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Mechanisms of genotypic differences in tolerance of iron toxicity in field-grown rice

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ABSTRACT

Iron (Fe) toxicity is a major constraint to rice yields in much of the world due to the greater solubility of reduced ferrous Fe in paddy soils compared with ferric Fe in aerobic soils and resulting excess uptake into the plants. There is genotypic variation in tolerance in *Oryza* gene pools, but so far only weak-effect alleles have been identified, largely because multiple critical physiological processes determine the tolerance. Most past research has been done in nutrient solution screens at the seedling stage, and not under field conditions over the full life cycle. We investigated tolerance mechanisms in a diverse set of genotypes under field conditions in a highly iron toxic soil in the Central Highlands of Madagascar. We made repeated plant samplings of young and old tissues throughout the growth period until maturity. Multiple mechanisms were involved, and the importance of different mechanisms changed between growth stages. Higher grain yields were mainly due to healthy vegetative growth, achieved either by reducing Fe uptake (exclusion) or by minimizing the effect of excess uptake through compartmentalization in older tissues and tissue tolerance. Exclusion mechanisms were relaxed during reproductive growth, leading to increased Fe accumulation in shoots. But tolerant genotypes were nonetheless able to grow well through a combination of Fe compartmentalization and tissue tolerance, so that grain filling could proceed relatively unimpeded. Tissue phosphorus (P) and potassium (K) concentrations were close to or below deficiency limits throughout growth. Exclusion by ferrous Fe oxidation in the rhizosphere will impede access of P and K ions to roots, but the differences in their tissue concentrations were much smaller than differences in growth rates, so growth rates evidently drove the uptake differences and responses to Fe toxicity were the more important constraints. There was no relation between grain yield and visual symptoms. To identify useful donors and markers for breeding it is important to develop screening protocols that capture the individual tolerance mechanisms, allowing for the effects of growth stage on their relative importance and expression, and possible interactions with other factors such as mineral nutrition. Selection for tolerance based on visual symptoms, particularly at the seedling stage, is overly simplistic, though it can be useful in the study of specific tolerance mechanisms.

1. Introduction

Iron (Fe) toxicity is a major constraint to rice production globally, causing severe yield losses (Dobermann and Fairhurst, 2000; Becker and Asch, 2005). It occurs in submerged paddy soils because the biogeochemical changes following submergence cause large increases in the concentration of ferrous iron in the soil solution, potentially leading to excessive Fe uptake into rice plants (Becker and Asch, 2005). It is a problem in rice soils across the globe, but particularly in the highly weathered, nutrient-depleted soils of inland valleys in Sub-Saharan

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Africa where much of the African rice production takes place (Rodenburg et al., 2014; van Oort, 2018). It is less of a problem in the young alluvial soils of the Asian rice lowlands, which have very different properties. Since that is where most rice research has been done, Fe toxicity has received less attention than other soil constraints to production. However, it is an increasingly important topic as attention is turned to increasing African rice production (Wopereis, 2013).

Water and nutrient management can mitigate Fe toxicity where resources permit (Becker and Asch, 2005; Rakotoson et al., 2019), but use of tolerant genotypes is more practicable. There is well-established variation in tolerance in both *Oryza sativa* and *Oryza glaberrima* gene pools for identifying donors and markers, yet progress in breeding has been slow (Kirk et al., 2022). To date, genome-wide association studies have revealed only small-effect alleles for tolerance (Meng et al., 2017; Diop et al., 2020; Melandri et al., 2021). This reflects multiple tolerance mechanisms, and difficulties in germplasm screening under field conditions due to large genotype-by-environment effects, and weak correlation between visible symptoms and beneficial stress response strategies.

Visible symptoms include leaf bronzing due to formation of reactive oxygen species (ROS), causing chlorophyll oxidation and impairment of photosynthesis (Pereira et al., 2013). Leaf bronzing score (LBS) and loss of grain yield are often used for germplasm screening, but the relationship between these two is often weak (Sikirou et al., 2015; Rakotoson et al., 2019), though there are exceptions to this (Audebert and Fofana, 2009). Much of the work on tolerance mechanisms has been done in culture solutions or pots, but these poorly represent field conditions and the complex interactions between Fe toxicity and soil physiochemical conditions.

