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Genetics/ Original Article

Identification of rice mutant families with chilling tolerance

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Abstract – The objective of this work was to characterize chilling tolerance in rice mutant families of the M$_4$ generation, at the seedling stage. Two experiments were carried out: chilling tolerance was evaluated in 43 mutant families, in the 'BRS Querência' original genotype, and in 19 commercial genotypes. In Experiment II, 8 mutant families from Experiment I, 'BRS Querência', and a mutant of the M$_5$ generation were tested. In both experiments, seedlings were evaluated under two conditions: 10°C for seven days and 25°C for seven days. In Experiment I, the induced mutations in rice led to varied responses in chilling tolerance traits, with some M$_4$ mutant families outperforming the original genotype. Experiment II highlighted the impact of mutations on chilling-tolerance, particularly in terms of leaf discoloration and plant recovery. Mutant families of the M$_4$ generation differ from the original genotype 'BRS Querência' in chilling tolerance at the seedling stage. The mutant families M36, M54, and M56 and 'BRS Querência' show genetic similarity, indicating a lack of chilling tolerance during the seedling stage. The mutant families M17, M21, M22, and M26 are promising for rice breeding programs because they present chilling tolerance. The M30 mutant family exhibits the best performance for all analyzed traits, indicating chilling tolerance at the seedling stage.

Index terms: *Oryza sativa*, genetic variability, induced mutation, low temperatures, mutagenic.

Identificação de famílias mutantes de arroz com tolerância ao frio

Resumo – O objetivo deste trabalho foi caracterizar a tolerância ao frio em famílias mutantes de arroz na geração M$_4$, no início do estágio vegetativo. Foram realizados dois experimentos. No experimento I, a tolerância ao frio foi avaliada em 43 famílias mutantes, no genótipo original 'BRS Querência' e em 19 genótipos comerciais. No Experimento II, 8 famílias mutantes do Experimento I, 'BRS Querência' e um mutante proveniente da geração M$_5$ foram testados. Em ambos os experimentos, as plântulas foram avaliadas em duas condições: 10°C por sete dias e 25°C por sete dias. No Experimento I, as mutações induzidas em arroz levaram a respostas variadas nas características de tolerância ao frio, com algumas famílias mutantes M$_4$ tendo superado o genótipo original. O Experimento II destacou o impacto das mutações na tolerância ao frio, particularmente em termos de descoloração das folhas e recrescimento da planta. As famílias mutantes no estágio M$_4$ diferem do genótipo original 'BRS Querência' em tolerância ao frio, no início do estágio vegetativo. As famílias mutantes M36, M54 e M56 e 'BRS Querência' apresentam similaridade genética, o que indica falta de tolerância ao frio durante o início do estágio vegetativo. As famílias mutantes M17, M21, M22 e M26 são promissoras para programas de melhoramento genético de arroz, pois apresentam tolerância ao frio. A família mutante M30 apresenta o melhor desempenho em todas as características analisadas, o que indica tolerância ao frio no início do estágio vegetativo.

Termos para indexação: *Oryza sativa*, variabilidade genética, mutação induzida, baixas temperaturas, mutagênico.

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Introduction

Although rice (*Oryza sativa* L.) is considered a staple food for the global population (Vinci et al., 2023), its crop constantly faces constraints that cause yield losses. Abiotic stresses, such as low temperature or chilling stress, nutritional deficiency, iron toxicity, submergence, and drought create a suboptimal environment for rice production (Jeyasri et al., 2021). Temperature is undoubtedly one of the major factors that control rice distribution across the globe, since it is originated from tropical or subtropical areas, the reason rice is sensitive to chilling temperatures (Li et al., 2022).

When plants are subjected to low temperatures, damages are observed at all growth and development stages (Luo et al., 2022); however, at early growth stages, plants are most affected by cold weather, especially during germination and initial seedling stages (Unan et al., 2022). Chilling stress at the early seedling stage leads to wilting, yellowing, or withering leaves, also causing delayed seedling emergence, retarded plant development, yield loss, and poor grain quality. Therefore, chilling tolerance at the seedling stages is considered an important target in rice breeding programs (Ham et al., 2021).

