

Contribution For Improvement Of Rice Breeding Programs Through Morphoanatomical Traits

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Abstract

Breeding programs aim to increase grain yield and quality through selection oriented mostly by morphological and productive traits. Although morphoanatomical traits are preponderant for plant performance, they are usually time-consuming, demanding specific equipment and trained personnel. The aim of the study is to establish relationships among morphological, micromorphological and anatomical traits of rice genotypes, to support rice breeders in choosing variables to be assessed in the search for superior rice genotypes. We assessed morphological (2nd leaf and root lengths, tiller and root number), micromorphological (vein density, intervein distance, stomatal density and opening width) and anatomical (area of sclerenchyma, xylem and phloem vessels, and percentage of aerenchyma) traits. Root number and length, area of sclerenchyma and xylem and phloem vessels are proposed to be the most efficient traits to be assessed in detailed studies aiming to identify superior genotypes in rice breeding programs, from the morphophysiological point of view.

Keywords: anatomy, morphology, *Oryza sativa*, physiology, plant performance

1. Introduction

Rice is a staple food for about half the world, and together with crops as maize, wheat and potatoes, helps guaranteeing the human demand for calories and nutrients (Fukagawa & Ziska, 2019). Substantial gains in both rice grain yield and quality were reached due to improved breeding techniques and local adjustments of management practices (Alam et al., 2013; Khan et al., 2014).

Generally, breeding programs aim to increase crop grain yield and quality through selection procedures that seek at individual and community plant levels, oriented mostly by morphological and productive traits (Fukagawa & Ziska, 2019). It is, however, a consensus the remarkable contribution of morphoanatomical traits for plant performance (Abdusalam & Li, 2018). Traits of this nature are usually time-consuming, also demanding specific equipment and trained personnel to be assessed. It can be challenging to assess morphoanatomical traits of a large number of genotypes, if only limited staff and equipment is available.

Radosevich et al. (2007) described the importance of plant vigorous early development for competition against weeds and other plant species. In general terms, those who emerge first and grow faster will take the lead and use most of the environmental and input resources available (Fageria & Moreira, 2011; Zain et al., 2014), considering a community leading strategy based on dominance (Avolio et al., 2019). Although growth parameters can be inferred from biometric measures, as plant height and leaf area, most detailed assessments are demanded as breeding programs find even more challenging to keep increasing rice grain yield and quality beyond the current levels. Morphological aspects of rice plants as high growth rates, early tillering and high leaf area, thus, may be decisive for rice plant performance in production fields (Feldman et al., 2017; Bertolino et al., 2019).

Furthermore, micromorphological traits as stomatal density and caliber may affect gas exchange rates and, therefore, greatly contribute to superior plant physiological performance (Bertolino et al., 2019). Xylem and phloem caliber are also determinant on the ability of transporting substances as water, nutrients, proteins, sugars and informational molecules, from the anatomical point of view (Lucas et al., 2013; Bayley & LeeGood, 2016), working in close cooperation with stomatal regulation.

Caine et al. (2019) report the impact of plant micromorphology on rice performance, focusing on stomatal aspects. These authors present evidences that proper stomatal density and dimension may improve plant ability of gas exchange, reducing water loss with improved CO₂ assimilation. Furthermore, stomatal interaction with xylem and phloem ability in conducting substances, as well as with aerenchyma, may prove to affect gas exchange rates and as a result the overall plant physiological performance (Colmer, 2003).

Other morphoanatomical aspect known to interfere on plant physiology is the vein density in rice leaves (Feldman et al., 2017). According to these authors, rice genotypes with higher vein density in leaf blade tend to perform better, probably when coupled to adequate caliber on xylem and phloem vessels (Lucas et al., 2013; Eom et al., 2013; McAdam et al., 2017).

The main aim of the study is to establish relationships among morphological, micromorphological and anatomical traits of rice genotypes from the same breeding program, in a way to support rice breeders in choosing morphoanatomical variables to be assessed in the search for superior genotypes, and consequently, superior rice cultivars in grain yield levels and tolerance to abiotic factors.

2. Method

2.1 Experimental Design and Management

The experiment was carried out at Embrapa Clima Temperado, Terras Baixas experimental station, located in Capão do Leão, Rio Grande do Sul, Brazil, in the 2019/2020 growing season, in randomized blocks design with four replications. Each plot was constituted by a rectangular 500 L fiberglass water tank, equipped with independent valve-controlled water inlets and outlets for precise irrigation and drainage as needed. Tanks were filled with a 5 cm layer of crushed stone followed by a 5 cm layer of coarse sand, and a 50 cm layer of a Planossolo (Typic Albaqualf) previously corrected and fertilized for rice crop (Rice Commission, 2018).

Treatments consisted of seven commercially available rice varieties with recognized superior performance, plus 57 rice lineages from Embrapa's breeding program, planted in 1 m long rows spaced in 0.175 m, thinned to the density of 300 plants m⁻² ten days after emergence. The cycle length of the 64 genotypes (emergence to harvest) varied from 110 to 135 days. Genotypes were distributed in different tanks according to their cycle length, to standardize management practices. Planting was accomplished in 23/Sep./2019, and emergence occurred in 02/Oct./2019.

