

Genetics components of rice root architecture and carbon isotopic fractionation parameters: a tracer for breeding in a water saving irrigation management

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Abstract

Phenotyping is the major bottleneck in the effort to develop varieties of rice (*Oryza sativa* L.) suitable for grow under a water saving irrigation management, such as alternate wetting and drying irrigation technique (AWD). In order to analyze if the genotypic variability for carbon isotope discrimination (CID) in rice leaves could be used as a relatively high-throughput tracer to early select superior genotypes highlighting improved root architecture traits when submitted to AWD, a set of twenty varieties grown under semi-natural conditions were submitted to two water irrigation regimes, continuous flooding (CF) and AWD cycles. Coefficients of genetic variance (σ^2_g) obtained for root architecture, micro-morphological and physiological traits were significant for all of them regardless of adopted irrigation system, except to mean root diameter. The three significant principal components (PCs) with eigenvalue > 1, explain the most of the total variation across cycles and water regimes. For most of analyzed traits, the values of heritability coefficients were higher regardless of adopted irrigation management and trait category; for CID, the magnitudes of broad heritability at individual level (greater than 0.80) were similar in the two irrigation techniques, evidencing that the success of selection is independent of irrigation management. The higher CID values after three AWD cycles are associated to varieties with higher total root length and volume. To our knowledge, this is the first study demonstrating the potential application of CID as tracer to select root architecture traits in rice when water-saving irrigation management are of concern.

Introduction

A challenge of the current agriculture is to feed more than nine billion people in the next decades (Jacquemin et al. 2013; Fan and Brzeska 2014). Additionally, this challenge takes an even greater dimension if taken into account the negative effects of climatic changes predictions on annual rainfall regime, mean temperatures, heat waves, changes in pests dynamics and its acclimation, global change of atmospheric CO₂, ozone and sea levels (Brito et al. 2010; Diola et al. 2011; Raza et al. 2019). Predictions on the impact of these extreme climatic events on global population remain unclear; there are strong evidences, however, that its occurrence will probably influence plant species distribution and physiological resilience ability in specific environments, consequently affecting its productivity. Taken together the above mentioned topics and considering that rice (*Oryza sativa* L.), is a staple food for billions, the current use of up to 2500 L of water per 1 kg of rice produced (Bouman 2009) is almost unacceptable and justifies efforts in development of techniques for most rational water use. One of the tools to contribute for this aim is the construction of plant ideotypes specifically more efficient in water use.

In this sense, to allow farmers to either reduce water demand for rice paddy irrigation or to save water for other purposes, some water-saving irrigation techniques have been developed. AWD irrigation technique is one such technology, developed in the 1990s by the International Rice Research Institute (IRRI) (Price et al. 2013). In summary, when AWD is adopted, water management is carried out via intermittent flooding, which alternates cycles of saturated and unsaturated soil conditions. Under this management, during specific development phases and time, the water entry is closed allowing it to subside until the soil

reaches a threshold of water potential, after which the field is reflooded. In this context, the adoption of AWD in rice paddies could be used to increase water use efficiency and at the same time, reduce energetic costs involved in water capture, pumping and distribution, compared to the traditionally made by most farmers that adopt the continuous flooded system. Refinements in AWD could lead to an alternative for farmers to face the negative impacts of climate change on rain distribution regime in the future, besides contributing to decrease rice production costs.

Different studies has highlighted that the impacts of high water use by continuously flooded systems can be addressed with the implementation of AWD, but in many cases yields are reduced when this techniques is adopted (Carrizo et al. 2017). Meta-analysis carried out by these authors showed that the degree of soil drying during the drying cycles was critical to ensuring that yields were kept high. Additionally, they reported that with mild AWD managements, where soil water tension at root depth were below 20 kPa or where the water table in sub-superficial irrigation did not drop below 15 cm in soil depth, not significantly reduced rice grain yield. On the other hand, severe yield losses were verified at AWD where the soil water tension at root depth was beyond 20 kPa. In this sense, physiological breeding could elucidate the mechanisms involved in the plant responses during alternate wetting and drying cycles, leading to definition of some physiological tracer to supply the rice breeding programs with a tool to identify suitable genotypes to obtain cultivars better adapted to AWD management, without penalizing grain quality and productivity. The scarcity of suitable phenotyping protocols is a major bottleneck for rice breeding progress in the achievement of superior genotypes for AWD, what could increase the adoption of this technique by farmers. Physiological traits known to contribute to increase the plant performance under AWD are laborious to quantify and require resource intensive procedures to be measured; thus, unsuitable for some of the early stages of a breeding program where thousands of genotypes should be screened. Among these, stomatal conductance coordination, root and shoot plasticity and photosynthesis rate, besides the ability to develop deep roots, comprises important contributors for the improvement of plant responses under limited water availability.

There is a large set of information reporting contribution of certain root architecture traits and robustness leading to improvements in plant performance under drought, regardless of their constitutive or inducible expression way. Under different drought degrees and plant development phase, plasticity in root length and volume or even in mean diameter has been associated to increases in plant gas exchange performance, biomass production and even grain yield (Weber et al. 2014; Sandhu et al. 2016; Brito et al. 2019).

