ISSN: 1984-5529 Científica Jaboticabal v.47, n.2, p.199-209, 2019

http://dx.doi.org/10.15361/1984-5529.2019v47n2p199-209

Iron biofortification in rice: in search of morphological traits for indirect selection in breeding programs

Biofortificação com ferro em arroz: em busca de caracteres morfológicos para seleção indireta em programas de melhoramento

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Recebido em: 17-12-2018; Aceito em: 06-03-2019

Abstract

Iron (Fe) deficiency is an important cause of health concern in developing countries, demanding mitigation strategies to fight this disorder. The biofortification of staple foods, such as rice (*Oryza sativa* L.), could be achieved via the development of improved cultivars. Iron quantification is relatively costly and time-consuming, making its routine use impracticable in breeding programs. Therefore, the identification of traits to be used in indirect selection strategies would be of high interest. This study aimed to find promising traits for use in indirect selection programs aiming Fe biofortification in rice grains. A diverse set of 95 rice genotypes, mostly Brazilian, was grown at Southern Brazil, in 2016/2017, and Fe content in brown and polished grains, as well as other 12 morphological traits, were assayed. Analysis of variance, linear correlation, and path analysis were carried out. Different levels of association between traits were found, being panicles per plant and caryopsis width the most promising for use in indirect selection aiming increased Fe content in both brown and polished rice. However, secondary traits, such as caryopsis length, have also to be considered when performing selection aiming Fe biofortification in the cereal.

Additional keywords: breeding strategies; correlation; mineral content; nutritional quality.

Resumo

A deficiência de ferro (Fe) é uma importante causa de preocupação em termos de saúde em países em desenvolvimento, demandando estratégias de mitigação desta desordem. A biofortificação de alimentos-base da dieta, como o arroz (*Oryza sativa* L.), pode ser alcançada através do melhoramento genético de cultivares. A quantificação do teor de ferro é relativamente cara e demorada, fazendo seu uso rotineiro impraticável em programas de melhoramento. Assim, a identificação de caracteres para serem utilizados em estratégias de seleção indireta é de elevado interesse. Este estudo objetivou encontrar caracteres promissores para seleção indireta, objetivando biofortificação de ferro em grãos de arroz. Um painel diverso com 95 genótipos de arroz, a maioria brasileiros, foi cultivado no Sul do Brasil, em 2016/2017, e foi avaliado o conteúdo de Fe em grão integral e polido, assim como outros 12 caracteres morfológicos. Foram realizadas análise de variância, correlação linear de Pearson e análise de trilha. Diferentes níveis de associação entre os caracteres foram observados, sendo panículas por planta e largura da cariopse os mais promissores para uso em seleção indireta objetivando incremento no teor de Fe, tanto em arroz integral como polido. Entretanto, caracteres secundários, como comprimento da cariopse, também devem ser considerados ao realizar a seleção, visando à biofortificação com Fe nos grãos do cereal.

Palavras-chave adicionais: estratégias de melhoramento; conteúdo de minerais; correlação; qualidade nutricional.

Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods for humankind, comprising the largest part of the diet of more than half of human population (Mohan et al., 2017). In rice breeding programs, the development of cultivars with improved yield potential has been the primary target. However, quality and nutritional aspects of the grain have risen in relevance, for many reasons (Sharma et al., 2017).

Nutritional quality of food, particularly mineral content has become more important over the years (Saltzman et al., 2013). Among nutrients with high importance in the human diet, iron (Fe) receives high attention, as it is crucial to many biological functions (Khush et al., 2012; Murgia et al., 2012; Camaschella, 2015; Vasconcelos et al., 2017). The deficiency of this element is one of the most critical health problems in developing countries, most places where, at the same time, rice is the staple food (Stein, 2010; Hackl et al., 2017). Biofortification with Fe in rice grains, through breeding improved cultivars, is the most convenient and impacting mitigation strategy against the deficiency of this micronutrient (Bouis & Welch, 2010; Sperotto et al., 2012).

There is important genetic variability for Fe content in rice grains (Zhang et al., 2004; Anandan et al., 2011). However, it is a complex quantitative trait, showing intermediate heritability, thus profoundly affected by the environment (Huang et al., 2015). It is also a trait of difficult evaluation, i.e., relatively costly and time-consuming, requiring specific analytical analysis to determine the content in food. Additionally, it involves the use of mature grain for analysis, thus direct selection at early development stages of the plant is not possible. In this regard, indirect selection is a convenient alternative (Carvalho et al., 2004).

Indirect selection is based on one or more secondary traits aiming to select and thus improve another trait, the main object of selection, exploring the association among traits (Falconer, 1981). For a successful indirect selection, the secondary traits must be easier or cheaper to evaluate in comparison to the main trait. Furthermore, more convenient is the selection, when the secondary traits are also of agronomic interest, so the crop can be improved for many traits simultaneously. Phenotypic, genotypic or environmental correlations can exist between traits, being the genotypic correlation the most informative for breeders, as it captures trait heritabilities. Genotypic correlations are explained mainly by pleiotropy or gene linkage genetic phenomena (Falconer & Mackay, 1996; Nogueira et al., 2012).