The success of rice breeding programs depends on genetic variability among parental genotypes (Akter et al., 2022), presenting germplasm that can tolerate chilling stress, with a wide range of variations in chilling tolerance. However, rice breeding programs are challenging for various reasons: japonica rice subspecies are more tolerant to chilling stress than indica rice (Glaszmann et al., 1990; Thapa et al., 2020), but crosses between them usually present spikelet sterility due to cross incompatibility (Akter et al., 2022); in addition, a linkage between related genes to chilling tolerance and low amylose content is suggested (Magalhães, 2003), but low amylose from japonica rice is undesirable to Brazilian consumers, who prefer indica rice with high amylose content and its specific culinary and industrial grain quality traits (Streck et al., 2018). Another challenge for developing new chilling-tolerant genotypes is its polygenic nature (Yongbin et al., 2023).

Therefore, mutations can be used to create genetic variability and support functional genomics studies (Hernández-Soto et al., 2021), but spontaneous mutation rates in higher plants are low, ranging from 10^{-5} to 10^{-8} (Jiang & Ramachandran, 2010). Consequently, mutagenesis became a valuable strategy to increase mutation frequency and to support the development of novel genotypes (Luz et al., 2016; Hernández-Soto et al., 2021), and many studies show success in DNA changes mediated by mutagenesis, presenting the improvement of desirable agronomics traits in rice (reviewed in Viana et al., 2019).

The objective of this work was to characterize chilling tolerance in M_4 mutant rice families at seedling stage.

Materials and Methods

The mutant families of the studied rice belong to the germplasm collection of the Plant Genomics and Breeding Center at Universidade Federal de Pelotas and were obtained using 0.15 M ethyl methanesulphonate (Luz et al., 2016). The treated seeds were sown in the experimental field at Terras Baixas Experimental Station (ETB), located at Embrapa Clima Temperado, in the municipality of Capão do Leão, in the state of Rio Grande do Sul, Brazil (31.48ºS, 52.24ºW, at 15 m of altitude) to advance generations until M_3. In the M_3 generation, the evaluations were conducted for agronomic traits and chilling tolerance at the germination stage. Only families that demonstrated chilling tolerance during germination in the M_3 generation had the experiment advanced. The generation advances of the families to M_4 and M_5 were conducted in a greenhouse at ETB, in 2016. Experiments I and II were conducted to identify the mutant families with genetic variability to chilling tolerance.

In Experiment I, a total of 43 M_4 mutant families were evaluated, among which are the genotypes: 'BRS Querência', the original one; 'Nourin Mochi', a chilling-tolerant one; 'BR/IRGA 409', a chilling-sensitive one; and other 17 commercial ones. Three replicates of five seedlings each were used, following a completely randomized design.

In Experiment II, the eight M_4 mutant families that presented contrasting results to chilling tolerance in Experiment I were evaluated, they were: 'BRS Querência' and the M5 16A3 mutant of M_5 generation. Four replicates of five seedlings each were employed. Additionally, a control treatment with plants kept at 25°C was carried out, and seedling evaluations were also conducted at days 14 and 28. The methodology to obtain the seedlings was consistent for both experiments and followed specific steps.

At day 0, seeds were placed in a plastic tray with filter paper moistened with distilled water in BOD at 25°C. At day 7, seedlings were selected and transferred to a pot containing 1.5 L of modified Yoshida nutrient solution, according to Singh et al. (2010) methodology, in BOD at 25°C. At day 14, the Yoshida nutrient solution was renewed, and the seedlings were transferred and subjected to chilling exposure in BOD at 10°C. For Experiment II, seedling evaluation was performed. At day 21, the Yoshida nutrient solution was renewed again, and the seedlings were transferred to a 25°C environment (BOD) for a recovery period. At day 28, seedling evaluation was conducted for both Experiments I and II.

The relative performance (RP) of the traits evaluated in Experiment II was calculated using the following equation: $\mathrm{RP} = \bar{X}_{10°C} / \bar{X}_{25°C} \times 100$, where $\bar{X}_{10°C}$ is the mean of the variable after chilling recovery and $\bar{X}_{25°C}$ is the mean of variable in the control treatment.