Topdressing fertilization consisted of 125 kg ha⁻¹ of N as commercial urea split in three applications: 15 kg ha⁻¹ of N at the 2-leaf stage (V2); 60 kg ha⁻¹ of N at the 4 to 5-leaf stage, prior to flooding, and the remaining 40 kg ha⁻¹ of N applied at panicle initiation. This stage was estimated using the software Planejarroz (Embrapa/UFSM, 2019) by supplying location, emergence date and indicating a commercial variety with approximately similar cycle length.

Irrigation was managed in the alternate wetting and drying (AWD) system (Sriphirom et al., 2019). All plots were irrigated daily to keep soil moist, from rice sowing to the beginning of tillering (4 to 5-leaf stage). In this phase, a 7 cm water layer was established and maintained for 10 days aiming not to interfere in nitrogen uptake by rice plants. Then, the first AWD cycle started. For that, the plots were not irrigated again until soil water tension reached 20 kPa. At this water tension, a new 7 cm water layer was established and maintained for three days, starting a new soil drying cycle, always having the soil water tension of 20 kPa as the threshold for reflooding (Pinto et al., 2020). AWD cycles were repeated until ripening begun. Soil water tension was monitored by installing Watermark® sensors (Irrometer inc., USA) in each plot. These were wired to electronic data loggers for continuous follow up on soil water status. Data was reviewed twice a day.

Spontaneous weeds in the plots were eliminated by hand; insects were controlled by a single application of 14 g ha⁻¹ of zeta-cypermethrin, eight days after emergence (DAE), and no later

insecticide application was needed. Diseases occurrence were also negligible for all genotypes, not demanding any control measures.

2.2 Morphological Assessments

Morphological analysis of plants were done 22 DAE. For each replication and genotype, 2 rice plants (8 plants per treatment) were collected, deposited into a glass with water, and immediately taken to the lab where the 2nd leaf (first expanded leaf) and root lengths were measured, and tiller and root number were counted. Leaf length was measured with graduated ruler from leaf collar to its tip; root length was measured from seed insertion to the longest root tip. After assessments, the second leaf (first expanded leaf) was removed for the micromorphological analysis.

2.3 Micromorphological Assessments

The middle section of the adaxial (upper) face of the second leaf was observed and photographed in optical microscope (Nikon e200) in two magnifications: 0.5 x / 4 x and 0.5 x / 40 x, respectively for camera and objective lens, resulting in 2 x and 20 x total magnifications. Leaf was gently pressed between microscope slide and the glass cover slip, held together by lateral adhesive tapes, to keep leaf flat open. Photography scales were set by previously capturing images of a microscope calibration ruler, in both magnifications. Two images with resolution of 2 048 x 1 536 pixels (3.1 MP) were taken per leaf, in different areas, for later analysis.

Images were saved in hierarchical naming structure for later processing, which was accomplished using the software ImageJ v.1.53c (NIH, 2020). After the proper software scale calibration, the vein density, inter-vein distance (2 x magnification – Figure 1), stomatal density and opening width (20 x magnification – Figure 2) were measured using the proper software tools. Stomatal related data were obtained after applying the filter “relief” to the images to increase stomatal border and opening contrasts (Figure 2). Stomata number was counted in an area correspondent to 25 % of the captured image, as this was the area into each picture where the microscope was better focused. This area was properly considered in stomatal density calculations. Scales were reported into images by ImageJ.

2.4 Anatomical Assessments

Leaf samples were collected for anatomical assessments 110 DAE. A section of 10 cm of leaf was cut from the middle third of the second leaf of rice plants, being obtained two samples per replication and genotype. These samples were put into acrylic flasks and fully covered with alcohol 70 %, sealed and stored into refrigerator at 5 ± 2 °C. The solution was exchanged weekly during four weeks, to remove part of the green pigment chlorophyll, and later the assessments were performed.

Leaf sample was cut by hand with good quality razor blade, after insertion in maize stem cortex, to facilitate cuttings by immobilizing the rice leaf sample. About 30 thin straight cuttings, transversal to leaf length, were obtained per sample. Cuttings were dispersed into a Petri dish containing clean water; the two thinner cuttings of the rice leaf were chosen,

transferred to a microscope glass slide and added a drop of clean water, being later covered with a glass cover slip and taken to the microscope for image capture.

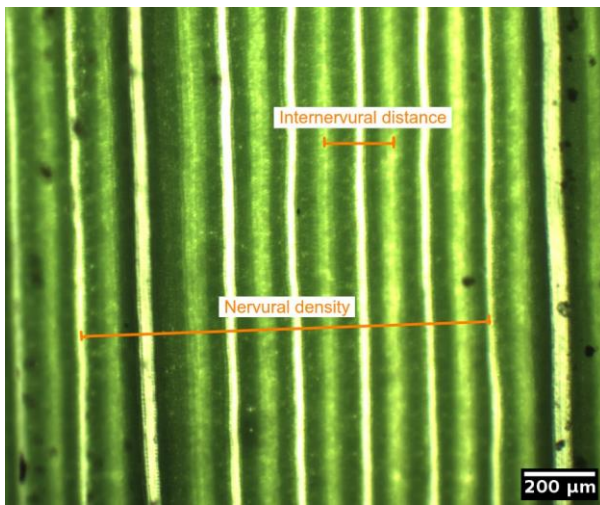


Figure 1a. Magnification of the adaxial (upper) surface of the second rice leaf, collected 22 days after emergence, through a 4x microscope objective lens.