Despite the enormous potential that indirect selection offers, some aspects deserve attention. In many cases, the correlation among traits has not a "cause and effect" nature, not allowing a direct interpretation of a linear correlation. In this regard, a strong association between two traits can happen due to the indirect effect of a third trait or even more traits. Thus, special analysis, such as the *Path analysis* (Wright, 1923) allow a better understanding of the real association between the traits under analysis and thus a better application of the results (Wright, 1921, 1923; Nogueira et al., 2012; Barbosa et al., 2017).

Therefore, the present study aimed to investigate the association between morphological traits, including of agronomic importance, and Fe content in rice grains, verifying the most promising ones for use in indirect selection in breeding programs aiming iron biofortification in rice.

Materials and methods

Rice panel and field experimental procedures

A diverse rice (*Oryza sativa* L.) panel consisting of 95 accessions, from *indica* (mainly) and *japonica* subspecies, composed especially by Brazilian elite cultivars (released by different breeding programs), cultivars from other countries, lines obtained through mutation breeding, and even one hybrid cultivar, was phenotyped regarding grain iron content and diverse morphological traits (Table 1).

The panel was grown, both for harvesting grains for iron content analysis and evaluating the morphological traits, in lowland conditions, at Capão do Leão – Rio Grande do Sul, Brazil, during the 2016/2017 crop season. The experimental design was randomized blocks, being each plot constituted by a one-meter row, spaced 0.20 m. Forty-five viable seeds were sown per row. The flooded irrigation system was applied. All agronomic practices followed the technical Brazilian recommendation for rice (SOSBAI, 2016). Harvesting by hand was performed at the grain physiological maturity.

Table 1. Genotypes assayed at Southern Brazil conditions for Fe content in brown and polished rice and agronomic traits, and their origin.

Genotype	Origin	Genotype	Origin	Genotype	Origin
BRS 358	Brazil	EMPASC 100	Brazil	M1341[1]	Brazil
BRS 6 Chui	Brazil	EMPASC 101	Brazil	M443[1]	Brazil
BRS 7 Taim	Brazil	EMPASC 102	Brazil	M1410[1]	Brazil
BRS AG	Brazil	EMPASC 103	Brazil	Basmati 370	Pakistan
BRS Agrisul	Brazil	EMPASC 104	Brazil	Bluebelli	USA
BRS Atalanta	Brazil	EMPASC 105	Brazil	Brilhante	Chile
BRS Bojuru	Brazil	EPAGRI 106	Brazil	Cacho Grande	Brazil
BRS Firmeza	Brazil	EPAGRI 107	Brazil	Carnaroli	Italy
BRS Formoso	Brazil	EPAGRI 108	Brazil	Carolina	USA
BRS Fronteira	Brazil	EPAGRI 109	Brazil	Japonês de Várzea	Unknown
BRS Ligeirinho	Brazil	SC 173	Brazil	Jasmine	Thailand
BRS Pampa	Brazil	SC 460	Brazil	Jasmine 85	Thailand
BRS Pampeira	Brazil	SCS 112	Brazil	Koshihikari	Japan
BRS Querência	Brazil	SCS 114 AndoSan	Brazil	Lemont	USA
BRS Sinuelo CL	Brazil	SCS 116 Satoru	Brazil	Meio Chumbinho	Unknown
BRSA 701 CL	Brazil	SCS 117 CL	Brazil	Nowrin Mochi	Unknown
Cachinho	Brazil	SCS 118 Marques	Brazil	Puitá Inta CL	Argentina
IAS 12-9 Formosa	Brazil	SCS 119 Rubi	Brazil	Rexoro	USA
BRS Pelota	Brazil	SCS 121 CL	Brazil	Sambuc	France
Qualimax	Brazil	SCS 115 CL	Brazil	Selenio	Italy
BR IRGA 409	Brazil	SCS BRS 111	Brazil	Soulanet	France
BR IRGA 410	Brazil	IRAT 124	France	Tetep	Vietnam
BR IRGA 412	Brazil	IRAT 162	France	Tomoe Mochi	Japan
BR IRGA 413	Brazil	BRS CIRAD 302	Brazil		
BR IRGA 414	Brazil	SCS BRS Tio Taka	Brazil		
EEA 404	Brazil	OR 63-252	Philippines		
EEA 405	Brazil	MNA PB 0405	Brazil		
IRGA 417	Brazil	Zebu	Brazil		
IRGA 418	Brazil	Guri Inta CL	Brazil		
IRGA 419	Brazil	TOX 514-16-101-1	Nigeria		
IRGA 420	Brazil	Amarelo B	Unknown		
IRGA 424 CL	Brazil	Amaroo	Australia		
IRGA 427	Brazil	Arbório	Italy		
IRGA 428	Brazil	Austral	Unknown		
IRGA 429	Brazil	M1150[1]	Brazil		
BR IRGA 411	Brazil	M1313[1]	Brazil		

[1]Mutant lines.