The recovery evaluation (RE) was obtained with the equation: RE = a - b, where a is the evaluation after the recovery period on day 28 and b is the one after the chilling exposure on day 14.

Data from both experiments were subjected to residual analysis to verify normality and variance homogeneity to test if data transformation was necessary, which indicated the need to transform leaf discoloration after the recovery period in both experiments through the equation $\sqrt{x + 1/2}$. The other traits presented a normal distribution and were not transformed. Then, the data were subjected to analysis of variance at $p \leq 0.01$, and to Scott-Knott’s mean-value grouping test at $p \leq 0.01$.

Pearson’s correlation coefficient was used to establish the degree of association between evaluated characters, and, subsequently, the data were subjected to principal component analysis (PCA). The groups formed in the PCA were defined based on the following traits: leaf discoloration after recovery period, seedling survival rate, root length relative performance, root recovery, shoot length relative performance, and shoot recovery, after which the Euclidean distance was calculated, followed by lineage grouping using the UPGMA method. The number of clusters formed was defined by K-Means from the sum of squares within the cluster (WSS). The analysis was performed using the SAS Studio Software (SAS Institute Inc., Cary, NC, USA), Genes program (Cruz, 2013), PAST 3.18 and Orange Data Mining (Demsar et al., 2013).

Results and Discussion

There was variability among genotypes collected under stress for the following traits in Experiment I: leaf discoloration after recovery period, root length, and shoot length; and for Experiment II, the traits were: leaf discoloration after the recovery period, seedling survival rate, root and shoot relative performance, and root and shoot recovery (Table 1).

In Experiment II, the traits leaf discoloration after chilling exposure, capacity for shoot recovery leaf discoloration, and leaf discoloration of the control did not show significant differences. In the control treatment group, all the seedlings exhibited the same scale of one for green plants, as expected. These findings are consistent with a previous study that identified QTLs linked to rice chilling tolerance, particularly evident during repeated chilling stress cycles and recoveries, rather than immediately after the first period of chilling stress (Li et al., 2023), whose symptoms become more evident after temperature stabilization, during the evaluation of chlorophyll levels (Cruz et al., 2007). In seedlings, a reduction in chilling stress symptoms occurred in the second evaluation after plant recovery.

In Experiment I, a significant response to chilling after the recovery period was observed for the traits: leaf discoloration after recovery period, root length, and shoot length among the 43 mutant families, as well as in the comparison with 'BRS Querência', 'Nourin Mochi', 'BR/IRGA 409', and the 17 other commercial genotypes (Table 2). In all analyzed traits, there were families with higher averages than the original genotype, 'BRS Querência', which suggested that the induced mutation effectively altered the genotypes of the mutant families. Previous studies utilizing the tilling technique to detect mutants have demonstrated that chemically induced mutations can be obtained with the EMS treatment (Luz et al., 2021; Henry et al., 2014).

Regarding leaf discoloration after recovery period, 48.83% of the mutant families presented absolute mean value higher than 'BRS Querência' and they were clustered in groups C and D. Nevertheless, 41.86%

and 18.60% of the mutant families were clustered in group A for root and shoot length, respectively, being 'BRS Querência' found in group D. Different to other traits, leaf discoloration indicates chilling tolerance when the values are lower. Rice plants exposed to low temperatures show leaf discoloration or leaf rolling to chilling stress. Therefore, leaf discoloration has been used as an efficient trait for the selection of chilling-tolerant genotypes in rice (Ham et al., 2021; Akter et al., 2022).

Table 1. Mean squares and coefficients of variation for the traits evaluated of mutant rice families of M_4 generation, 'BRS Querência' and 19 commercial genotypes in Experiment I, as well as 'BRS Querência' and M516A3 of M_5 generation in Experiment II.