Figure 1b. Magnification of the adaxial (upper) surface of the second rice leaf, collected 22 days after emergence, through a 40x microscope objective lens, after application of the filter “relief” for edges sharpening.

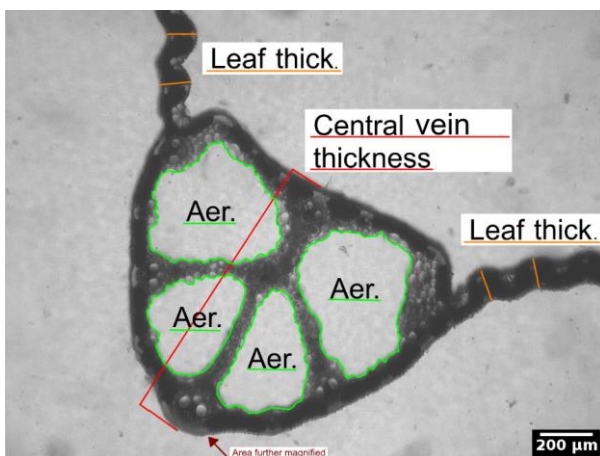


Figure 2a. Cross section of the central part of the second rice leaf, collected 110 days after emergence, through a 4x microscope objective lens.

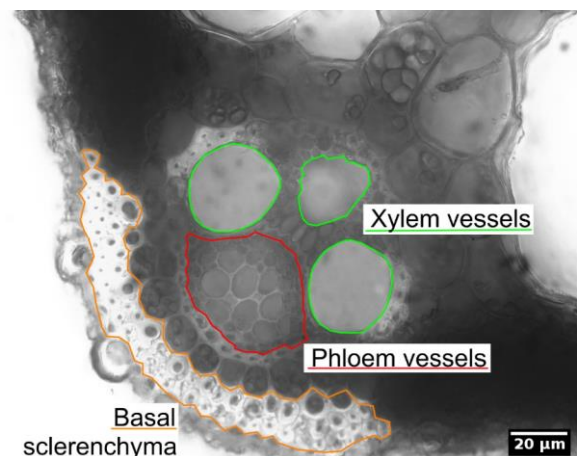


Figure 2b. Bottom vascular bundle of the cross section of the central part of the second rice leaf, collected 110 days after emergence, through a 40x microscope objective lens.

Images were obtained in the same equipment and magnifications described for

micromorphological analysis. Images were also assessed in the software ImageJ v.1.53c, after proper software scale calibration. The percentage of aerenchyma in the central vein of rice leaf (Figure 3) was assessed in pictures taken at 2 x magnification, while xylem and phloem vessels, as well as sclerenchyma areas, were reported from pictures with 20 x magnification (Figures 3, 4), for the basal vein of the central vein.

2.5 Statistical Analysis

All statistical analysis were run into the R environment (R development team, 2020), by using commands made available by the packages “ExpDes” and “stats”, respectively for the univariate and multivariate analysis.

Data mining began by investigating all data sets in terms of ANOVA assumptions – normality and variance homogeneity of errors. After elimination of a few outliers, these assumptions were met. All variables were submitted to analysis of variance by the F-test at 5 % probability. In case of significance, treatment means were grouped by Scott-Knott at 5 % and data was presented in Figures where green (■), yellow (■) and red (■) bars represented the first, second and third mean groups, respectively.

After the univariate analysis, each variable into the data set was transformed into percent basis (0 - 100), verified again for normality of errors, and submitted to multivariate analysis of principal components (PC's), by the command “prcomp”. The multivariate analysis were executed respecting their requirements, as reported by Fendler et al. (2001). Data was presented in pattern plot, where the first and second PC's were mapped to “x” and “y” axis, respectively.

3. Results

All variables were significant at $p \leq 0.05$ in the univariate ANOVAs. Furthermore, the pattern PCA analysis was able to group variables as function of the two main principal components.

3.1 Morphology

The 2nd rice leaf blade length measured 22 DAE was superior only in 14 genotypes, out of 64, with average value corresponding to ~ 8.3 mm for the superior group, and ~ 7.9 mm for the remaining genotypes (Figure 3a).

Rice genotypes were pooled in two groups in terms of root length (Figure 3b), with 26 genotypes presenting roots ~ 40 % longer than the remaining 38 genotypes. Similar behavior was reported for root number (Figure 3c), where 28 genotypes presented longer roots at magnitudes of ~ 30%. Only 16 genotypes (25 %) presented both longer and more abundant roots (Figures 3b;c).

Only four genotypes, among the 64 tested ones, were able to tiller earlier in the experimental conditions (Figure 1d), with two of them presenting three tillers and the other two presenting 2.5 tillers on average.

3.2 Micromorphology

Rice leaf veins (set of vascular bundles) are arranged in parallel, longitudinally to leaf length

(Figure 1a). Between veins there is a region with high density of bulliform cells. As leaves are most transparent in the strip of bulliform cells, veins are visible by passing a calibrated light beam through the leaf.