Carbon isotope discrimination (CID) has been extensively used as a proxy for physiological breeding of many plant species, aiming to improve its water use efficiency or its effective capacity of water use under dryland condition (Farquhar and Richards 1984; Farquhar et al. 1989; Brito et al. 2014; Dixon and Carter 2019). Nevertheless, there is scarce knowledge about its usability to identify desirable genotypes when AWD is adopted; especially considering the soil dynamic changes created by AWD and its complex interactions among genotypes. As proposed by Farquhar and Richards (Farquhar and Richards 1984), plants with C₃ metabolism discriminate against ¹³C in favor of ¹²C during the conversion of CO₂ into

carbohydrates. According to these authors, the comparison of plant isotopic composition among and between species led to definition of CID, which was defined as the deviation from unity of atmospheric $^{13}\text{C}/^{12}\text{C}$ divided by plant $^{13}\text{C}/^{12}\text{C}$. The main efforts to understand the drivers of carbon isotopic discrimination led to the finding that CID shares a positive and linear relationship with the ratio of internal leaf CO_2 (C_i) and the atmospheric CO_2 concentrations (C_a) (G D Farquhar et al. 1989). This is strongly impacted by stomatal conductance and photosynthetic performance, what allow researchers to make conclusions regarding principles of plant physiology from CID analysis (Brugnoli et al. 1988). When considered gas exchange performance over time, especially for photosynthesis and stomatal conductance in the tissue, where these parameters are analyzed, a high CID in leaf may be assumed to have experienced high stomatal conductance with low photosynthetic capacity or high performance for stomatal conductance and high photosynthesis, whereas the opposite may be assumed for low CID in plant tissue (Dixon and Carter 2019). Taken together, these assumptions and mode of dynamic progress of flooding and drying cycles via AWD, could lead to identify and classify genotypes based in traits known to be related to water deficit tolerance. But if considered the labor and time demanded to do direct phenotyping such as root robustness and architecture, deep roots assessment, stomatal conductance and anatomical plasticity, the leaf CID plasticity can serve as an initial practical and feasible tool for screening genotypes exhibiting these traits. At follow, preliminary data for the use of leaf CID as tool for breeding programs are highlighted, aiming to establish them as a potential tracer in plant ideotype construction for the AWD technique.

Material And Methods

Genetic material and growth conditions

A trial test was conducted under semi-natural conditions using a structure facility constructed specifically for this purpose (Figure 1). The study was accomplished during the 2019/2020 cropping season, at Low-Land Embrapa Experimental Station, located near Pelotas city, Rio Grande do Sul state, Brazil. Soil from this region is classified as Haplic Planosol (Albaqualf) (Santos et al. 2018), with geographic coordinates (31°46'19" S, 52°20'33" W), 17 m ASL, a traditional region for rice production under continuous flooding. Detailed description of the facility structure will be described in the next subtopic.

The analyzed data sets were collected from a set of 20 genotypes (table 1) consisting of rice traditional varieties and elite breeding lines from the breeding program of Brazilian Agricultural Research Corporation (Embrapa), plus a hybrid (XP 113) from a private company.

Table 1 Genetic materials and genealogy of crosses made between conventional parents, with the respective status within breeding program, origin, genotype per cross e subtype plants used for the study

Genotypes	Status	Origin.	Cross combination	Year	type
AB 14738	Line	Embrapa	BRA Pampa x Irga 424	2014	Indica
BRA 050151	Line	Embrapa	BRS 7 Taim/CL Sel 720	2005	Indica
BRS 358	Cultivar	Embrapa	GIZA 175/ MILYANG 49	2016	Japonica
BRS A705	Cultivar	Embrapa	BRA 01016/CNAi10393	2020	Indica
BRS Firmeza	Cultivar	Embrapa	BR IRGA 411/BLUEBELLE // LEMONT	1999	Indica
BRS Pampa CL	Cultivar	Embrapa	BRS Pampa (3)/Puitá INTA CL	2019	Indica
BRS Pampeira	Cultivar	Embrapa	IR 22/CNA 8502	2016	Indica
CNA 1003	Progeny S0:3	Embrapa	Multiples (Pop. SR CNA 11)	2010	Indica
CNA 1120	Progeny S0:3	Embrapa	Multiples (Pop. SR CNA 11)	2011	Indica
CNA 1121	Progeny S0:3	Embrapa	Multiples (Pop. SR CNA 11)	2011	Indica
CTB 1419	Progeny F5	Embrapa	Sel. TB 1211-2/IRGA 424	2014	Indica
CTB 1444	Progeny F5	Embrapa	Sel. TB 1211-1 BRA 051108	2014	Indica
CTB 1455	Progeny F5	Embrapa	Sel. TB 1211-5/BRA 051077	2014	Indica
LTB 13016	Line	Embrapa	BRS Firmeza/BRS Agrisul	2013	Indica
LTB 13036	Line	Embrapa	BRA 050055/BR-IRGA 409	2013	Indica
LTB 14002	Line	Embrapa	BRS Atalanta/Oro	2014	Indica
LTB 17033	Line	Embrapa	CL 113-4-1-1/CL 591	2017	Indica
LTB SEL 1211-2	Line	Embrapa	CL 113-4-1-1/CL 591	2012	Indica
LTB SEL 1211-3	Line	Embrapa	CL 113-4-1-1/CL 591	2012	Indica
XP 113	Hybrid	Rice Tec	Confidential information	2015	Indica