Morphological traits evaluated

Five plants per row were evaluated for the following twelve morphological rice descriptors and traits: flag leaf width (FLW) and length (FLL), main culm diameter (CD) and length (excluding the panicle) (CL), panicle length (PL) and weight (PW), number of panicles per plant (PP), number of infertile (IGP) and filled grains per panicle (FGP), weight of thousand grains (WTG), caryopsis length (CaL) and width (CaW). Correlation analyses between these traits and Fe content in brown (only husked) (FeB) and polished (FeP) rice grains were performed.

Iron quantification

After drying, the samples of each genotype were divided in two parts, being one part only husked and the other husked and polished for two minutes in a mill tests (SUZUKI, model S21, MT). Next, all samples were milled (MARCONI, model MA020, Piracicaba/SP). In general, the preparation of samples for quantification followed Batista et al. (2014). Approximately 170 mg of milled rice was weighted in a plastic tube (Falcon Corning, Tamaulipas, Mexico) and 1.2 mL of sub-distilled nitric acid (DST-1000, Savillex, EUA) (65% Synth, Brazil) was added. The tubes remained for 48 h at ~25°C, with ocasional shaking. Finally, they were heated (90 °C) for 2 h using a water bath (CIENTEC, model 246, Piracicaba/SP, Brazil). After cooling, the volume of the sample was made up to 14 mL with ultrapure water (MILLIPORE, Milipak Filter Unit, São Paulo/SP, Brazil).

For the Fe quantification, the samples were injected in an inductively coupled plasma - mass spectrometer (ICP-MS Agilent 7900, Hachioji, Japan). Blank samples and reference materials were analyzed in every lot of digestion and quantification. All operational parameters of the equipment are described in Table 2.

The calibration solutions were prepared by diluting the calibration standards of different elements (10mg L^{-1} PerkinElmer, EUA) in HNO_3 5% v/v. The calibration standards ranged from 10-1000 µg L^{-1}.

Statistical analyses

All analyses were carried out with the aid of the software *GENES* (Cruz, 2013). The data were subjected to analysis of variance (ANOVA) by the F test ($p \leq 0.05$ and $p \leq 0.01$). Next, genetic parameters of genotypic and phenotypic variances, the coefficient of experimental variance and broad sense heritability were determined. Rice quantification in brown (FeB) and polished rice (FeP) were transformed through the equation $x=\log_{10}$, aiming to fulfil the ANOVA assumptions.

In the sequence, genotypic and phenotypic correlations were analyzed for the evaluated traits. The significance of the phenotypic correlation was calculated by a t-test (with n-2 degrees of freedom), and the genotypic correlations by a Mantel's test and a bootstrap analysis with 10,000 simulations. A weak degree of multicolinearity (according to the number of conditions 86.42) was found among the variables, thus no variable had to be excluded (Montgomery & Peck, 1981). Even though, a constant k=0.1002 was adopted to minimize its effect. Next, the path analysis was carried out to indicate direct and indirect effects of the genotypic and phenotypic correlations on the morphological traits evaluated on Fe content in brown and polished rice.

Table 2 - Conditions and operational parameters for ICP-MS analysis.

Monitored Isotopes	
Helium Mode (LoD¹ in µg L⁻¹)	^{24}Mg (0.005), ^{31}P (32.9), ^{43}Ca (1.5), ^{52}Cr (0.006), ^{55}Mn (0.04), ^{57}Fe (0.3), ^{59}Co (0.0007), ^{63}Cu (0.01), ^{66}Zn (0.24), ^{75}As (0.11), ^{82}Se (0.25)
Internal standard (all modes)	^{193}Ir 10 µg L^{-1}
Peak pattern / Replicates / Sweeps	3 / 2 / 100
Radio Frequency Power	1550 W
Argon Flow Rate	15 L min^{-1}
Sample uptake / speed / stabilize	8 s / 0.4 rps² / 4 s
Nebulizer pump (acquisition)	0.25 rps (all modes)
Carrier Nebulizer Gas Flow rate	1.07 L min^{-1}
Nebulizer type	Mira Mist™
Spray chamber	UHMI Quartz Spray Chamber
Temperature	2 ºC
Torch (2.5mm) sample depth	8 mm
Interface	Nickel cones
Sampler cone	1.0 mm
Skimmer	0.9 mm
Collision cell	Helium > 99.999 %

¹LoD: Limit of detection; ²rps: rotation per second.

Results

The results of the analysis of variance are summarized in Table 3. All variables showed statistical significance for genotype factor, which evidences expressive genetic variability for this study. In general, the coefficients of heritability verified can be classified as from intermediate to high magnitude (Resende, 1995), ranging from 44.23% to 91.47%. Fe content in brown and polished rice presented heritability of 44.95% and 44.23%, respectively, which were the smallest coefficients observed in this work.

Analysis of phenotypic and genotypic linear correlation between traits were carried out, and the results are presented in Table 4. Although many significant correlations were observed, especial attention is given here to associations between all morphological traits and Fe content in rice. Between Fe in brown and polished grains, there was only found correlation of phenotypic nature, which was of positive direction, but of small magnitude (0.293).