Source of variation	DF	Experiment I(1)			DF	Experiment II(1)								
		LDR	RL	SL		LDR	LDE	CLR	LDC	SS	RLP	RR	SLP	SR
Genotypes	62	0.78(2)	6.76(2)	34.92(2)	9	0.90(2)	0.14(3)	0.49(3)	0.00	1,737.78(2)	276.04(2)	1.60(2)	332.49(2)	4.89(2)
Error	126	0.14	1.03	3.90	30	0.09	0.06	0.07	0.00	178.34	32.47	0.11	81.59	0.23
Overall mean	-	2.34	6.74	18.88	-	2.00	1.38	1.59	1.00	74.50	64.75	1.40	71.64	2.34
CV (%)	-	16.18	15.13	10.47	-	15.62	18.05	17.32	0.00	17.92	8.80	24.19	12.60	20.54

(1)LDR, leaf discoloration after recovery period; RL, root length; SL, shoot length; LDE, leaf discoloration after chilling exposure; CLR, capacity to shoot recovery the leaf discoloration; LDC, leaf discoloration of the control; SS, seedling survival rate; RLP, root length relative performance; RR, root recovery; SLP, shoot length relative performance; SR, shoot recovery. (2)Significant at p ≤0.01; (3)Nonsignificant.

Table 2. Clustered means for chilling tolerance evaluated traits in 43 rice mutant families at M_4 generation, 'BRS Querência' and 19 commercial genotypes(1).

Group	Higher mean per family	Lower mean per family	Identification of the family in descending order according to the average
			Leaf discoloration after recovery period
A	3.08	2.72	BRS Sinuelo CL, IRGA 428, IRGA 426, IRGA 417, BRS Atlanta, M57, BR IRGA 409, Epagri 108, BRS Fronteira, BRS Pelota, SCSBRS Tio Taka, M36, CICA8, M59, M54, M58, M53
B	2.61	2.27	M61, M37, M31, M25, IR 8, M4, BRS 6 Chuí, M43, M42, M62, BRS Firmeza, M39, M35, M63, M56, M38, M51, M40, BRS Querência, M23, M50
C	2.19	1.87	M32, M29, M21, M55, M41, M28, M47, M45, M34, M33, M27, M24, M20, M18, HSC16 I, M22, M19
D	1.81	1.22	M26, M16, M30, M17, Nino, Nipponbare, IAS-12-9-Formosa, Nourin Mochi
			Root length
A	10.59	7.12	M21, M26, M32, M23, M17, Nipponbare, M27, IAS-12-9-Formosa, M30, M38, M31, Nourin Mochi, M19, CICA8, M29, M37, M22, Nino, M18, M20, BR IRGA 417, M35, M24, M28
B	7.00	5.77	M42, M45, M39, M34, BR IRGA 409, M16, BR IRGA 426, M33, BRS Pelota, M62, Epagri 108, M40, M58, BR IRGA 408, BRS Firmeza, M41, M63, SCSBRS Tio Taka, HSC16 I, M36, M43
C	5.63	4.11	M61, IR 8, M4, M47, BRS 6 Chuí, M25, M55, M56, M53, BRS Querência, BRS Atlanta, M50, M54, M51, BRS Sinuelo CL, M57, M59, BRS Fronteira
			Shoot length
A	25.60	22.13	IAS-12-9-Formosa, Nipponbare, M30, M19, M27, M21, M31, M26, Nino, Nourin Mochi, M22, M17
B	21.81	19.30	M43, M18, M20, M23, M47, M29, M58, M50, M45, M25, HSC16 I, M59, M38, M51, M62, M16, M55, CICA 8, M37
C	18.73	15.56	BRS Firmeza, M63, M32, M39, BRS Sinuelo CL, M24, M35, M57, M56, IRGA 417, M53, IRGA 426, M61, M34, BRS Querência, M54, IRGA 409, M42, M41, M28, M33, M36, BRS Pelota
D	14.96	13.00	IRGA 428, BRS 6 Chuí, BRS Fronteira, M40, Epagri 108
E	12.47	9.87	SCSBRS Tio Taka, M4, BRS Atlanta, IR 8

(1)No significant difference between mutant families into the same group by Scott-Knott’s test at 1% probability.