The BRS Pampa CL, BRS 358 cultivars and AB 14738 elite line were selected based on results from a field study conducted by Brito and co-workers (Brito et al. 2019), where BRS Pampa showed a positive plasticity for total root volume and root length, whereas a negative plasticity was found for BRS 358 and AB 14738. The set of additional genotypes were included based on its use by regional farmers or as result of grain yield performance in field assessments by Embrapa's Rice Breeding Program. Sowing was accomplished in rows spaced in 0.175 m with 1m long, thinned to density of 300 plants m⁻² ten days after emergence. The set of genotypes used were similar for maturity group (125-135 days from emergence to maturity). Sowing was accomplished in September 23th, 2019, emergence occurred in October 02nd, 2019.

Topdressing fertilization consisted of 100 kg ha⁻¹ of N as commercial urea split in two applications: 70% of the dose was applied at the beginning of tillering of the plants (20 days after emergence – DAE), prior to flooding, and the remaining 30% of the dose was applied nearby to panicle initiation. This stage was estimated by using the software PlanejArroz (Steinmetz et al. 2020) by supplying location, emerge date and indicating BRS Pampa CL, a commercial variety with similar cycle length to the evaluated set of genotypes. Soil chemical parameters are shown in Table 02.

Table 2 Soil chemical characteristics from 0 - 20 cm of the soil layer in Estação Experimental Terras Baixas (EETB), Embrapa Temperate Agriculture, Pelotas, RS, Brazil.

EETB	O.M.	pH	P	K ⁺	Ca ²⁺	Mg ²⁺	Al ³⁺	Clay
Sample*	g dm ⁻¹	water	----- mg/dm ³ -----		----- cmol _c /dm ³ -----			g dm ⁻¹
22894	15	5.2	1.9	43.0	1.2	1.1	0.7	170

* Analysis were carried out according to Tedesco and co-workers (Tedesco et al. 1995)

The study comprised two water managements consisting of a well-watered set of plots, where plants were maintained under continuous flooding (control), and another treatment submitted to Alternate wetting and drying management (AWD) (stressed) imposed from V₄ plant stage (Counce et al. 2000) onwards (Price et al. 2013), consisting of three intermittent cycles of irrigation. A set of rectangular 500 L fiberglass water tanks was arranged in a split-plot design, with water treatments as the main plots and genotypes as subplots, in three replications. From rice sowing to the beginning of tillering, all plots were irrigated daily to keep soil moisture near field capacity. At tillering, a five cm flood depth was established for each plot and maintained under continuous flooding by 10 consecutive days. Subsequently, for each cycle of intermittent irrigation, those plots submitted to AWD were drained and not irrigated again, being monitored until soil water tension reached 20 kPa (7-11 days depending on prevalent climatic conditions), when the first AWD cycle was completed. At this water tension, plots were re-irrigated with a new five cm flood depth, and maintained by 72 h. Subsequently, a new AWD cycle was started by controlled drainage via independent valve-controlled water inlets and outlets of each plot. Three intermittent irrigation cycles were sequentially imposed. The progress of soil water tension was monitored by installing two Watermark sensors (Irrometer inc., USA) per plot at 10 cm depth, wired to electronic data loggers for continuous follow up on soil water status. Data were reviewed twice a day and mean values, excluding outliers, was used to define the time for re-irrigation at the end of each intermittent cycle (Figure 1).

Facility structure and measured phenotypic traits

Aiming an efficient control of the intermittent irrigation cycles for AWD treatment, a specific facility structure was idealized and constructed to allow the establishment of good plant growth conditions and, at same time, especially designed to permit the control of uniform drainage during application of AWD (Figure 1). This structure created the adequate conditions for applying the AWD technique due to the efficient control of water inlets and outlets, including the water layer height, and uniformity in the rapid drainage for AWD.

Figure 1 Overview of a low-cost structure facility specifically designed and constructed for monitoring and efficient control of the alternate wetting and drying cycles during the study; red arrows indicate rainwater reservoir (1), data loggers for soil tension monitoring and logging (2), float-controlled layer valve (3), water inlet controller (4) and water drainage controller (5)

Briefly, this structure consisted of a set of fiberglass tanks connected by PVC pipes to a 5000 L water reservoir fed by rainwater collected on the rooftops of two adjacent greenhouses. This set of rectangular 500 L fiberglass water tanks was equipped with independent valve-controlled water inlets and outlets for

precise irrigation and drainage as needed. These tanks were filled with a 5 cm layer of crushed stone followed by a 5 cm layer of coarse sand and, 50 cm layer of soil previously corrected and fertilized for rice (SOSBAI 2018). Soil was collected from a nearby area, with long history of rice cultivation under continuous flooding. The soil was collected between 0 and 20 cm depth, which represents the average root depth in local soils. Water layer height was controlled via installation of a float valve for each tank. For uniform and fast drainage, a perforated 25 mm PVC pipe with 70 cm long (5 cm higher than the height of the tank wall) were installed in the center of each tank in order to avoid the formation of water pockets into soil during application of the intermittent irrigation cycles, standardizing the drainage procedures. Water re-entry into the system after a drainage cycle occurred when the watermark sensors of the plot indicated 20 kPa of soil water tension (Pinto et al. 2016; Pinto et al. 2020).