For brown rice, both phenotypic and genotypic significant correlations were found with flag leaf width, panicles per plant, caryopsis length (positive), culm length and caryopsis width (negative). For polished rice, panicle length, panicles per plant, infertile grains per panicle (positive), panicle weight and caryopsis width (negative) were the traits which showed phenotypic and genotypic significant correlations. Thus, considering simultaneously the phenotypic and genotypic linear correlation, panicles per plant (positive) and caryopsis width (negative) were the morphological traits showing significant linear correlation with Fe content in both brown and polished rice.

Table 3. Summary of analysis of variance and estimative of genetic and phenotypic parameters of morphological traits and related to Fe content assayed in 95 rice genotypes grown at Southern Brazil.

SV[1]	DF	Main Squares						
		FLL	FLW	CD	CL	PL	PP	PW
G	94	42.79**	0.06**	0.005**	241.98**	15.45**	12.44**	1.07**
B	2	18.06	0.02	0.0007	35.20	1.65	5.02	0.10
r	188	11.52	0.01	0.002	78.99	4.65	6.73	0.35
μ	-	25.24	1.32	0.35	72.70	22.05	8.70	2.74
CV (%)	-	13.44	10.16	14.07	12.22	9.77	29.81	21.59
$\sigma^2{}_g{}^2$	-	10.42	0.02	0.001	54.33	3.60	4.14	0.24
$\sigma^2{}_p{}^2$	-	14.26	0.01	0.001	80.66	5.15	1.90	0.35
H^2 (%)	-	73.06	72.01	55.51	67.35	69.90	45.82	67.24
		FGP	IGP	WTG	CaL	CaW	FeB	FeP
G	94	2114.4**	480.95**	11.72**	2.25**	0.52**	0.01**	0.04**
B	2	463.30	380.97	3.67	0.03	0.24	0.16	0.62
r	188	570.37	140.27	5.13	0.39	0.14	0.10	0.25
μ	-	95.55	25.06	26.07	9.22	2.74	4.10	3.58
CV (%)	-	24.99	47.24	8.69	6.83	13.74	2.48	4.46
$\sigma^2{}_g{}^2$	-	514.69	113.55	18.36	0.61	0.17	0.002	0.006
$\sigma^2{}_p{}^2$	-	704.82	160.31	20.07	0.75	0.12	0.006	0.01
H^2 (%)	-	73.025	70.83	91.47	82.37	72.65	44.95	44.23

[1]SV – source of variation; DF – degrees of freedom; G – genotype; B – block; r – residue; μ–average; CV – coefficient of variation (%); $\sigma^2{}_g{}^2$ – genetic variation; $\sigma^2 p^2$ – phenotypic variation; H^2 – broad sense heritability (%); FLL – flag leaf length; FLW - flag leaf width; CD - culm diameter; CL - culm length; PL - panicle length; PP - number of panicles per plant; PW - panicle weight; FGP – number of filled grains per panilcle; IGP - number of infertile grains per panicle; WTG - weight of thousand grains; CaL - caryopsis length; CaW – caryopsis width; FeB - Fe content in brown rice; FeP – Fe content in polished rice grains. **significant at p ≤ 0.01 by the F test.

Table 4. Phenotypic and genotypic coefficients of correlation for 14 traits assayed in 95 rice genotypes grown at Southern Brazil.

Trait	Rf[1]													
	FLL	FLW	CD	CL	PL	PP	PW	FGP	IGP	WTG	CaL	CaW	FeB	FeP
FLL		0.1	0.1	0.1	0.3**	0.0	-0.1	-0.2	0.4**	0.1	-0.1	0.0	0.0	0.2
FLW	0.0		0.3**	-0.1	0.5**	0.1	0.3**	0.3**	0.4**	-0.2	0.3**	-0.3**	0.3**	0.2
CD	0.1	0.3		0.4**	0.1	-0.2	0.4**	0.1	0.3**	0.3**	0.1	0.2	0.1	-0.1
CL	0.1	-0.1	0.5^{+}		0.1	-0.4**	0.2*	0.0	0.1	0.3**	-0.2	0.5**	-0.3**	-0.3**
PL	0.3	0.5^{+}	0.2	0.1		0.1	0.4**	0.4**	0.4**	-0.2	0.4**	-0.3**	0.2	0.3**
PP	-0.1	0.3	-0.3	-0.6^{++}	0.2		-0.2*	0.0	-0.1	-0.4**	0.2	-0.5**	0.3**	0.3*
PW	-0.1	0.3	0.5^{+}	0.2	0.4^{+}	-0.4		0.7**	0.0	0.2	0.1	0.2	-0.1	-0.3**
FGP	-0.3	0.4^{+}	0.0	-0.0	0.4^{+}	0.1	0.6^{++}		-0.0	-0.4**	-0.2	-0.2	0.1	-0.2
IGP	0.4^{+}	0.4^{+}	0.4^{+}	0.1	0.5^{++}	-0.1	0.1	0.0		-0.1	0.2	-0.2*	0.1	0.2*
WTG	0.1	-0.2	0.3^{+}	0.4^{+}	-0.2	-0.6^{++}	0.3	-0.5^{++}	-0.1		0.2*	0.6**	-0.2*	-0.2*
CaL	-0.1	0.3	0.2	-0.1	0.5^{++}	0.4^{+}	0.2	-0.2	0.2	0.2		-0.4**	0.3**	0.3*
CaW	0.1	-0.4^{+}	0.2	0.5^{++}	-0.4^{+}	-0.8^{++}	0.2	-0.3	-0.3	0.7^{++}	-0.5^{++}		-0.4**	-0.4**
FeB	-0.1	0.6^{+}	0.2	-0.5^{+}	0.3	0.8^{++}	-0.1	0.1	0.2	-0.3	0.6^{++}	-0.6^{++}		0.3*
FeP	0.4	0.3	-0.1	-0.3	0.5^{+}	0.6^{+}	-0.5^{+}	-0.2	0.4^{+}	-0.3	0.4	-0.6^{++}	0.5	