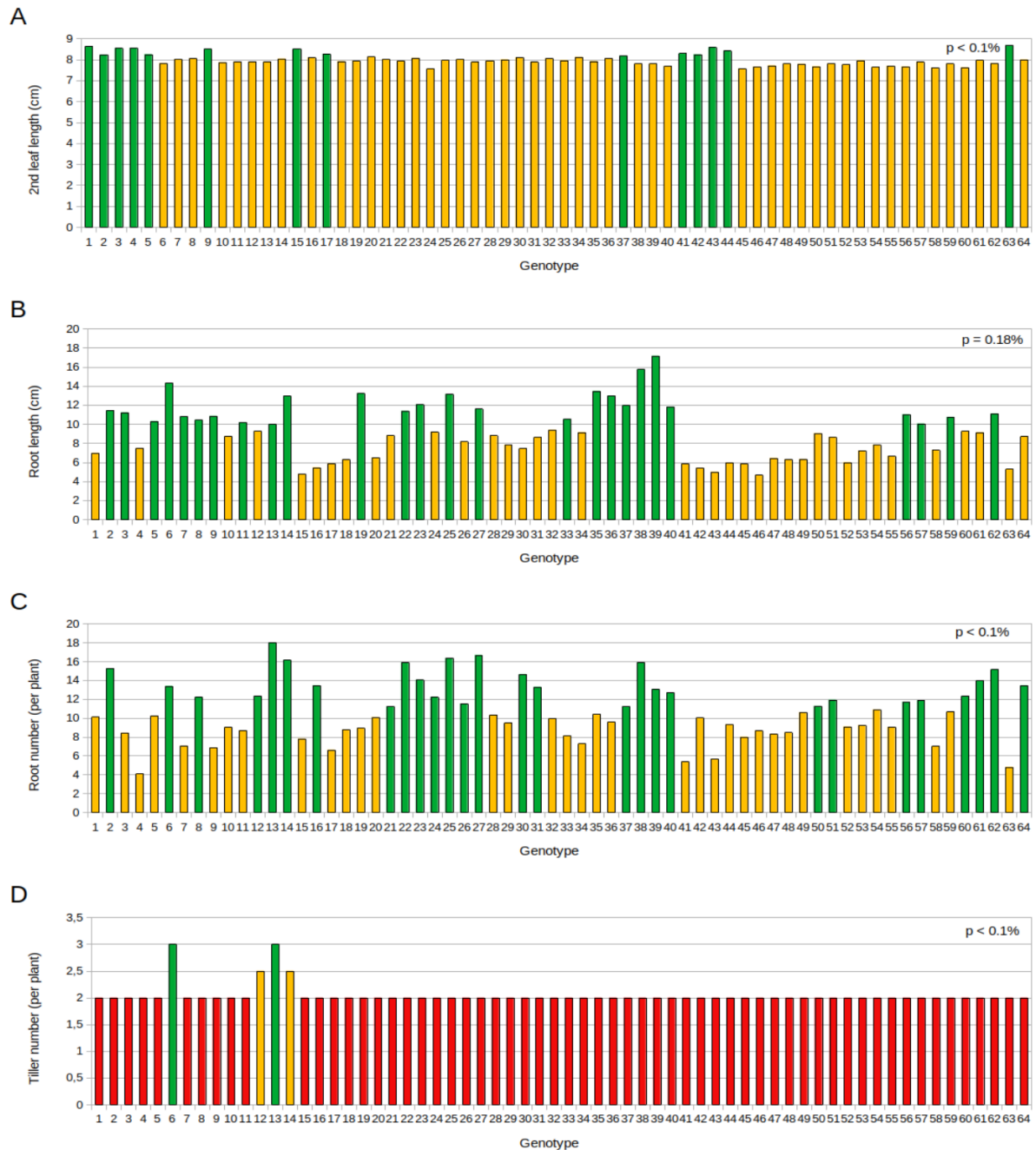


Figure 3. Morphological traits assessed 22 days after emergence in rice genotypes from the same rice breeding program at Embrapa Clima Temperado, Pelotas-RS, Brazil. Green (■), yellow (■) and red (■) bars represent the first, second and third mean groups, respectively, according to Scott-Knott at 5% probability.

Vein density differed among rice genotypes (Figure 4a), with 37 genotypes (58%) presenting ~ 6 veins per mm of leaf width, while the second group of genotypes presented ~ 5.4 veins per mm of leaf width (Figure 4a). Most of the genotypes presenting lower vein density, however, also had higher vein thickness (Figure 4b); the remaining width is fulfilled by bulliform cells. This seems to act as a compensation point, where lower vein density is mostly coupled to thicker veins. Genotypes 7, 14 and 54, however, were able to couple higher inter-vein density with higher caliber veins (Figures 4a;b), probably at the cost of lower volume of bulliform cells.

Our data highlight that stomatal density (SD) was higher for 21 genotypes (~ 500 stomata mm⁻²), while the remaining 43 genotypes presented ~ 408 stomata mm⁻² of leaf (Figure 4c) – a reduction of ~ 20 % on SD compared to the first statistical group, according to Scott-Knott's.

