57′57″S and 48°48″01′W, southern Brazil), pre-germinated rice seeds of three varieties were sown: Rubi (red pericarp), Onyx (black pericarp), and Marques (white pericarp) in two periods: (*i*) September to December-2015 and (*ii*) January to April-2016. Along the vegetative stage of the plants, samples of adult leaves were collected in regular intervals (3) and taken to the laboratory for the biochemical analyzes.

The radiation data for the studied period were provided by the Information Center for Environmental Resources and Hydrometeorology of Santa Catarina (CIRAM/EPAGRI).

2.2 Biochemical Analyzes - Total Phenolic and Flavonoid Compounds and Antioxidant Activity (DPPH Assay)

Samples of rice leaves (1 g, fresh weight, n = 4) were macerated in crucible with liquid N_2 and added of 5 V methyl alcohol (MeOH). The organosolvent extract was recovered by filtration on cellulose filter under vacuum, followed by the biochemical analyzes. The total content of phenolic compounds was determined by the Folim-Ciocalteau colorimetric method [11], recording the absorbance of the reactions in an UV-visible spectrophotometer (Gold Spectrum lab 53 UV-Vis spectrophotometer, BEL photonics, Brazil) at $\lambda = 750$ ηm.

To determine the total flavonoid contents, the methodology described by Zacarias *et al*. (2007) was adopted, with modifications. An aliquot of 0.5 mL of the MeOH extract was added to 0.5 mL of methanolic aluminum chloride solution (2% w/v) and to 2.5 mL analytical standard ethanol. After one hour of incubation, the absorbance was measured at 420 ηm. The results were expressed as mg of quercetin per g of dry mass.

The reduction potential of the DPPH radical by the MeOH extracts of the leaf samples was determined as described by Kim et al. (2002). To that end, the absorbance of a DPPH methanolic solution (1 mM in 80% methanol) was measured at wavelength 530 nm. The DPPH-methanolic extract mixture was incubated for 30 min in the dark and the antioxidant reaction measured at 530 ηm.

2.3 Extraction and Quantification of Total Chlorophylls and Carotenoids

Rice leaf samples (100 mg, fresh weight, n = 4) were incubated in a water bath at 65° C with 7 mL dimethylsulfoxide (DMSO) for two hours. The extract was recovered by filtration and the final volume adjusted to 10 mL with DMSO (Hiscox & Israelstam, 1979). The absorbance values at $\lambda = 480$, 649, and 665 ηm were obtained through an UV-vis spectrophotometer (Gold Spectrum lab 53 UV-Vis spectrophotometer, BEL photonics, Brazil). For purpose of calculation of the amounts of chlorophylls *a* and *b,* the *Wellburn* formulas (1994) were used, being the data expressed as mg/g dry mass.

2.4 Extraction and Quantification of Total Soluble Sugars

The extraction of soluble sugars was done as proposed by Shannon (1968). The rice leaf samples (100 mg, fresh weight, n = 4) were crushed in liquid nitrogen and macerated in MCW solution (methanol: chloroform: water, 12:5:3, v/v/v). Total soluble sugars were measured according to Umbreit & Burris (1964). The absorbance readings were taken at 630 ηm in an UV-vis spectrophotometer (UV-2000A, Instrutherm). The content of total soluble sugars was calculated from the standard glucose curve (1 to 200 μg mL^{-1}, $y = 0.008x$, $r^2 = 0.99$). The results were expressed as mg glucose per g dry mass.

2.5 UV-Visible Scanning Spectrophotometry

DMSO extracts of leaf samples were UV-vis scanned (Gold Spectrum lab 53 UV-Vis spectrophotometer, BEL photonics, Brazil) in their absorbances over the spectral window ($\lambda = 480 - 665$ ηm). The data set was exported as a.*csv* file format for further chemometrics analysis.