Rg

[1]Rf – Phenotypic coefficient of correlation; Rg - genotypic coefficient of correlation; FLL – flag leaf length (cm); FLW - flag leaf width (cm); CD - culm diameter (cm); CL - culm length (cm); PL - panicle length (cm); PP - number of panicles per plant; PW - panicle weight (g); FGP – number of filled grains per panilcle; IGP - number of infertile grains per panicle; WTG - weight of thousand grains (g); CaL - caryopsis length (cm); CaW - caryopsis width (cm); FeB - Fe content in brown rice (ng g^{-1}); FeP – Fe content in polished rice grains (ng g^{-1}). **, *: Significant at p ≤ 0.01 and p ≤ 0.05, according to t test; $^{+}$, $^{++}$: significant at p ≤ 0.01 and p ≤ 0.05, according to Mantel's test. For a better visualization coloring of the significant coefficients followed the classification of Pearson's coefficients of correlation revised by Mukaka (2012).

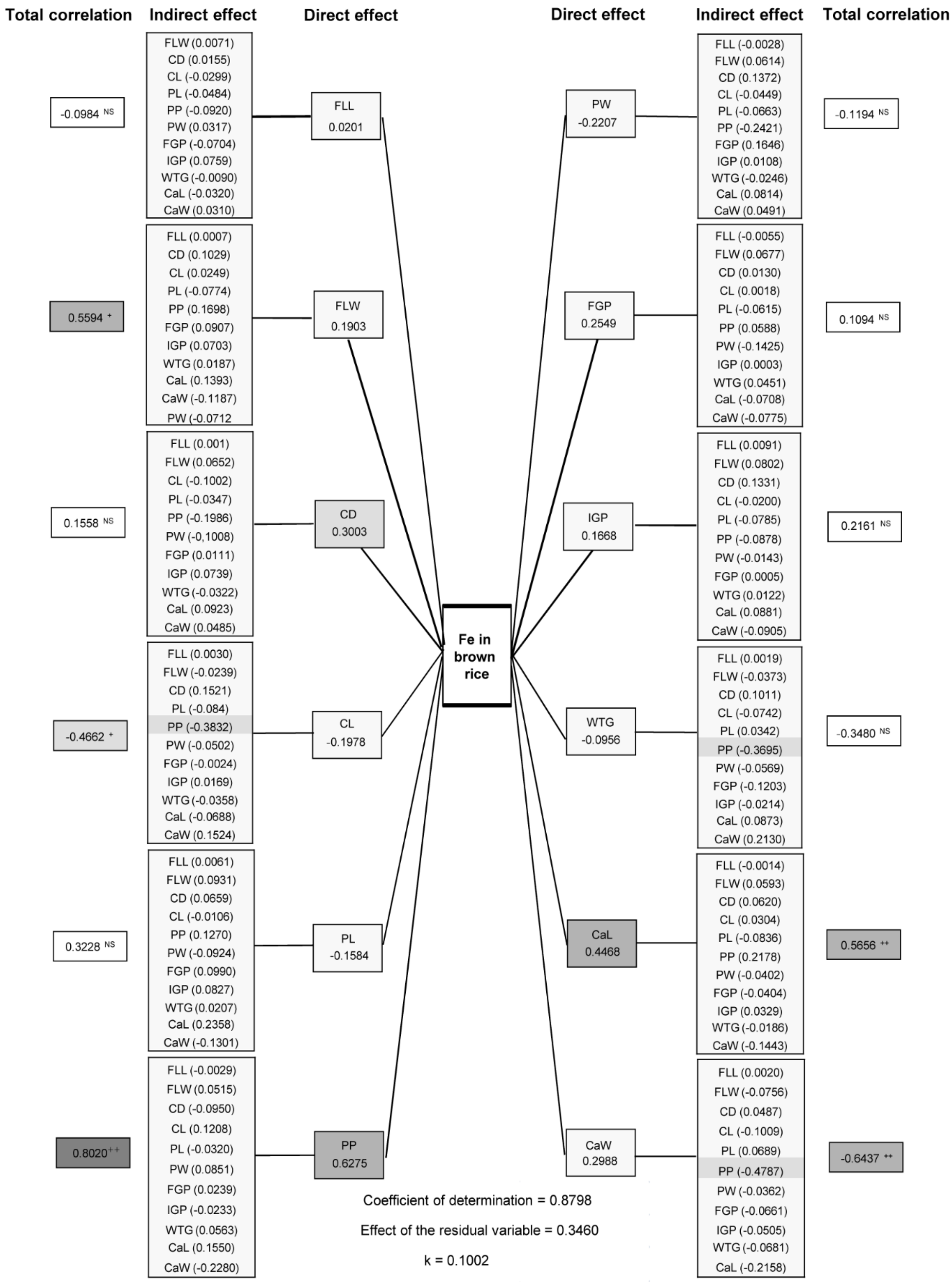

Figure 1 - Estimative of the direct and indirect effects of the path analysis coeficients of correlation from the phenotypic and genotypic correlations on the trait Fe in brown rice grains. FLL – flag leaf length (cm); FLW - flag leaf width (cm); CD - culm diameter (cm); CL - culm length (cm); PL - panicle length (cm); PP - number of panicles per plant; PW - panicle weight (g); FGP – number of filled grains per panilcle; IGP - number of infertile grains per panicle; WTG - weight of thousand grains (g); CaL - caryopsis length (cm); CaW - caryopsis width (cm). $^{+}$, $^{++}$: significant at $p \leq 0.05$ and $p \leq 0.01$, according to Mantel's test. ^{NS}: Not significant. For a better visualization coloring of the coefficients followed the classification of Pearson's coefficients of correlation revised by Mukaka (2012).