Four types of tolerance mechanism are recognised (Engel et al., 2012; Wu et al., 2017; Aung and Masuda, 2020; Kirk et al., 2022): (1) exclusion of Fe from roots by oxidation of soluble ferrous iron (Fe(II)) to insoluble ferric iron (Fe(III)) by oxygen released from the roots; (2) retention of Fe in root cell vacuoles and plastids, decreasing translocation to shoots; (3) partitioning of excess shoot Fe into old or less-critical tissues to prevent damage to the youngest leaves; and (4) ROS detoxification through antioxidant enzymatic reactions. Hence genotypes are broadly distinguished as Fe 'excluders' and 'includers'. There has been recent progress in understanding the molecular physiology of tolerance mechanisms, including below-ground processes controlling Fe retention in roots and root-shoot transport, as well as above-ground partitioning and tissue tolerance (Aung and Masuda, 2020; Kirk et al., 2022). However, there is a lack of data and understanding under true field conditions. There is a need for integrated understanding of the complex tolerance response under field conditions, with which to identify markers for use in breeding.

A further point is the role of plant mineral nutrition and interactions between Fe toxicity and nutrient deficiencies. Nutrient deficiencies both compound the Fe toxicity and are exacerbated by it (Yamauchi, 1989; Sahrawat, 2005; Kirk et al., 2022). Given that nutrient deficiencies are typical of highly weathered Fe toxic soils, particularly deficiencies of phosphorus, potassium, calcium and magnesium, studies of Fe toxicity under field conditions need to consider interactions with these deficiencies.

Our aim in this study was to characterise genotypic differences in tolerance mechanisms under real field conditions, with a view to developing tailored screening methods for individual mechanisms and their interactions. We did this with a field experiment on a strongly iron-toxic soil in Madagascar with a set of contrasting genotypes provisionally identified as having a range of tolerances to Fe toxicity.

2. Materials and methods

2.1. Genotypes

There were six lowland rice genotypes in the first year of the experiment with a seventh added in the second year. Table 1 gives a tentative classification of the genotypes as tolerant or sensitive to Fe toxicity based on yield and LBS in an initial screening of 40 genotypes at three different Fe-toxic sites in Madagascar (results in Fig. S1 and images of the selected genotypes in Fig. S2), combined with the results of earlier research (Rakotoson et al., 2019).

2.2. Experimental set up

A two-year field experiment was conducted at Sambaina, Manjakandriana District in the Central Highlands of Madagascar (18°53′12.77′'S, 47°47′6.79′'E) during the wet seasons (December to May) of 2019–20 and 2020–21. The soil is a Gleysol with clay loam texture, aerobic pH (in H_2O) = 4.5, anaerobic pH (in H_2O) = 6.7, organic carbon = 62 g kg^{-1}, cation exchange capacity (cobalt hexamine method) = 2.4 $cmol_c$ kg^{-1} and total Fe = 57 g kg^{-1} of which 10 g kg^{-1} is easily-soluble on soil reduction (Rakotoson et al., 2019). Weather data was collected using a Watchdog station (Spectrum Technologies Inc., Plainfield, IL, USA). In Year 1, average temperature over the season was 16.6 °C with range 10.0–20.1 °C; in Year 2, average temperature was 16.8 °C with range 8.7–21.9 °C (Fig. S3). Cumulative rainfall over the season was 242 mm in Year 1 and 742 mm in Year 2 (Fig. S3).