In the present study, the stomatal width (SW) was also measured, with 23 genotypes presenting SW about 25 % higher than the others (Figure 4d). Superior SD is coupled to smaller SW for 14 genotypes out of 64 (Figures 4c;d), supplying a set of genotypes for breeding, considering the new evidences on SD and SW relationships and consequences for water use efficiency (Bertolino et al., 2019; Caine et al., 2019).

Xylem vessel area was one of the variables with greater difference among genotypes, that were pooled into three groups (Figure 5a). Xylem vessels caliber were on average ~ 800; ~ 580 and ~ 385 mm² per vessel, respectively, for the superior, middle and inferior groups formed by Scott-Knott's (Figure 5a). Our analysis, however, did not consider possible differential vessel densities among genotypes.

3.3 Anatomy

Phloem is the main responsible for carbohydrates reallocation and movement of signaling substances from source to sink organs, supporting plant growth and development (Eom et al., 2012). The area of phloem vessels bundle (Figure 5b) was also stratified in three groups, whose areas were ~ 2 050, ~ 1 478 and ~ 1 116 mm², for the superior, middle and inferior groups, respectively.

As xylem and phloem widely cooperate in an orchestrated organization to supply the demand for water, carbohydrates, proteins, signaling substances and others in different parts of the plant, their dimensions should be compatible to each other. In these terms, coupled superior caliber for xylem and phloem vessels bundles were reported to be present in 17 genotypes, out of 64 (Figures 5a;b).

The percentage of aerenchyma in the central leaf vein (Figure 5c) differed among genotypes, with two distinct groups. The first group, with 14 genotypes, presented ~ 34 % aerenchyma, while the second group, with 50 genotypes, presented ~ 29 % (Figure 5c). Aerenchyma is preponderant on rice ability to deal with waterlogged or flooded soil and plays an important role in gas diffusion to parts of the plant located into the water zone (Colmer, 2003; Xu et al., 2018).

The genotypes were pooled into three groups regarding the area of sclerenchyma in the basal portion of leaf vein: respectively with ~ 3 500; ~ 2 500 and ~ 1 300 mm² of sclerenchyma for the first, second and third genotype groups (Figure 5d).