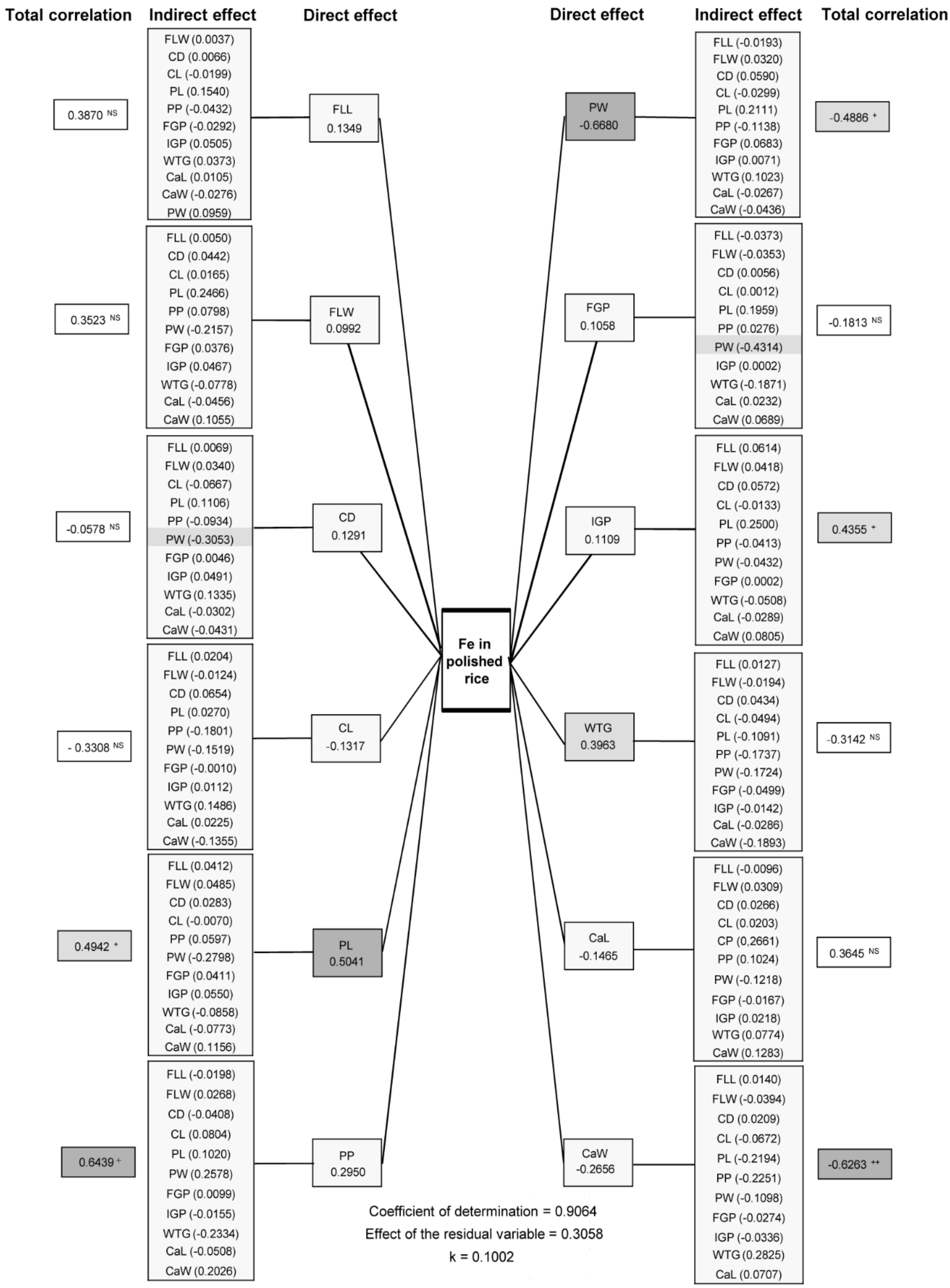

Figure 2 – Estimative of the direct and indirect effects of the path analysis coeficients of correlation from the phenotypic and genotypic correlations on the trait Fe in polished rice grains. FLL – flag leaf length (cm); FLW - flag leaf width (cm); CD - culm diameter (cm); CL - culm length (cm); PL - panicle length (cm); PP - number of panicles per plant; PW - panicle weight (g); FGP – number of filled grains per panicle; IGP - number of infertile grains per panicle; WTG - weight of thousand grains (g); CaL - caryopsis length (cm); CaW - caryopsis width (cm). $^{+}$, $^{++}$: significant at $p \leq 0.05$ and $p \leq 0.01$, according to Mantel's test. ^{NS}: Not significant. For a better visualization coloring of the coefficients followed the classification of Pearson's coefficients of correlation revised by Mukaka (2012).

In order to better understand the real association between traits under analysis, finding secondary traits, i.e., of indirect effect which have also to be taken into account in the indirect selection approach, *Path analysis* (Wright, 1923) was applied. As the coefficient of determination of the model for the phenotypic effect showed low magnitude, only the genotypic correlation was considered in this study (Figures 1 and 2). In general, the genotypic correlations between traits showed both negative and positive effects with different magnitudes, allowing to identify critical secondary traits. The estimative of the direct and indirect effect of the analyzed traits on the main variable Fe in brown rice evidenced panicles per plant as the most influent trait, with a high direct effect (0.6275), illustrating a true association between these traits, being caryopsis width the variable with the most expressive secondary effect on the path (-0.2280). Secondly, caryopsis width, which has shown a negative linear (total) correlation of strong magnitude on Fe in brown rice, presented a considerable direct positive effect (0.2988) and panicles per plant (-0.4787) and caryopsis length (-0.2158) presented the most significant indirect impact. Other traits, such as culm and caryopsis length and flag leaf width also deserve consideration, as well as the traits which influenced them indirectly.

The results of the path analysis for Fe in polished rice as a function of explanatory traits also evidenced both positive and negative associations between the variables. Panicles per plant expressively contributed in a direct form (0.2950) and was influenced indirectly via panicle weight (0.2578) and caryopsis width (0.2026). Caryopsis width showed a direct negative effect (-0.2656) similar to the indirect effects via panicles per plant (-0.2251) and