A set of twenty genotypes consisting of modern cultivars, progenies, lines and a hybrid grown under semi-natural conditions, submitted to two water irrigation regimes (AWD cycles and continuous flooding), were evaluated to assess the variation in phenotypic traits. In total, 24 phenotypic traits/derivatives, distributed in three categories (Root architecture, physiological/shoot morphological and micro-morphological traits), were evaluated during plant vegetative phase (Table 3).

Table 3 List of analyzed and derived phenotypic traits broadly classified into three categories (A-C) with trait acronyms and units

Trait	Trait acronym	Unit
1. Root architecture traits		
<i>Total root length (cm)</i>		
Total root length under continuous flooding in first cycle	TRL in CF - 1 st cycle	cm
Total root length under continuous flooding in second cycle	TRL in CF - 2 nd cycle	cm
Total root length under continuous flooding in third cycle	TRL in CF - 3 rd cycle	cm
Total root length under alternate wetting drying in first cycle	TRL in AWD - 1 st cycle	cm
Total root length under alternate wetting drying in second cycle	TRL in AWD - 2 nd cycle	cm
Total root length under alternate wetting drying in third cycle	TRL in AWD - 3 rd cycle	cm
<i>Total root volume</i>		
Total root volume under continuous flooding in first cycle	TRV in CF - 1 st cycle	cm ⁻³
Total root volume under continuous flooding in second cycle	TRV in CF - 2 nd cycle	cm ⁻³
Total root volume under continuous flooding in third cycle	TRV in CF - 3 rd cycle	cm ⁻³
Total root volume under alternate wetting drying in first cycle	TRV in AWD - 1 st cycle	cm ⁻³
Total root volume under alternate wetting drying in second cycle	TRV in AWD - 2 nd cycle	cm ⁻³
Total root volume under alternate wetting drying in third cycle	TRV in AWD - 3 rd cycle	cm ⁻³
<i>Mean root diameter</i>		
Mean root diameter under continuous flooding in first cycle	MRD in CF - 1 st cycle	μm
Mean root diameter under continuous flooding in second cycle	MRD in CF - 2 nd cycle	μm
Mean root diameter under continuous flooding in third cycle	MRD in CF - 3 rd cycle	μm
Mean root diameter under alternate wetting drying in first cycle	MRD in AWD - 1 st cycle	μm
Mean root diameter under alternate wetting drying in second cycle	MRD in AWD - 2 nd cycle	μm
Mean root diameter under alternate wetting drying in third cycle	MRD in AWD - 3 rd cycle	μm
(B) Physiological and shoot morphological traits		
Carbon isotope discrimination under continuous flooding	CID in CF	‰
Carbon isotope discrimination under alternate wetting drying	CID in AWD	‰
Shoot dry weight under continuous flooding	Shoot DW in CF	g
Shoot dry weight under alternate wetting drying	Shoot DW in AWD	g
(C) Micro-morphological traits		
Stomatal density	SD	n ^o /mm ⁻²
Stomatal pore width	SPW	μm

Carbon fractionation analysis

An isotopic mass spectrometer at the Stable Isotopes Center of the Universidade Estadual Paulista - UNESP, Brazil was used to determine the leaves carbon isotope percentages. Leaves samples were dried in an oven at 50°C for 48h to homogenized in a cryogenic mill (Geno / Grinder 2010 - SPEX SamplePrep, USA) using liquid nitrogen at -196°C. A 50 to 70 µg aliquot of each sample was weighed in a tin capsule using a 1 µg resolution scale (XP6 - Mettler Toledo, Switzerland). The homogenization of the samples increases the representativeness of the small sample rate. The capsules were analyzed in a CF-IRMS continuous-flow isotope ratio spectrometry system using an IRMS (Delta V Advantage, Thermo Scientific, Germany) coupled to an Elemental Analyzer (Flash 2000, Thermo Scientific, Germany) using a gas interface (ConFlo IV, Thermo Scientific, Germany). The CF-IRMS determined the isotopic ratio of Carbon R ($^{13}\text{C} / ^{12}\text{C}$) and the values were expressed in relative difference of the isotopic ratio ($\delta^{13}\text{C}$), in mUr (Brand and Coplen 2012), from the V-PDB standard according to the equation 1 (Coplen 2011). The standard uncertainty of the CF-IRMS is ± 0.15 mUr and the results were normalized from the IAEA-NBS-22 standard, as shown by the following formula: $\delta^{13}\text{C} = [R(^{13}\text{C} / ^{12}\text{C})_{\text{sample}} / R(^{13}\text{C} / ^{12}\text{C})_{\text{VPDB}}] - 1$. For carbon isotopic discrimination (Δ), the following formula was used: $\Delta = (\delta^{13}\text{Ca} - \delta^{13}\text{Cp}) / (1 + \delta^{13}\text{Cp})$, where $\delta^{13}\text{Ca}$ and $\delta^{13}\text{Cp}$ are the carbon isotope compositions of atmosphere and plant samples, respectively (Farquhar et al. 1989). As a convention, the $\delta^{13}\text{Ca}$ values was assumed to be -8.0 mUr (Hall et al. 1994). As pointed before, carbon isotope composition values at most of time expressed in terms of 'per mil' aiming indicate that the original value was multiplied by 10^3 .