The japonica genotypes 'Nino', 'Nourin Mochi' and 'Nipponbare' were grouped in group D for leaf discoloration, and in group A for shoot length and root length. Japonica subspecies are more tolerant to chilling than indica ones (Glaszmann et al., 1990; Feng et al., 2023), because japonica genotypes accumulate more chlorophyll under low temperatures, leading to higher carbohydrate accumulation for grain production, as well as enhancing shoot growth. In the present study, carbohydrate accumulation was not measured, but genotypes showed weak development (Table 2), which indicates that chilling tolerance might have different mechanisms (Fukuda & Terao, 2015). Some not discolored genotypes tolerate the cold stresses better than other ones, but grow more slowly, which means, on the other hand, that not always they could be the better field varieties. The genotype 'BR/IRGA 409', known as chilling-sensitive one, clustered in the groups with lower means for all traits analyzed.

In Experiment II, there was a significant response to leaf discoloration after the recovery period for the M_4 mutant families analyzed, and only M36, M54 and M56 showed lower means, repeating the results found in Experiment I (Table 3). This suggests that genetic changes improving the performance under chilling were obtained by the mutation-inducing technique.

The M_4 mutant families M21, M26, M30, M22, M17, and the M_5 M5 516A3 mutant were able to regrow after low-temperature exposure, as detected in traits such as seedling survival rate as well as root and shoot recovery. In contrast, the mutant families M56, M36, M54, and 'BRS Querência' did not show this ability. However, only the M30 mutant was clustered in group A for shoot length relative performance and root relative performance, along with M22 and M17 mutants. The traits shoot and root recovery consistently produced positive results, indicating that Evaluation A, after the recovery period, yielded superior results compared to Evaluation B, after chilling exposure, which suggests that seedlings can resume growth after exposure to low temperatures. Similar results were found for the trait root regrowth in studies of chilling tolerance in rice, in which improved chilling tolerance was achieved through root regrowth, as evidenced by the difference between after the recovery period and after the chilling exposure measurements (Cruz et al., 2007; Pouramir Dashtmian et al., 2013; Lone et al., 2022).

The M5 516A3 mutant is a chilling-tolerant genotype during the germination period, whose mutation was identified by tilling in the *Os03g0103300* gene (Luz et al., 2021). However, it was clustered in the groups of low averages for the other analyzed traits, except leaf discoloration (Table 3), which after recovery period and chilling tolerance at different growth stages are controlled by independent genes (Li et al., 2023). This mutant has been recently registered as a breeding line by Universidade Federal de Pelotas at Ministério da Agricultura, Pecuária e Abastecimento in Brazil, named 'LFAEM140'.

In Experiments I and II, negative correlations were observed between leaf discoloration after recovery

Table 3. Grouped means for chilling tolerance evaluated traits in eight rice mutant families at M_4 generation, 'BRS Querência' and M5 516A3 of M_5 generation$^{(1)}$.

Group	Higher mean per family	Lower mean per family	Identification of the family in descending order according to the average
			Leaf discoloration after recovery period
A	2.64	2.44	M56, M36, M54
B	2.23	1.99	BRS Querência, M17, M21
C	1.83	1.22	M26, M22, M5 516A3, M30
			Seedling survive rate
A	100.00	80.00	M21, M26, M30, M22, M17
B	72.50	45.00	M5 516A3, BRS Querência, M54, M36, M56
			Root length relative performance
A	2.64	2.44	M30, M22, M17
B	2.23	1.99	BRS Querência, M54, M21, M56, M26
C	1.83	1.22	M5 516A3, M36
			Root recovery
A	2.64	2.44	M17, M30
B	2.23	1.99	M21, M26, M22, M5 516A3, M56
C	1.83	1.22	BRS Querência, M36, M54
			Shoot length relative performance
A	2.64	2.44	M30
B	2.23	1.99	M17, M26, M21, M56, M22, M54, M5 516A3, M36, BRS Querência
			Shoot recovery
A	2.64	2.44	M21
B	2.23	1.99	M30, M17, M5 516A3, M22
C	1.83	1.22	M26
D	1.83	1.22	BRS Querência, M36, M56, M54