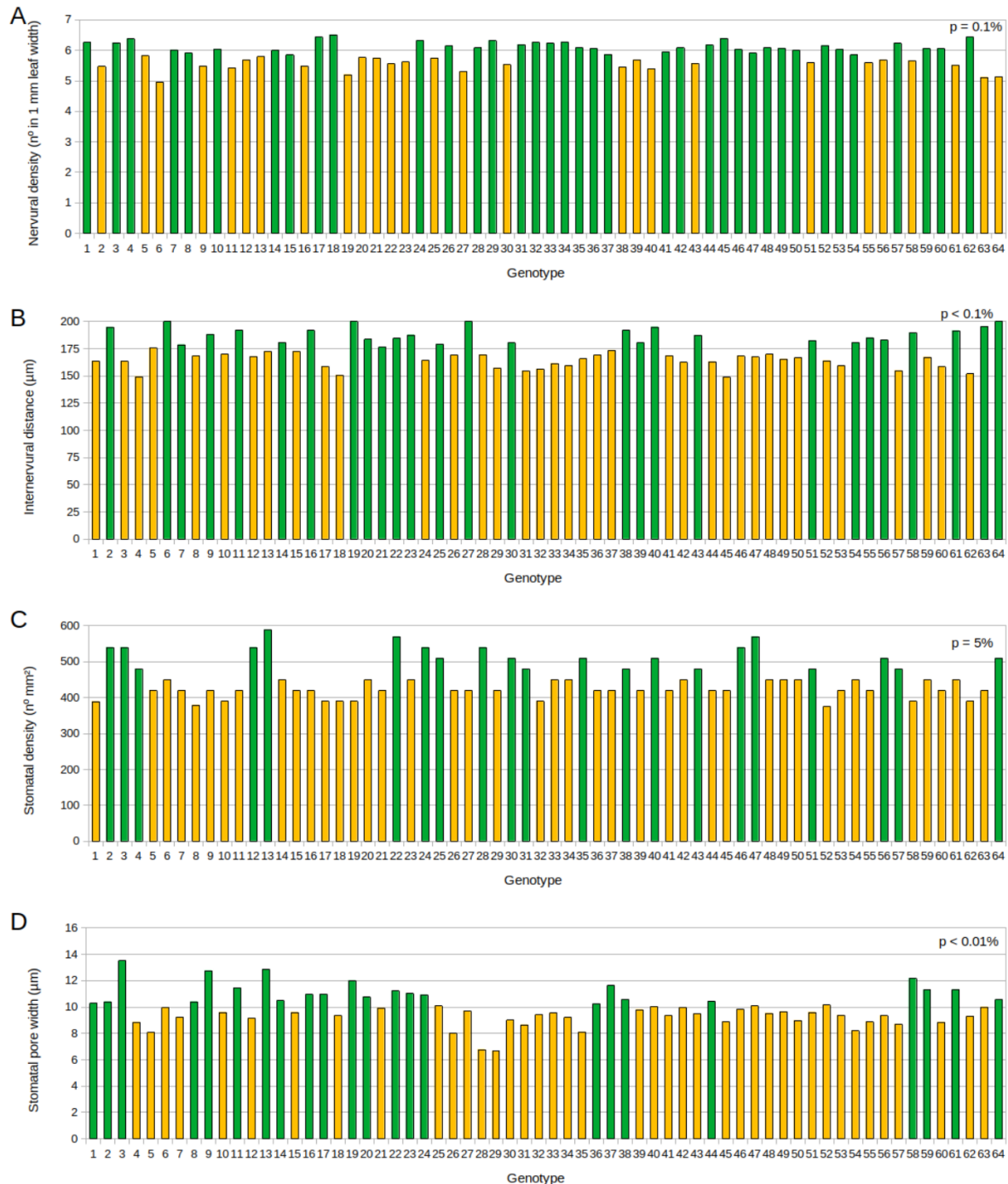


Figure 4. Micromorphological traits assessed 22 days after emergence in rice genotypes from the same rice breeding program at Embrapa Clima Temperado, Pelotas-RS, Brazil. Green (■) and yellow (■) bars represent the first and second mean groups, respectively, according to Scott-Knott at 5% probability.

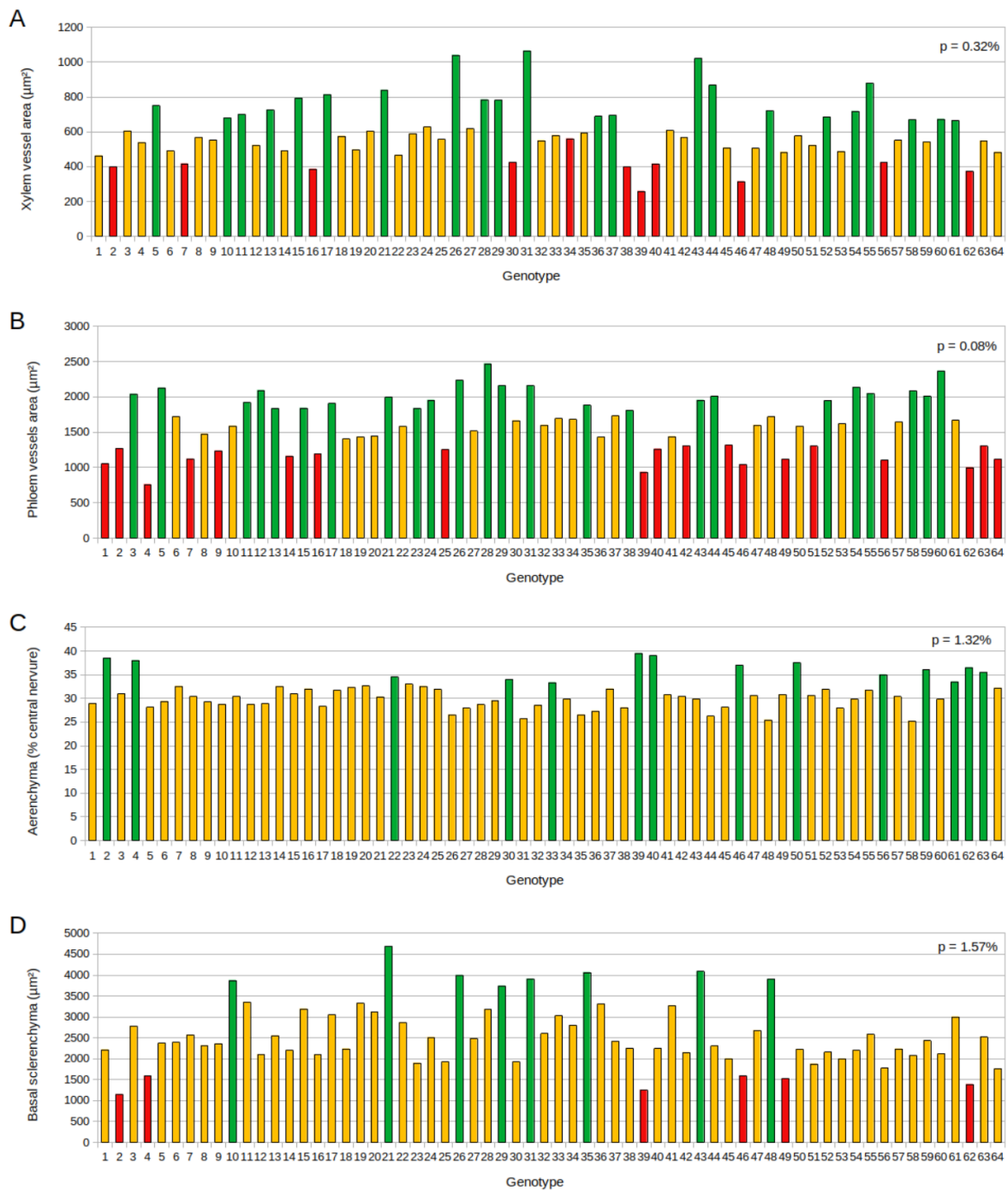


Figure 5. Anatomical traits assessed 110 days after emergence in rice genotypes from the same rice breeding program at Embrapa Clima Temperado, Pelotas-RS, Brazil. Green (■), yellow (■) and red (■) bars represent the first, second and third mean groups, respectively, according to Scott-Knott at 5% probability.