panicle length (-0.2194). Another expressive trait, panicle weight, which has shown significant negative correlation with Fe in polished rice, presented the highest direct effect of this study (-0.6680) on the main variable, which highlight the relationship between these traits. On the other hand, number of infertile grains per panicle showed the smallest direct effect on the main variable, thus all the other traits contribuited to the total correlation observed.

Discussion

The present study aimed to investigate the association between a set of morphological traits, mostly of agronomic importance, and Fe content in rice grains, with the final objective of finding among the former the most promising ones for use in indirect selection strategies in breeding programs aiming iron biofortification of rice. Indirect selection is an approach in which the selection is performed on one or more secondary traits with the final aim to improve another main trait, based on the correlation between them. Before carrying out this approach, many aspects about the traits have to be firstly considered, being the heritability one of them.

Heritability can be defined as the degree of correspondence between the phenotype and the breeding value of an individual for a given trait (Hallauer et al., 1988). It is influenced, at least in part, by the amount of loci controlling a given trait, and once known, helps the selection of this and other associated traits (Amorim et al., 2008; Silva et al., 2011). Its coefficient is considered low when between 0.01 and 0.15, intermediate when between 0.15 and 0.50 and high when the values are higher than 0.50 (Resende, 1995). The coeficients of heritability of Fe in brown and polished rice were only of intermediate magnitude, however, most of the other studied traits showed a high heritability, illustrating the potential application of the indirect selection aiming improvement in Fe grain content in the studied genotypes.

Despite the valuable information obtained through the analysis of linear correlation, it has to be considered that it is only a method of simple linear association (Vencovsky & Barriga, 1992), thus due the importance of the cause and effect relationships, the path analysis (Wright, 1923) was carried out separately for each Fe content trait, i.e., Fe in brown and in polished rice. This analysis allows identifying which traits have a more pronounced direct or indirect effect on a primary (main) trait. Moreover, allows a more profound analysis regarding the influence of the traits present in the study, that can help to explain the existence of positive and negative correlations, with different magnitudes among the studied variables (Silva et al., 2005).