$^{(1)}$No significant difference between mutant families into the same group by Scott-Knott's test at 1% probability.

period, shoot length, and root length, while positive correlations were noted between shoot length and recovery and root length and recovery (Figures 1 and 2). These findings suggest that genotypes with high plant survival rates and less leaf discoloration exhibit a better ability to restore growth and cellular elongation after chilling stress and recovery periods. The M_4 mutant family M30 exhibits this correlation, as it was clustered in group D for leaf discoloration after the recovery period with a lower absolute mean and in group A for seedling survival rate. In Experiment II, positive correlations were found between seedling survival rate, shoot regrowth, root regrowth, and negative correlations between plant survival rate and leaf discoloration. Previous reports have demonstrated correlations among these traits and indicate that the recovery rate indeed reflects the true tolerance to low-temperature stress at the seedling stage (Akter et al., 2022; Singh et al., 2022).

In the PCA, the first principal component was the most significant, explaining 69.56% of the total variation (Figure 3). This component was primarily attributed to traits such as root and shoot length relative performance, root and shoot recovery, and seedling survival rate. Reddy et al. (2021), in their study characterizing diverse rice genotypes for seedling stage high and low-temperature tolerance, similarly found that root and shoot traits played a crucial role in separating rice genotypes in a PCA analysis, supporting the findings of the present study. The variation among the mutant families, accounting for 13.62%, was attributed to the second principal component, primarily associated with leaf discoloration after the recovery period. Using the same traits as the PCA, the hierarchical grouping analysis, UPGMA, led to the

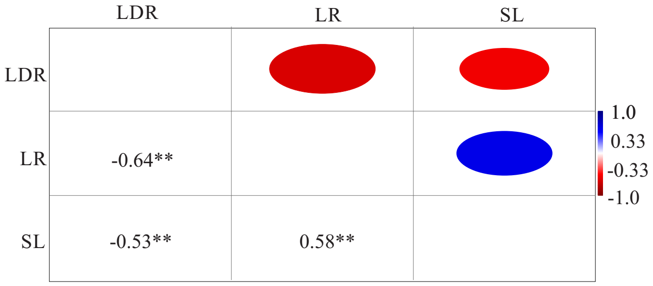

Figure 1. Correlation coefficients between leaf discoloration after recovery period (LDR), root length (RL) and shoot length (SL), in 43 mutant families, 'BRS Querência' and 19 commercial genotypes in Experiment I. **Significant at 1% probability.