2.6 Statistical and Chemometric Analysis

The biochemical and UV-vis data sets of the leaf extracts investigated were processed considering the respective wavelengths of interest. Further, the data matrix was exported as a.*csv* format file and subjected to univariate and multivariate statistical analysis, using principal component analysis (PCA). PCA can help one to extract relevant features from a given dataset, minimizing the redundant information and characterizing the relationship between the variables studied.

For that, scripts were written in R language using tools defined by our research group, through the *specmine* package, and some functions from the packages Chemospec [11] and HyperSpec [12]. The scripts, raw data, and chemometrics analysis are available in supplementary material, at http://darwin.di.uminho.pt/pacbb2017/rice-cultivars. The report of analysis generated from the scripts provided by the R Markdown is also available at this site, allowing the computational experiments details to be analysed in detail and fully reproducible.

3 Results and Discussion

The results from the spectroscopic and biochemical analyzes of the primary (sugars) and secondary (chlorophylls, carotenoids, phenolic compounds, and flavonoids) metabolites, as well as the antioxidant activity allowed identifying discrepancies of leaf's metabolic profiles of the three varieties investigated regarding the effect of accumulated solar radiation and the average daily radiation over each experimental interval studied, *i.e.*, September to December-2015 and January to April-2016.

The one-way analysis of variance (ANOVA) of the biochemical data revealed discrepancies ($p < 0.05$) among the rice varieties, mostly for the contents of phenolic and flavonoid compounds, followed by the antioxidant activity (DPPH assay), sugars, and chlorophylls. On the other hand, the rice genotypes do not differ significantly in their carotenoids concentrations over the years (see report in supplementary material for the details).

For radiation data, linear regression analysis was performed first starting with the mean daily radiation, and then considering their accumulated values. The most visible effects occurred in the variables carotenoids, chlorophyll, and soluble sugars, followed by DPPH Inhibition. For carotenoids, the R2 values show that over one third of the variance can be explained by the radiation levels (regression analysis results are available in supplementary material). The phenolics and flavonoids showed high p-values, thus do not seem to be affected by the radiation levels.

In a follow-up experiment, PCA was applied to the biochemical data aiming to discriminate the rice genotypes. PCA shows that mostly of the data set variability (65.6%) has been explained by the first two principal components.

In this analysis, the variables contents of sugars, phenolics, and flavonoids, as well as the inhibition (%) of DPPH are in line with PC1, whereas chlorophylls and carotenoids spread over the PC2 axis. The results revealed a clear separation of the genotypes according to their metabolic profiles. Ônix samples grouped in PC1+/PC2+, influenced by the higher concentration of sugar. On the other hand, Rubi genotype grouped in PC1− due to their higher amounts of chlorophylls, carotenoids, phenolic compounds and higher inhibition activity of the DPPH radical. The Marques variety was found between the groups of the other two cultivars at PC1+ and PC2− (Fig. 1).