The design was completely randomised with six genotypes in Year 1 and seven in Year 2, and four replicates. Seeds were sown in a nursery bed next to the experimental plots and grown for 21 days. Before transplanting, the soil was saturated with irrigation water pumped from the nearby river, and then hand-ploughed to a depth of 20 cm, harrowed and manually levelled. The size of subplots containing one genotype was 2 m^2 (1 m x 2 m) in Year 1 and 1.6 m^2 (0.8 m x 2 m) in Year 2. The plots were randomised within one block per replicate, separated by bunds. The fertilizer was broadcast and incorporated into the blocks with 50 kg ha^{-1} nitrogen (N) as urea, 20 kg ha^{-1} phosphorus (P) as triple superphosphate and 20 kg ha^{-1} potassium (K) as potassium sulphate. The 21-d old seedlings were transplanted into the plots with single plants per hill and 20-cm spacing between and within rows. The plots were then submerged with at least 10 cm of standing water and this depth was maintained throughout the experiment by addition to the standing layer or removal from it. Given the lower rainfall in Year 1 (Fig. S3) roughly twice as much irrigation from the river was required as in Year 2. The field was drained a few days before harvest to make harvesting easier. The plots were weeded manually twice before booting stage.

In Year 1, the soil solution was periodically sampled through Rhizon samplers (Rhizosphere Research Products, the Netherlands) permanently installed in the plots between plant rows to 10-cm depth below the floodwater-soil boundary. There was one sampler in each of four plots on a transect perpendicular to the sloping land next to the site. Solution was withdrawn into pre-evacuated 12-cm^3 glass vials and kept refrigerated until analysed by atomic absorption spectrometry (Thermo Scientific iCE 3000 Series AAS) at LRI.

2.3. Plant measurements

Sampling was done at each of the following growth stages: tillering, booting, flowering and maturity. The following tissues were sampled at each stage: youngest leaf (YL), middle leaves (ML), old leaves (OL) and stem/leaf sheath (ST). Additionally, flag leaves (FL) were sampled at the flowering stage and panicles (PN) at maturity. Plants were collected from two randomly selected hills per plot. They were separated into component tissues immediately after sampling in the field, the tissues stored in paper bags, then oven-dried at 60 °C for 48 h and weighed.

Leaf symptoms of Fe toxicity were visually scored as a percentage of affected leaf area in the whole plant canopy on a scale from 0 (no symptoms) to 10 (100% of the leaf area affected) (Wu et al., 2014). Leaf scoring was done for each sampling time except at the maturity because of difficulties in differentiation with plant senescence.

Iron, P and K concentrations were analysed in each tissue separately.

Table 1
Rice genotypes used in the study, their origin, their putative response to iron toxicity based on past research (Rakotoson et al., 2019) and a preliminary screening at field sites in Madagascar (Materials and methods), and grain yields in the experiments reported here. Data are means of 4 replicates. Similar letters in a column indicate no significant difference by HSD-test.

Full name	Short name	Origin	Supposed response to iron toxicity	Growth duration (days)		Grain yield (g m^{-2})	
				Yr 1	Yr 2	Yr 1	Yr 2
B14339E-KA-28	KA-28	Indonesia	moderately tol.	153	144	277[b]	548[a]
Bahia*	Bahia	Spain	Tolerant		166		320[c]
Ciherang	Ciherang	Indonesia	Sensitive	166	166	77[d]	274[d]
IR64	IR64	IRRI	Sensitive	153	144	160[c]	317 [cd]
NERICA-L-43	L-43	AfricaRice	Tolerant	166	166	207[c]	309 [cd]
Tsipala 421	Tsipala	Madagascar	Tolerant	153	144	421[a]	503[a]
X265	X265	Madagascar	moderately tol.	153	144	446[a]	392[b]

*Year 2 only.

Oven-dried samples were ground to a powder (Retsch ZM 200, 0.2-mm sieve), portions digested in concentrated HNO_3 and H_2O_2 in a microwave digestion system (MARSXpress, CEM Corporation, Mathews, NC, USA), and concentrations of Fe, P and K in the digests determined by ICP-MS (PerkinElmer NexION 350, Boston, MA, USA).