Root sampling and image analysis

At the end of each of the three AWD cycles, three plants per genotype were randomly collected independent of applied irrigation system. For this propose, a whole plant core sampler (50 mm diameter by 70 cm long) tube, inserted from the top to the bottom of the cultivation tanks. Subsequently, the whole plants (shoots and roots) were individually prepared; samples consisted of shoot and roots, separated by means of a cut in the crown region aiming facilitate its washing and whitening. Subsequently, these roots were taken to the photographic studio of Embrapa Temperate Agriculture to obtain the root images; these were then placed into a black-bottomed plastic tray containing a water film approximately 2 mm thick, thus facilitating root dispensal. Roots were spread evenly with the aid of tweezers to avoid root overlapping in the photography. Images was captured by using a Canon EOS 7D photographic camera, 40 mm lens, at a fixed distance of 1.20 m from the target, positioned in an L-shaped support, using LED lighting.

From the obtained root images, morphometric parameters were analyzed by using the software WinRHIZO PRO 2013a (Regent Instruments. Inc., Quebec, Canada). Mean root diameter, total root volume and total root length were quantified and considered for calculation of root architecture traits.

Micro morphological analysis

Leaves were collected 23 days after emergence during the first AWD irrigation cycle. For measurements, immediately after cutting, the middle section of the adaxial (upper) face of the second leaf was observed and photographed via an optical microscope (Nikon e200) with 0.5 x magnification for camera and 40 x for microscope objective lens. Leaf sections were gently pressed between two microscope glass slides, held tight together by adhesive tapes, to keep leaf flat open. Photography scales were set by previously capturing images of a microscope calibration ruler, in both magnifications. Subsequently, two images with resolution of 2048 x 1536 pixels (3.1 MP) were randomly taken per leaf for analysis.

Captured images were saved in hierarchical naming structure for later processing via the software ImageJ v.1.53c (NIH, 2020). After software scale calibration, the stomatal density and stomatal pore width (20 x magnification) were quantified by using software tools. Stomatal related data were obtained after applying the filter "relief" to the images to increase stomatal border and opening contrasts. Stomatal density was quantified 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 appropriately considered in stomatal density calculations. Scales were marked into images by ImageJ software.

Statistical Procedures

For estimation of genetic parameters were used the 'R package sommer' (Covarrubias-Pazaran 2016; Covarrubias Pazaran 2018). Firstly, individual analysis were carried out for each irrigation system; genotypes being considered as random effects, testing the significance of the genetic variance (σ_g^2) and residual variance (σ_e^2) components via Wald test (Z ration). Additionally, estimate of broad sense heritability (h^2) at individual level were done according to (Holland 2006). An univariate unstructured variance model, in which consider the genotypes as random effect and irrigation system as fixed effect were used to estimation of co-variances with subsequent calculation of genetic correlations to a same variable in the two irrigation systems. The test procedures adopted for genetic correlations were identical those used for heritability calculations. All used procedures are integrated into the R Core Team (Core R Team 2020).

The heatmap was constructed using the R package Pheatmap' (Kolde 2019); variables were standardized individually being the distance between variables calculated via Pearson's correlation for a given variable in each irrigation system via Ward's test. For genotypes grouping formation, the estimation of Euclidean distances were calculated using Ward's procedures also using Ward's procedures. Taken into account that experiment was comprised by three intermittent irrigation cycles, opted as cut point by three genotypes groups or variables, as shown in Figure 5.

Subsequently, after normality test by Henze-Zinkler, the obtained data were subjected to multivariate statistical approaches, via principal components analysis (PCA) in order to generalize, reduce the overlapping among evaluated variables and characterize the germplasm more comprehensively. This

statistical approach is extensively used in situations where a large number of variables should be evaluated, allowing into major components and total variation. In order to facilitate the identification of genotype traits which make it possible to differentiate them from the others in terms of root architecture and its derivatives, physiological/morphological shoots and micro-morphological traits, during PCA analysis some procedures were previously determined during the graph scales definitions. For this propose, scale values on the X axis and on the Y axis were gradually increased leading to maximum genotypes scattering, without loss of any genetic materials and information. The procedures allow an easy overview of those genotypes that were positioned near the centre. These genotypes are similar and not show significant differences between themselves for evaluated variables. On the other hand, those genotypes positioned near and at extremes of two scales can be thought as differing from the others, with superior characteristics closely associated with their positioning across the biplot graph. As cited by Dallastra and co-workers (Dallastra et al. 2014) these genotypes are those that should receive special attention and are likely to be selected. The choice of principal components was based on eigenvalues higher than one (1.0), as by Kaiser's study (Kaiser 1958). According to this author, eigenvalues greater than this threshold can generate components having a relevant amount of information of the original variables. Multivariate analysis via PCA and data plotting were carried out using tools of the SigmaPlot 14.0, from Systat Software (SigmaPlot Version 14.0).