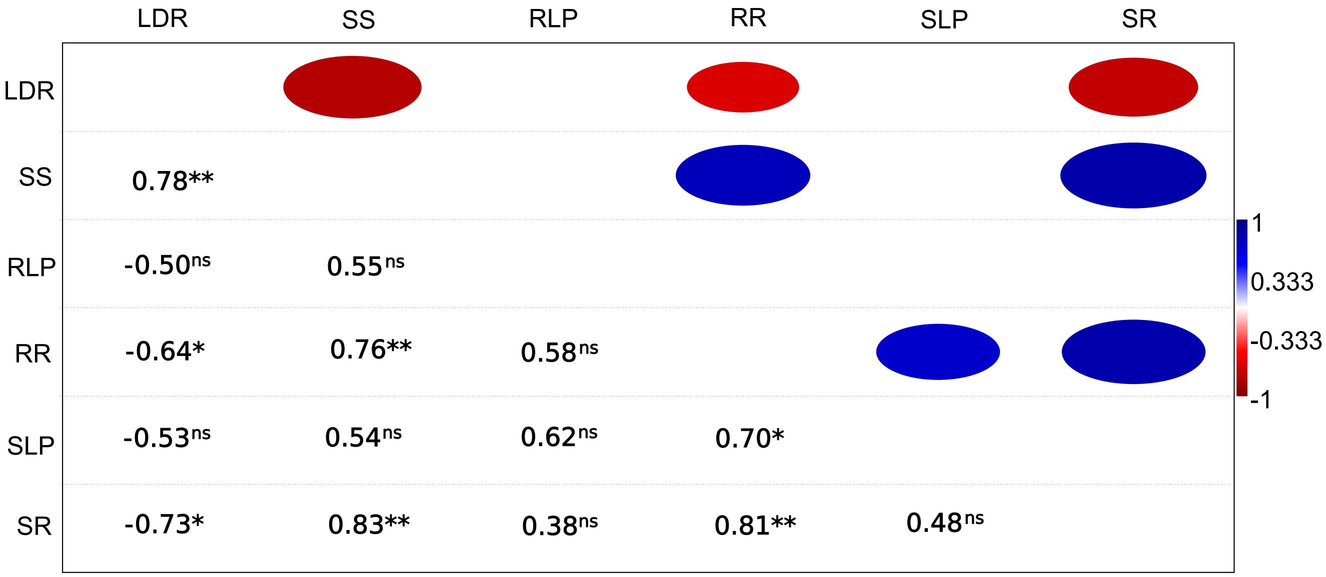

Figure 2. Correlation coefficients between leaf discoloration after recovery period (LDR), seedling survive rate (SS), root length relative performance (RLP), root recovery (RR), shoot length relative performance (SLP), shoot recovery (SR) in eight rice mutant families at M_4 generation, 'BRS Querência' and M5 516A3 of M_5 generation. * and **Significant at 5 and 1% probability. ^{ns}Nonsignificant.

formation of three distinct clusters (Figure 4), aligning with the principal component analysis results.

In both multivariate analyses, the mutant M30 was grouped into cluster 2, a separate one (Figure 3). The mutant families M36, M56, M54, and 'BRS Querência' were clustered together in cluster 1 in the PCA analysis, while M22, M26, M21, M5 516A3, and M17 were in cluster 3. In the UPGMA analysis, the same clusters were formed; however, the M5 516A3 mutant was placed in cluster 1 (Figure 4). The M_5 mutant family, M5 516A3, exhibited chilling tolerance during the germination period (Luz et al., 2021) and was grouped with M_4 mutant families with lower averages for the analyzed traits during seedling stage. 'BRS Querência'