2.4. Data analysis

Tissue Fe contents were calculated from concentrations multiplied by dry weights (DW). Whole shoot Fe concentrations were calculated from the sum of the tissue contents divided by the whole shoot DW. Average growth rates were calculated for the vegetative stage (i.e., transplanting to booting) and reproductive stage (i.e., booting to maturity) from the changes in shoot DW divided by the time interval. Average rates of shoot Fe, P and K uptake over the vegetative and reproductive stages were calculated from the change in total shoot contents divided by the time interval. The ratio of Fe concentrations in different shoot tissues was also calculated at the booting and flowering stages, to assess the potential partitioning of Fe between shoot tissues during vegetative and reproductive growth.

Statistical analyses were performed with the R program (Version 4.2.0 https://www.R-project.org/). Genotype effects of all measured parameters at each growth stage were assessed by one-way analysis of variance (ANOVA) and means were compared by Tukey's HSD (Honestly Significant Differences) test ($p \leq 0.05$). The AGNES hierarchical algorithm in R was used for grouping of genotypes based on the degree of dissimilarity between them and clusters calculated from Euclidian distances in a matrix of concentrations and contents (Fig. S4). To allow for co-linearity, relations between grain yield or LBS and other variables were also assessed using lasso regression with the caret package (Kuhn, 2008). To explain each phenotype with the least of the other phenotypes, a model was generated using 51 samples out of the 52 total (6 genotypes in Yr 1 and 7 genotypes in Yr 2, both with 4 replicates), and one sample was used for cross-validation. This process was repeated 52 times to obtain average coefficients and the relative importance of each variable.

3. Results

Iron concentrations in the whole shoot (calculated from the sum of individual tissue contents divided by the whole shoot DW) ranged from approx. 600 to > 3000 mg kg^{-1} (Fig. 1A for booting stage; Fig. S5 for other stages). There were differences between genotypes, growth stages and years. But in all cases, the values far exceeded the threshold of 300 mg kg^{-1} reported for Fe toxicity in rice (Dobermann and Fairhurst, 2000). The concentration of dissolved Fe, which we take to be predominantly Fe(II) given the much lower solubility of Fe(III), increased rapidly in the first 15 d following soil submergence and then decreased somewhat by 45 d but remained > 120 mg L^{-1} until the field was drained before harvest (Fig. S6). There were no significant differences between the plots.

Genotypic differences in grain yield were consistent between years and largely agreed with the tentative classification in Table 1 with 'sensitive' genotypes Ciherang and IR64 having lowest yields in both years. Yields were poorer in Year 1 and maturity was delayed by 9 days in the shorter duration genotypes (Table 1). This does not appear to have been due to difference in temperature regime (Fig. S3) or water regime given that the same water regimes were maintained in both years by supplementing rainfall with irrigation. The likely explanation is that the plants in Year 2 benefited from residual P and K from Year 1. The greater rainfall in Year 2 might have brought more Fe(II) into the plots from upwelling groundwater flow, but this was not apparent in the yield or plant Fe data.

3.1. Genotypic differences during vegetative growth

During vegetative growth, represented by the booting stage (approx. 12 weeks after transplanting), there were large genotypic differences in shoot Fe concentration, DW and Fe content (Fig. 1A–C). Based on shoot Fe concentrations and content, we defined Tsipala, L-43 and Bahia as Fe 'excluders' (concentrations < 1400 and 850 mg kg^{-1} in Yr 1 and 2, and contents < 20 and 15 mg $plant^{-1}$ in Yr 1 and 2, respectively) and the other four genotypes as 'includers' (concentrations > 1700 and 1100 mg kg^{-1} in Yr 1 and 2, respectively). Shoot Fe concentrations in IR64, for example, were twice those in the excluders in both years. Differences in shoot DW between excluders and includers were not as marked as the differences in Fe concentration and content. We did not measure root Fe because, in puddled flooded soils, there is no satisfactory method for unequivocally separating true root Fe from that adsorbed on external root surfaces as 'plaque' or other soil constituents (Mori et al., 2016). By exclusion we are therefore referring to exclusion from the shoots, either by retention in roots or exclusion from them.