Among the possible causes of the correlation between traits is pleiotropy, a phenomenon in which one gene defines the expression of two or more traits, and linkage disequilibrium among pairs of genes, which can be maintained along many generations (Carmona et al., 2015). In case of grain quality traits of rice, they exhibit complex relationships mainly due to the mentioned genetic forces, making most of the time, the breeding process an arduous task. It is clearly illustrated in this study, when the genotypic correlation between Fe in brown and polished rice was not significant, justifying the analysis and further employment of other traits in more elaborated breeding schemes.

In addition to a strong association and high heritability, a trait has a promising use in indirect selection if it is cheaper, easier and time-saving to evaluate in comparison to the main trait or even if the trait is important *per se*, i.e., agronomically relevant, so breeding would lead to improvements in more than one trait simultaneously (Carvalho et al., 2004). Taking all these criteria into account, the most promising traits to be used in indirect selection aiming Fe biofortification in rice are panicles per plant and caryopsis width, i.e., when using the studied germplasm as base.

Panicles per plant presented the highest coefficient of linear correlation with both brown and polished rice, in the positive direction, and also showed one of the highest direct effects in the path analysis, being thus, less affected by other traits, showing a true association with the main traits (Vencovsky & Barriga, 1992). In terms of convenience, it is a yield component, therefore using it for selection would possibly benefit the productive potential of the genotype, and it is considerably cheap to evaluate, as well as simple and fast. The only drawback of using panicles per plant when breeding using this germplasm as the base, is the heritability verified, which was only of intermediate magnitude, as it is a polygenic trait and thus influenced by the environment (Rebolledo et al., 2016).

Caryopsis width has also been shown as a convenient trait for indirect selection. The coefficient of correlation observed was negative, indicating that the narrower the grain, the higher the Fe content in rice. Considering that in Brazil the more consumed kind of grain is the long and thin type, the so-called “agulhinha” (CONAB, 2015; Streck et al., 2017), breeding programs can maintain their ideotype of plant, thus, not being necessary to change and selecting for other grain types when targeting higher Fe content. Oppositing to panicles per plant, caryopsis width presented high coefficient of heritability, however, smaller direct effect in the path analysis, thus making the indirect selection more complicated, as other traits have to be considered simultaneously.

After uptaken by roots, Fe is transported to leaves and other storage organs (Gao et al., 2016; Kim & Guerinot, 2007). The association among the flag leaf and Fe content in rice grains was here found and has been already documented, e.g., it was already verified the remobilization of Fe from the flag leaf to rice grains (Sperotto et al., 2010). On the other hand, however, another study verified that removing the flag leaf did not have effect on the Fe content in rice grains (Sperotto et al., 2013). Thus, further research is required before moving efforts to improve flag leaf size with the final objective of Fe biofortification in this cereal.

The lenght of the caryopsis was also associated, in this case positively, with higher Fe content, especially in brown rice. This correlation was previously verified by Sellappan et al. (2009) and studies carried out by Zhang et al. (2004) on a rice genotype with black pericarp have also found positive correlations between caryopsis lenght and Fe content in grain. Other traits, which deserve attention in this study and in breeding programs, are culm length and panicle length. The results found here suggest that breeding for smaller plants, which has been already a target worldwide for decades, as well as for longer panicles, which can contribuite to yield potential, can also contribuite to the increase of Fe content in rice grains. All these results encourage breeding Fe biofortified rice, as this improvement would not impair most of other important breeding targets.

In this regard, some exceptions found here were for number of infertile grains per panicle and panicle weight, which associated with higher Fe content but at the same time usually in an opposite way to yield, thus, must not be considered in indirect selection. Given the importance of the approach but also the possible drawbacks applying indirect selection, similar work has been already carried out on rice in other countries (Anandan et al., 2011; Ajmera et al., 2017; Kalyan et al., 2017; Rathod et al., 2017; Sowmiya & Venkatesan, 2017). Differences in the results reported in these studies were found, which can be mainly attributed to the different germplasms assayed, but also environmental conditions.

Other breeding approaches have been applied aiming Fe biofortification in rice. Efforts have been carried out, for example, in the development of transgenic plants, and different genes were assayed. Even though the strategies using genetic transformation resulted in the increase of Fe content, milling is still responsible for many losses (revised by Santos et al., 2017). Thus, the conventional selection of genotypes with high Fe content followed by hybridization continue to be an interesting alternative. It is important to emphasize that mineral content in rice grain are quantitative traits, strongly affected by the environment, thus the indirect selection can assist this process. Although important research has been carried out worldwide, to the best of our knowledge, this is one of the largest studies of this kind using Brazilian elite rice germplasm, which emphasizes the importance of this work.

Conclusion

Panicles per plant and caryopsis width are the most promising traits for use in indirect selection aiming Fe biofortification in brown and polished rice, however, in the case of the latter, traits of secondary effect have to be considered. Other traits, such as caryopsis length also deserve attention in breeding programs with this objective.

Acknowledgments

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Brazil, under grant no. 401902/2016-1. CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) also provided fellowships for the investigators and students. This work is part of the dissertation (Master degree in Sciences) of the first author.

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