Results And Discussion

Estimating genetic variance components and heritability

The authors decide begins showing the genetic components issues because we would have arguments to drive a sequential discussion across of the manuscript, besides help in the definition of used statistical approaches for subsequent phases of the analysis. Coefficients of genetic variance (π^2g) obtained for root architecture, micro-morphological and physiological traits were significant for all of them (table 4), regardless of adopted irrigation regime, except to mean root diameter at the end of third irrigation cycle for both the water managements. It should be noted that the existence of genetic variability is *sine qua non* to success of the selection. Thus, the data highlighted the existence of real probability to obtain improvements for evaluated traits, except for mean root diameters in these two conditions. In general, the values of heritability coefficients were higher regardless of adopted irrigation system and trait category (table 3). The heritability for shoot dry weight under continuous flooding was greater than under AWD, indicating that the selection is most favorable when carried out under continuous flooding. On the other hand, for carbon isotope discrimination, the magnitudes of heritability were similar in the two irrigation techniques, demonstrating that the success of selection is independent of irrigation technique. Analyzing root architecture traits such as total root length and volume, superior magnitudes of heritability was demonstrated under AWD, pointing that selection of these traits under intermittent irrigation might contribute to success.

Table 4 Estimate of genetic variance components (σ^2_g), residual variance components (σ_e^2) and heritability (h^2) in broad sense heritability for analyzed traits in a set of rice genotypes subjected to alternate wetting and drying and to continuous flooding at vegetative phase

Traits	AWD					CF					rg (AWD,CF)	sd (rg)
	Mean	σ_g^2	σ_e^2	h^2	sd(h^2)	Mean	σ_g^2	σ_e^2	h^2	sd(h^2)		
CID	22,54	0,54 **	0,07	0,883	0,042	22,95	0,2392 **	0,0438	0,845	0,055	0,546	0,175
MRD - 1 st cycle	272,99	0,00 n.s.	30,92	0,000	0,131	282,09	4,24 **	50,84	0,077	0,140	-	-
MRD - 2 nd cycle	270,10	0,00 n.s.	80,92	0,000	0,131	271,74	6,31 **	50,60	0,111	0,143	-	-
MRD - 3 rd cycle	269,82	0,00 n.s.	88,09	0,000	0,131	277,53	0,00 n.s.	86,09	0,000	0,131	-	-
TRL - 1 st cycle	2976	223781 **	18232	0,925	0,028	2942	92237 **	65842	0,583	0,118	0,104	0,250
TRL - 2 nd cycle	2759	94043 **	33930	0,735	0,086	2697	40918 **	20792	0,663	0,103	0,390	0,233
TRL - 3 rd cycle	3074	99043 **	19590	0,835	0,058	3442	107159 **	34047	0,759	0,080	0,068	0,248
TRV - 1 st cycle	2,451	0,0734 **	0,0362	0,670	0,101	2,460	0,1088 **	0,0341	0,762	0,079	0,513	0,201
TRV - 2 nd cycle	2,146	0,0309 **	0,0137	0,693	0,096	2,117	0,0295 **	0,0244	0,547	0,125	0,080	0,276
TRV - 3 rd cycle	2,442	0,0781 **	0,0198	0,798	0,069	2,796	0,064 **	0,028	0,696	0,096	0,170	0,249
Shoot DW	5,33	0,8884 **	0,710	0,556	0,123	6,03	2,124 **	0,173	0,925	0,028	0,087	0,253
SD	478,88	11967 **	3983	0,750	0,082	-	-	-	-	-	-	-
SPW	9,28	2,522 **	0,453	0,848	0,054	-	-	-	-	-	-	-

** Significant by Wald test ($P \geq 0.05$); ns means non-significant. Traits labels are in according to category (uppercase letters) in Table 3; together with their acronyms.

Nevertheless, the success of selection in a given water regime does not guarantee that genetic gains will also be obtained in the other water management, since a premise depends on the magnitude of the genetic correlations between evaluation conditions for a specific trait. In the same way, selection for a specific trait could lead to indirect gains or undesirable losses in others, depending on the magnitude of the present genetic correlations.