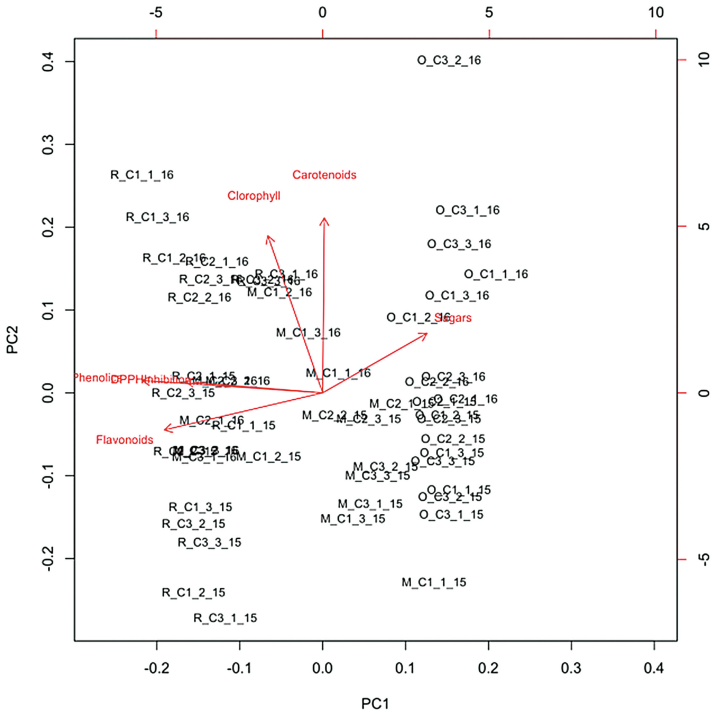

Fig. 1. Resulting bi-plot of the PCA results (PC1-39.3% and PC2-26.3%) showing the quantitative data variables (carotenoids, chlorophylls, phenolics, flavonoids, and inhibition of DPPH radical) in red, and the different scores of the samples (the reference is given in black for each sample).

In order to obtain a better understanding of the data dispersion (rice harvest seasons in southern Brazil), the results of the PCA were further analysed, considering the effects of the years of collection on those variables (Fig. 2A). The most of the 2016-collected samples grouped in PC2−, as the opposite has been detected for the samples collected in 2015. In a second approach, PCA results were interpreted aiming to correlated them

with the rice genotypes. Interestingly, as already seen above, PCA showed marked discrimination between Ônix (black pericarp, PC1+) and Rubi (red pericarp, PC1−) genotypes over the PC1 axis, while Marques (white pericarp) appears to be intermediate (Fig. 2B).

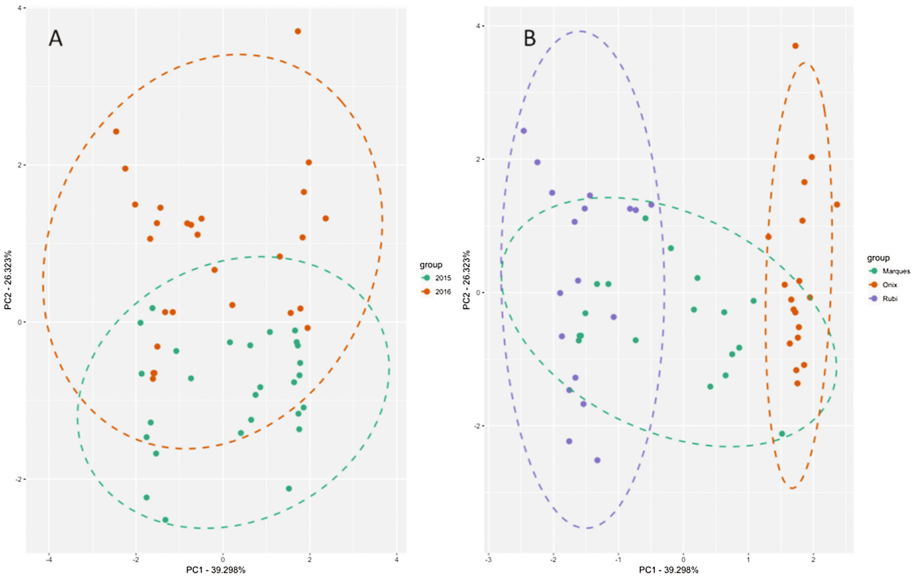

Fig. 2. Principal component analysis scoring scatter plots showing the effects of the year of collection on the biochemical variables of rice leaves (A) and among the rice varieties Marques, Ônix, and Rubi (B).