4. Discussion

4.1 Morphology

The 2nd rice leaf blade length is correlated to plant height (Ranawake et al., 2013) and possibly to plant's superior ability in competing against weeds (Thiel et al., 2018), but it is hardly correlated to rice grain yield (Ranawake et al., 2013). Tivet et al. (2001) report that rice leaf length often represents superior ability in growth rates, and depends greatly on its position in the plant. Furthermore, longer leaves in the upper third of rice plants may also increase its inter- and intra-specific competitive ability (Galon et al., 2015), which can be further increased by adequate levels of nitrogen fertilizer (Zhou & Wang, 2011).

Thicker and longer roots in rice, mainly when associated to deeper growth, may help avoiding drought stress in rice in areas where irrigation is managed in the alternate wetting and drying (AWD) system (Kim et al., 2020). Rice root length is also known to be correlated to rice grain yield levels (Fageria & Moreira, 2011). Roots are the main plant-soil interface, and rice mineral nutrition depends mostly on its roots ability in reaching and extracting adequate amounts of nutrients from the soil (Veçozzi et al., 2018). The same authors reported that possible nutrient limitations may come not only from insufficient applied amounts, but also from the lack of coupling N availability with rice demand timings. Liu et al. (2018) report that for hybrid rice breeding programs, both root length and volume were correlated to grain yields, and that increases in these features also increase rice response to N fertilization, being thus highly desirable in rice breeding programs.

On the same way, superior tillering in rice plants may increase its competitive ability against weeds (Galon et al., 2015), and possible volunteer plants of other rice varieties (Schreiber et al., 2018), and increase the use efficiency of environmental resources (Nawaz & Farooq, 2017). Vigorous tillering also allows rice to reach high leaf area index (LAI) earlier in the crop cycle, depending on N availability (Zhong et al., 2002). Zain et al. (2014) also reported that drought stress reduces rice tillering; thus, genotypes with superior tillering ability under intermittent drought stresses, as the AWD irrigation system, would perform better in grain yield. Our tillering results, however, are considered as evidence only and later studies on the theme will be conducted with the same genotypes to confirm this evidence.

4.2 Micromorphology

Bulliform cells are highly vacuolated and play the role of controlling leaf rolling as they inflate or dry out (Nawaz & Farooq, 2017), helping plants to overcome drought periods. Anatomically, they can be used as markers for determining the connection point between different leaf veins.

Leaf venation is structured to take substances to and from virtually any part of the plant, depending on the source-sink relationship (Feldman et al., 2017; McAdam et al., 2017). In this sense, vein density (VD – also called venation density) and veins thickness (VT) are preponderant in constituting adequately scaled pathways for substance movement into the plant (Tabassum et al., 2016). The same authors report that, under water deficit conditions, photosynthesis rate and water conductance ability are related to the major veins' thickness.

On the surface of the vein strips of the leaf, there are the stomata, whose main role is to act as gatekeepers in the control of gas exchange; the entry of CO₂ and water loss rates through stomata are particularly important for plant metabolism (Brito et al., 2016). Stomata play a critical role in regulating plant effective use of water, what can be related to high capacity to capture water for transpiration or even acting in increase/decrease of water use efficiency (WUE); they are also involved in nutrient uptake from roots by transpiratory fluxes.

For rice, new findings supply evidence that greater stomatal density in leaves may not always be positive for the plant (Caine et al., 2019). These authors report that under simulation of climate change, an engineered rice cultivar with fewer stomata proved to be most conservative in water use, with equivalent and even higher grain yields than the original cultivar. This result, however, is hypothesized to be linked to the expected grain yield levels since it is unlikely that genotypes with grain yields superior to 10 t ha⁻¹ would be benefited by lower stomatal density. This is yet to be deeper studied. Bertolino et al. (2019) report that smaller size stomata may contribute for a faster stomatal opening / closure process, also contributing for improved gas exchange equilibrium. There seems to be no clear idea if stomatal size (SS) being smaller really contributes for improved gas exchange rates, although there is a common consensus on a naturally inverse SD : SS relationship in plants (Dittberner et al., 2018). SS is often estimated from stomatal width (SW) (Bertolino et al., 2019). In this scenario, it seems most appropriate to consider as superior those genotypes with high stomatal density associated to smaller stomatal opening ($> SD / < SW$).

For rice paddies grown under continuous flooding irrigation, or even when the alternate wetting and drying management is adopted under deep soil profile, prevalent traits such as higher stomatal density and stomatal opening width are associated to deep and functional roots, higher hydraulic conductance of root and shoots, lower root apoplastic barriers, plant architecture and great osmotic adjustment capacity, on the improved grain yield performance (Kato & Okami, 2010; Eom et al., 2012; Steduto et al., 2012; Farally et al., 2019; Kim et al., 2020). These traits are not only related to genotype but interact with the environment (Poli et al., 2018); for this reason, these aspects should be taken into account by breeders in the definition of the environment target where the new genotypes will be grown. In other words, for breeding, environment matters.

In such conditions, prioritizing selection of plants carrying such traits could increase the probability to obtain cultivars with improved effective use of water capacity, which lead to maximize soil water capture for transpiration, and also can be associated to a decrease in the non-stomatal transpiration and minimizing water lost by soil evaporation.