Phenotypic traits variation and their interrelations

Multivariate statistical procedures, via principal component analysis (PCA) among root architectures and physiological traits were done across the three irrigation cycles and water irrigation regimes, among the set of genotypes. For total root length (TRL) and their derivatives (Figure 2), this approach identified three significant principal components (PCs) with eigenvalue > 1 , which cumulatively explain approximately 70% of the total variation for all cycles and water regimes. The first PC represent more than 32% of the total variation; which is associated with genotypic variation in the majority of this root traits and their derivatives, independently of irrigation cycle and water regimes. The eigenvalue for the second component was 1.7, which was associated with total root length in alternate wetting and drying regime in first irrigation cycle (TRL in AWD - 1st cycle) and TRL in continuous flooding in third irrigation cycle (TRL in CF - 3rd cycle). Additionally, these derivative variables were correlated with each other, even from distinct irrigation cycle and water regime. This PC retained a variance proportion greater than 21%, with main variables being carbon isotope discrimination in continuous flooding (CID in CF), TRL in AWD - 1st cycle and TRL in CF - 3rd cycle. For instance, CID in AWD highlighted a positive correlation with TRL in CF - 1st cycle ($r = 0.60$) and CID in CF ($r = 0.49$). Taken together, these results clearly suggest that selecting plants based on CID, independently of the adopted water regime, could result in plants with increased root length. It could reduce the costs involved in their suitability in routines

of breeding programs, since only a water regime could be enough to select superior genotypes. In Figure 2, it is also highlighted the dispersion of evaluated traits according to score and the correlation between them. Taken as an example, selecting BRS Pampeira and BRS A705 cultivars and LTB 17033 line, that show high CID in the two water regimes, could result in plants with greater performance for this desirable root trait.

Figure 2 PCA of the eight variables/derivatives involving total root length (TRL in cm) at three irrigation cycles and carbon isotope discrimination subjected to two-water regime treatments. The first two components show majority of variation with their vector projections for each trait. Biplot graph highlight the genotypes distribution (in dark blue) across worked scales. The traits marked by circles/ellipses contributed more to the variation explained by PC1, and to the variation explained by PC2. Blue and Red arrows denote carbon isotope discrimination under continuous flooding and under alternate wetting and drying water regimes, respectively; other trait labels are in according to category (uppercase letters) in Table 3,; together with their acronyms

When PCA was used to explain variation for total root volume (TRV) trait/derivatives, three significant principal components (PCs) with eigenvalue > 1 , explaining greater than 59% of the total variation across cycles and water regime were identified (Figure 3). The first PC represents more than 25% of the total variation, being associated to the genotypic variation for TRV in alternate wetting and drying at the first cycle (TRV in AWD – 1st cycle) besides TRV in AWD – 2nd cycle and CID at both irrigation regimes. The eigenvalue for the first and second components were 2.02 and 1.50, respectively. The second PC was associated with total root volume in continuous flooding at the second cycle (TRV in CF – 2nd cycle). Moreover, TRV in AWD – 2nd cycle was closely correlated with CID, regardless of the irrigation regime. This PC retained a variance proportion nearly 19%. For instance, CID in AWD showed positive correlation with CID in CF ($r = 0.62$). Again, high correlation found between CID values in both irrigation regimes clearly indicate that selecting plants based on CID, independently of the adopted water regimes, could result in plants with increased root volume. As pointed before, similarly found to root length, the costs involved in the selection for root volume could be significantly reduced, increasing their usability as a tracer to be included in routine of a breeding program, since only a water regime could be enough to select superior genotypes for this purpose. The Figure 3 supplies an overview of dispersion behavior of evaluated traits according to the score, and the correlation between them. For instance, selecting BRS A705 cultivar and CTB 1455 line with high CID capacity, the total root volume will link them, independently of adopted irrigation technique.

Figure 3 PCA of the eight variables/derivatives involving total root length (TRV in cm^{-3}) at three irrigation cycles and carbon isotope discrimination (CID) subjected to two-water regime treatments. The first two components show majority of variation with their vector projections for each trait. Biplot graph highlight the genotypes distribution (in dark blue) across worked scales. The traits marked by circles/ellipses contributed more to the variation explained by PC1, and to the variation explained by PC2. Blue and Red arrows denote carbon isotope discrimination under continuous flooding and under Alternate wetting and

drying water regimes, respectively; other trait labels are in according to category (uppercase letters) in Table 3,; together with their acronyms

Heat mapping of root architecture and carbon isotope discrimination traits of rice

Taken into account the genetic components analysis (table 4), the mean root diameter (MRD trait and derivatives, which showed non-significant genetic variance) were excluded from the next step, in which heat maps were generated based on standardization of phenotypic values (Figure 5 A and B). Thus, heatmap analysis is indicating a relative performance of a set of genotypes relative to the mean response for a given trait; and according to color scale, the positive dark blue represent those genotypes showing higher performance for a given root architecture and/or micro-morphological and physiological trait (positive values from standard phenotypic procedures).

From top down, the first cluster (Figure 5 A), it is comprised by most genotypes expressing positive and greater responses for carbon isotope discrimination (CID) in alternate wetting and drying (AWD), compared to response of these same genotypes in continuous flooding (CF). Interestingly, under AWD, BRS Pampeira and LTB 17033 displayed above average values for carbon isotope discrimination closely to total root volume and root length, all of them at the end of the third irrigation cycle. Moreover, most genotypes from this cluster highlight highest values for stomatal density and stomatal pore width. Even arranged in other clusters, these genotypes keep positive responses for these same root traits since the first irrigation cycle, indicating their constitutive character (Figure 5 B). As emphasized in the methodological topic, stomatal density and stomatal pore width were measured only at AWD in first intermittent cycle due to their high demand in human resources and time.