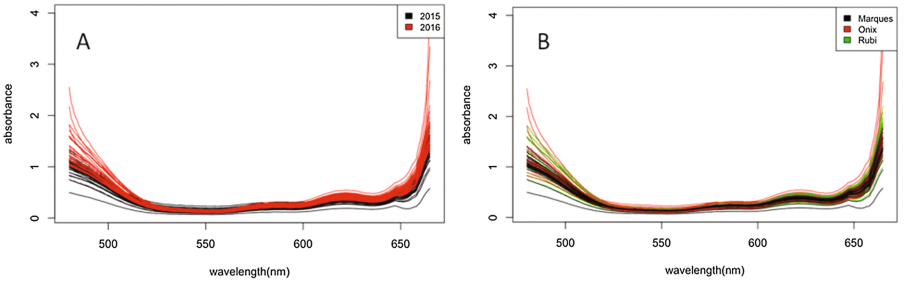

Fig. 3. UV-vis spectrophotometric profiles ($\lambda = 480 - 665$ ηm, DMSO) of leaf samples of rices genotypes. **A** – years 2015 and 2016. **B** – cultivars Marques, Onix, and Rubi.

Regarding the UV-vis spectroscopic profiles ($\lambda = 480 - 665$ ηm), a general vision to the class and contents of secondary metabolites is allowed, also revealing differences resulting from the genotypes and harvest times. All the studied samples showed intensive absorbance signals in the corresponding wavelengths of chlorophylls, carotenoids, and anthocyanins, with higher peaks for the 2016-harvest samples (Fig. 3A). Among the rice

genotypes, higher amounts were found in the Rubi leaf samples, followed by Ônix and Marques (Fig. 3B).

Taking into account the similarity of the UV-vis profiles among the samples and the eventual occurrence of redundant information, PCA was adopted again as a data reduction technique, in order to extract latent information from the spectroscopic data set. Again, the UV-vis spectral profile of 2015-collected samples seems to differ from that of 2016-collected ones (Fig. 4A), as a less clear separation has been found for the rice genotypes through the spectroscopic data set (Fig. 4B).

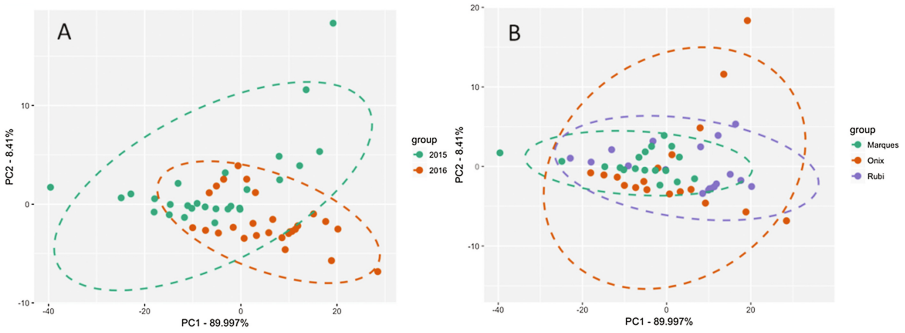

Fig. 4. Principal component analysis scores scatter plots (principal components 1 and 2) of the spectral data set (UV-vis, $\lambda = 480 - 665$ ηm, DMSO extract) colored according to the year (2015- and 2016-collected samples) (A) and to the rice genotypes Marques, Onix and Rubi.

4 Conclusions

In rice cultivation, climatic factors such as temperature and levels of solar radiation are determinant for the yield of the crop. Biochemical parameters may reflect possible physiological changes, *e.g.,* energetic and metabolic gains of plants throughout the crop cycle. The results obtained from the biochemical and UV-vis spectroscopic analyzes revealed the influence of the solar radiation on the metabolic profiles of the rice cultivars investigated.

For example, higher levels of chlorophyll, carotenoids, and sugars, important compounds associated to the photosynthetic apparatus, were shown in the 2016 harvest, when the solar radiation accumulated was larger than that found in 2015. Additionally, the rice genotypes respond differently to the insolation as noted for their discrepant secondary metabolites composition over the years. The chemometrics approach adopted allowed us to better discriminate the genotypes behavior over the years, by applying unsupervised multivariate statistical methods to the biochemical and UV-vis spectroscopic dataset. Taken together, the PCA findings suggest that different compounds may be used for building statistic monitoring models to better understand the rice genotypes answers to the solar radiation over the harvesting times in southern Brazil.

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