There was strong genotypic variation in LBS at booting stage and the differences were consistent between the years (Fig. 1D). However, despite the clear separation between excluders and includers in shoot Fe concentrations and contents, this was only partially reflected in bronzing scores. Includers KA-28 and IR64 had essentially the same Fe concentration and content, but differed greatly in bronzing scores, IR64 being much more heavily affected. Evidently KA-28 and other includers with low bronzing scores had some internal tolerance mechanism or mechanisms.

We assessed Fe uptake rates (Fig. 1E) and growth rates (Fig. 1F) between transplanting and booting. Includer genotypes had significantly higher Fe uptake rates than excluders, but there were no corresponding differences in growth rates. Growth rates were similarly low in L-43, IR64 and KA-28 but excluder L-43 had half the Fe uptake rate of the two includers. Growth rates differed between includers, with Ciherang and X265 having greater rates than KA-28 and IR64. The smaller shoot Fe concentrations in these genotypes were therefore possibly due to a dilution effect with greater growth. This may also explain the intermediate LBS of X265. However, Ciherang had consistently high LBS, comparable to includer IR64 which did not show any growth dilution

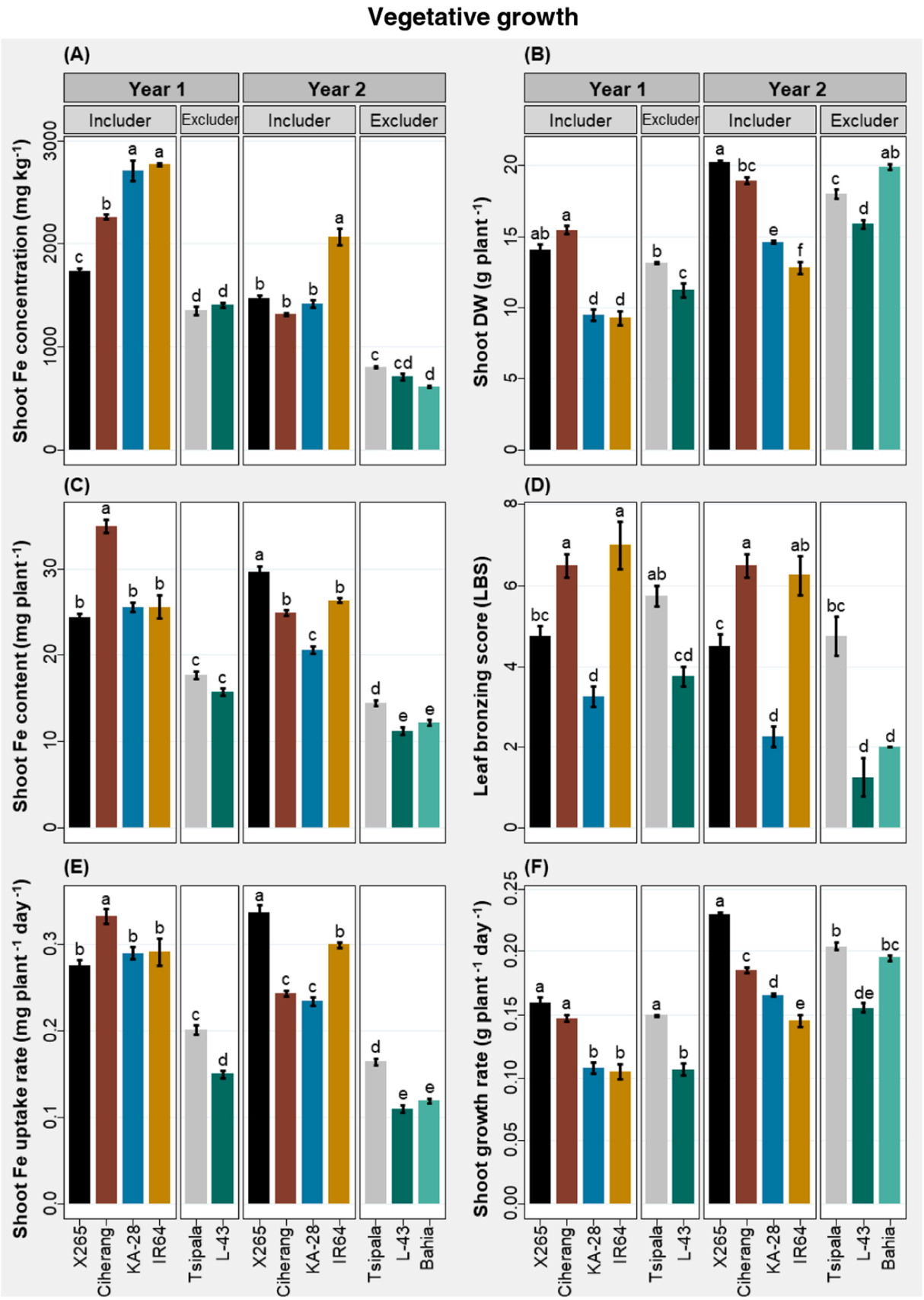

Fig. 1. Vegetative stage data for genotypes classified as Fe 'includers' and 'excluders': (A) shoot Fe concentration (sum of individual tissue contents divided by whole shoot DW), (B) shoot DW, (C) shoot Fe content, (D) leaf bronzing score, (E) shoot Fe uptake rate and (F) shoot growth rate. A–D are at booting stage; E–F are from transplanting to booting. Data are means ± standard errors ($n = 4$). Common letters indicate no significant difference by Tukey's HSD test.

effect.

3.2. Partitioning between shoot tissues during vegetative growth

During vegetative growth, Fe concentrations were much greater in stems and old leaves (> 2000 mg kg^{-1} in Yr 1 and 1200 mg kg^{-1} in Yr 2) than in young leaves (typically 500–800 mg kg^{-1}) (Fig. 2A). Excluders L-43 and Bahia had significantly lower Fe concentrations in all tissues compared to includers, whereas Tsipala only had lower concentrations in the stem. This may explain Tsipala's higher LBS because its leaf Fe concentrations were high. There were corresponding differences in Fe content. About 70% of total shoot Fe was in stems, whereas young leaves only contained 3% on average (Fig. 2B). The differences between excluders and includers were most evident for stem Fe content, which was