4.3 Anatomy

Rice vascular system is of primary importance for plant development as it serves as pathway for environmental resources as water, nutrients and sugars, as well as acting in long distance signaling (Lucas et al., 2013). Xylem is important also for transport of inorganic and organic forms of nitrogen, amino acids and amides from roots to shoots (Bailey & Leegood, 2016).

Transport of substances through xylem depends roughly on the difference of water potentials

in soil and in free air, whose effect is the flux of sap through the vases, from soil to roots, shoots, leaves and finally to the air as water vapor (Marenco & Lopes, 2008). For the transport to be effective, xylem vessels also need to be intact, free from leakage points, and vessel caliber is one of the determinants of sap flux rate through xylem (Bayley & Leegood, 2016).

The proportion of aerenchyma in leaves is also correlated to the water management system; plants submitted to intermittent flooding, as in the AWD water management system, tend to present lower aerenchyma than continuously flooded plants (Steffens et al., 2010). Furthermore, two main mechanisms of cell lysis for aerenchyma formation are reported (Parlanti et al., 2011); cell lysis may come by production of reactive oxygen species (ROS) as H₂O₂, or alternatively by superior ethylene production. Both substances tend to impact several other morphophysiological processes into the rice plant (Steffens et al., 2010).

The amount of sclerenchyma in the central vein of rice leaf blade is another anatomical trait with links to physiological performance (Aohara et al., 2009). Sclerenchyma is the harder type of fiber that promote support for an erect leaf position (Crang et al., 2018). Erect leaves in rice may allow higher plant density in production fields while reducing intraspecific competition for light compared to decumbent leaves (Chen et al., 2015), from a community point of view.

4.4 Multivariate Analysis

The principal components of multivariate analysis were able to attribute 62.4% of the total variation for PC1 and 17.5 % for PC2, where variables were pooled in three major groups (Figure 6). The group marked in green is smaller and includes only morphological traits, particularly root number and length, that accounted the most for both principal component axis (Figure 6). The blue marked group, on the other hand, included only anatomical traits, particularly the sclerenchyma, xylem and phloem areas at the basal vascular bundle of the central vein of rice leaf blade (Figures 2b; 6). The red marked group included traits from a mixture of sources (morphological, micromorphological and anatomical).

Regarding the X / Y distances among groups (Figure 6), morphological traits accounted more both for PC1 and PC2, while the anatomical ones accounted heavily only for PC1. Considering that PC1 accounted for most of the variation (62.4 %), morphological traits are most preponderant in pooling rice genotypes, followed by anatomical traits. So, in this sense, both the blue and the green groups are proportionally more distant from the diverse (red) group considering PC1, than they are from each other, considering PC2 (Figure 6), as PC1 accounts for a much higher part of the variation.

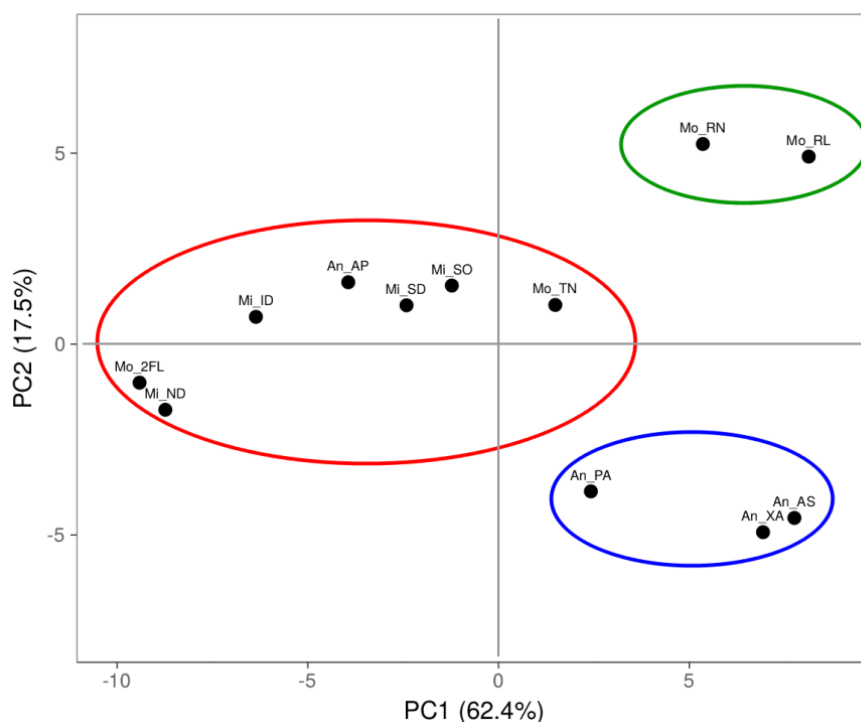


Figure 6. Multivariate analysis of principal components (PCA) pooling all assessed variables. Embrapa Clima Temperado, Pelotas-RS, Brazil, 2020.

5. Conclusions

Considering the nature of every set of variables previously discussed (Figures 3 - 5), as well as their interactions (Figure 6), root number and length, area of sclerenchyma and xylem and phloem vessels are proposed to be the most efficient traits to be assessed in detailed studies aiming to identify superior genotypes in rice breeding programs, from the morphophysiological point of view.

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