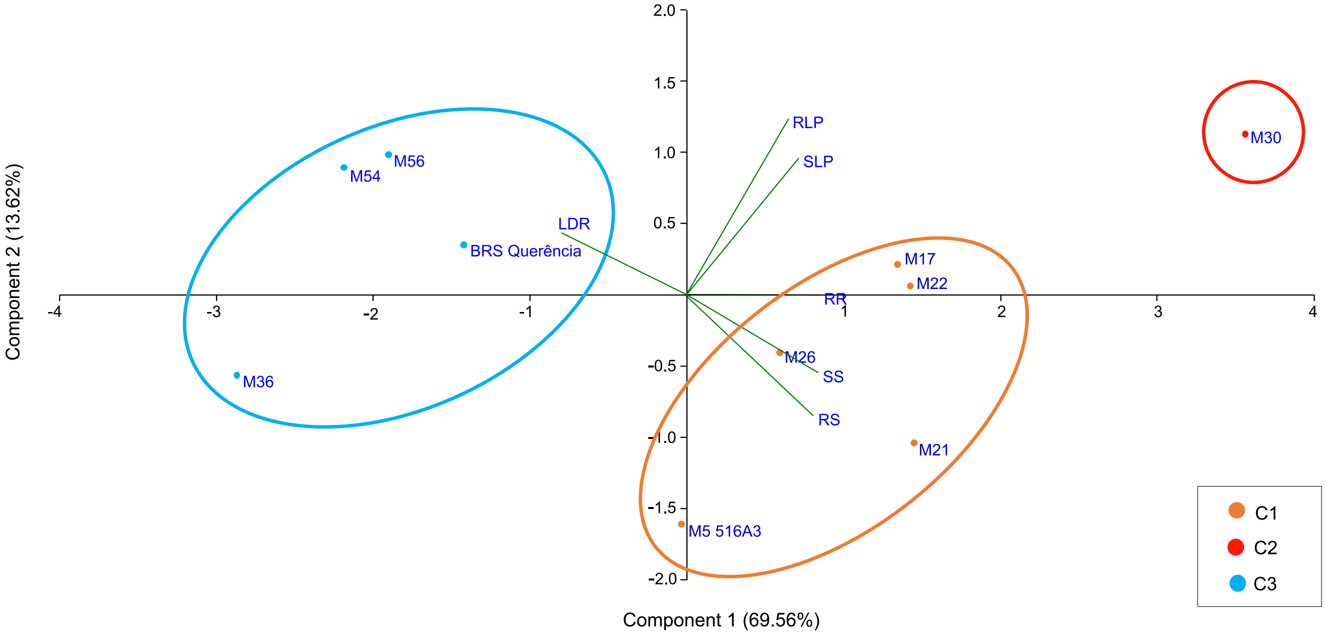

Figure 3. Principal component analysis (PCA) of eight mutant families, 'BRS Querência' and the M5 516A3 mutant of M_5 generation based on leaf discoloration after recovery period (LDR), seedling survive rate (SS), root length relative performance (RLP), root recovery (RR), shoot length relative performance (SLP) and shoot recovery (SR).

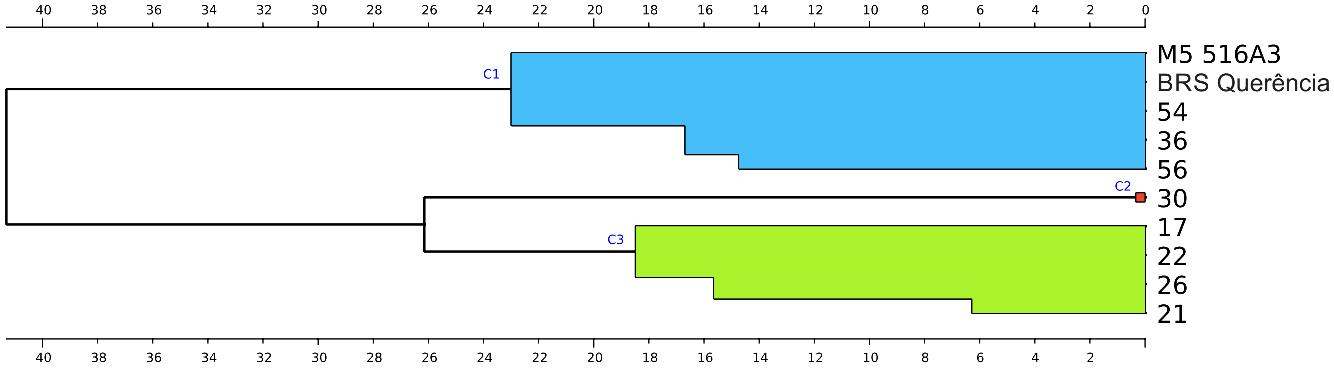

Figure 4. Hierarchical grouping obtained by the method of Unweighted Pair Group Method Arithmetic Mean (UPGMA) from the Euclidean distance of eight mutant families, 'BRS Querência', and the M5 516A3 mutant of M_5 generation based on leaf discoloration after recovery period, seedling survive rate, root length relative performance, root recovery, shoot length relative performance and shoot recovery.

was sensitive to chilling during the germination period (Teixeira et al., 2021), and this study revealed that this genotype did not display chilling tolerance during the seedling stage, clustering with M_4 mutant families with lower means, such as M5 516A3. On the other hand, the M_4 mutant family, M30, displayed the best performance for all evaluated traits, suggesting chilling tolerance during the seedling stage. The present research also suggests that cluster 2 in PCA analysis and cluster 3 in UPGMA analysis could be promising sources of chilling tolerance during the seedling stage for rice breeding programs.

Conclusions

1. Rice (*Oryza sativa*) mutant families of the M_4 generation differ from the original genotype 'BRS Querência' in chilling tolerance at the seedling stage.
2. The mutant families M36, M54, M56, and 'BRS Querência' show genetic similarity, indicating a lack of chilling tolerance during the seedling stage.
3. The mutant families M17, M21, M22, and M26 are promising for rice breeding programs because they present chilling tolerance.
4. The M30 mutant family exhibits superior performance in all analyzed traits, indicating chilling tolerance at the seedling stage.

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