CTB 1419, BRS 358, LTB 1306, LTB 13016, CAN 1120 and CTB 1144 comprise the second cluster for genotypes subjected to AWD. In this cluster (under AWD – Figure 4 A) most of the genotypes show negative values relative to stomatal density and stomatal pore width with reduced performance for most evaluated root traits, besides of their reduced capacity for effective use of water as shown by low carbon isotope discrimination responses. Differently from the second cluster, the third included most genotypes, even those with reduced CID capacity (with positive responses for total root volume and root length at first irrigation cycle), their root responses changed at third evaluation with negative performance for most of measured root traits.

Figure 4 Heat map analysis of root architecture, physiological, shoot morphological and micro-morphological traits, subjected to alternate wetting drying (A) and to continuous flooding (B) regimes

Obviously, attributing to only one or a few traits to justify the superior performance of a given genotype, subjected to AWD, would lead to a somewhat reductionist conclusion. On the other hand, it is well documented that genetic differences in rice root architecture and rooting depth may contribute to maintain higher stomatal conductance and, at the same time, allow them an increase in CO₂ influx helping to sustain a higher photosynthetic rate in plant grown under limited water availability. Taken together, geneticists, breeders and physiologist are in consensus that the breeding plants showing root

traits that result in improvements of grain productivity under limited water availability, should include long specific root length, root volume and changes in their diameter (plasticity), especially at depth with available water at deep soil profile (Yang et al. 2012; Comas et al. 2013).

In summary, standardized phenotypic values presented by heatmap indicates that genotypes with increased root length and volume at the end of the third irrigation cycle, were the ones who also showed positive responses to stomatal conductance, as pointed by their greater carbon isotope discrimination. From top down, the first cluster under AWD (Figure 4 A), draws attention to the fact that most genotypes highlighting positive values for stomatal density and stomatal pore width, were the ones that also showed increased total root volume and total root length, regardless of the irrigation cycle.

Stomata plays a fundamental regulation role in the trade-off between CO₂ influx and water efflux. However, recent advances have shown also their interaction with below-ground development or their contribution in the diffusion of other gases (Mohammed et al. 2019). These authors surprisingly found that rice root cortical aerenchyma is formed constitutively in plants overexpressing *OsEPF1*, a gene involved in the reduction of stomatal densities. In this sense, it should be considered that in the short-term, water loss is controlled as a dynamic change in the degree of stomatal openness; on the other hand, the stomatal density will determine this water loss in the long-term. As pointed before, positive root architecture responses (cluster 1 genotypes from top down, under AWD) were associated to superior physiological performance (roots with increased capacity to capture soil moisture). Furthermore, higher carbon isotope discrimination capacity leads to a definition of the traits that may promote significant contributions to plant ideotypes when AWD is adopted.

When breeders aim to select plants based on higher CID under continuous flooding irrigation ($h^2 = 0.85$), at least one of these, such as root total volume ($h^2 = 0.75$) and/or their total root length ($h^2 = 0.70$), will be probably improved. Under AWD, prioritizing the selection of those showing higher CID will also select increased root volume and length, AWD cycle number. Under AWD, BRS Pampeira and LTB 17033 are highlighted with positive responses for carbon isotope fractionation in the two irrigation methods. Interestingly, while BRS Pampeira shows greater stomatal density with lower stomatal pore width, LTB 17033 exhibits larger stomatal pore width associated to smaller stomatal density (Figure 4 A). Pore size has been the main determinant in the stomatal conductance (Fanourakis et al. 2015). It is already well consolidated that variation in anatomical features of stomata, as stomatal pore width and their regulation, may lead to impacts on gas exchange (Aliniaiefard and van Meeteren 2014; Giday et al. 2014). Moreover, stomatal conductance performance can be changed by variation in stomatal density, size or even by percentage of pore area per stomatal area. Increased stomatal size generally leads to increases in stomatal conductance due to associations with larger pores (Franks and Farquhar 2007). The contrasting behavior evidenced by these two genotypes (BRS Pampeira and LTB 17033) for water use regulation are also adopted by CNA 1003 and BRS Pampa CL, from the same cluster (Figure 5 4), even that in different magnitudes.

Conclusions

There is significant genotypic variability for CID in rice leaves, among the evaluated genotypes, regardless of cycles number in AWD irrigation. This approach demonstrated the plausibility of using CID as tracer with relatively high-throughput to early select genotypes with increased root volume and length, and higher stomatal conductance. Although this is a preliminary evidence in supporting this idea, the next step will focus on contrasting CID with the other traits in field conditions. In the next step, this validation will provide important insight into the robustness of the hypotheses presented in this work. We believe that it will be confirmed the root morphology behavior and gas exchange performance as being associated to CID in rice leaves, as well as to grain yield components.

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