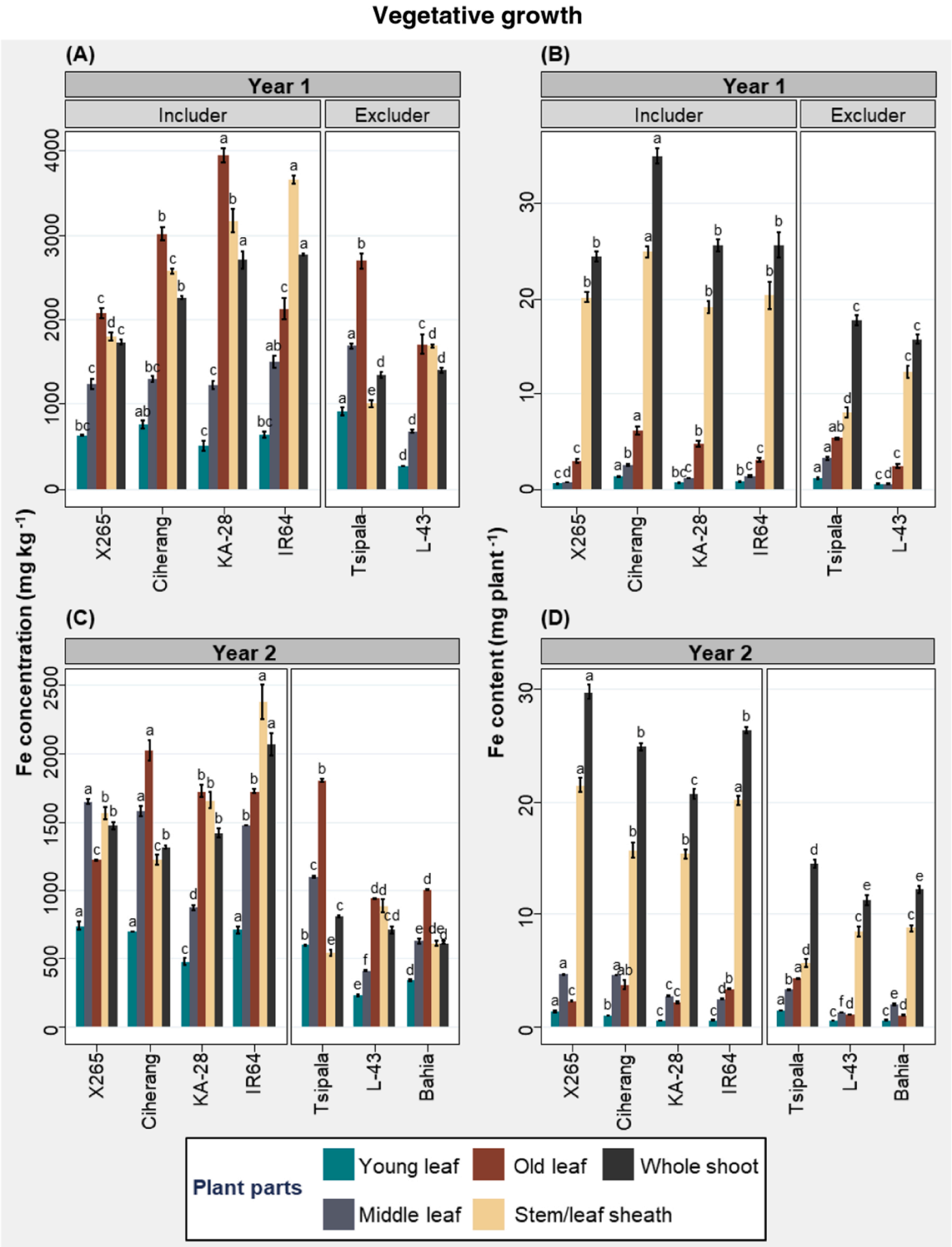

Fig. 2. Iron concentrations and contents in above-ground tissues at booting stage. Whole shoot Fe concentrations were calculated from the sum of the individual tissue contents divided by the whole shoot weight. Data are means ± standard errors ($n = 4$). Common letters indicate no significant difference between genotypes for a given tissue by Tukey's HSD test.

typically 50% smaller in excluders.

Table 2 shows the ratios of Fe concentrations in different shoot tissues between genotypes. KA-28 and L-43 consistently had the lowest ratio of Fe in young to old leaves and, to a smaller extent, in middle to old leaves. KA-28, L-43 and IR64 had the lowest ratios in young and middle leaves and stem, suggesting that those genotypes prevent Fe accumulation in their young and middle leaves by storing it in old leaves and stems. In addition, the lower bronzing scores of KA-28 than IR64 (Fig. 1D), despite similar Fe concentration, content and partitioning, suggests some additional tissue tolerance in KA-28.

3.3. Genotypic differences during reproductive growth and at maturity

There were genotypic differences in the shoot Fe concentration, DW and Fe content, as during vegetative growth (Fig. 3A–C). Whole shoot Fe concentrations at maturity remained very high (Fig. 3A) but differences between genotypes decreased and the distinction between includers and excluders detected at the booting stage was no longer apparent as KA-28 had lower and Tsipala higher than expected concentrations. Although shoot Fe content did not differ between includers and excluders (Fig. 3C), shoot biomass has a stronger influence on it (Fig. 3B). Over both years, genotypes Ciherang and IR64 consistently had the lowest shoot biomass (< 20 g plant^{-1}) and shoot Fe content (< 42 and < 33 mg plant^{-1} in Yr 1 and 2, respectively).

Leaf symptoms differed between genotypes during reproductive growth (Fig. 3D) and includers Ciherang and IR64 had the highest LBS, as they did during the vegetative stage, whereas includer KA-28 maintained its apparent tissue tolerance and showed little bronzing. Tsipala, classified as an excluder during the vegetative stage but not being able to maintain this during the reproductive stage, had a very high bronzing score.

The reversal between includers and excluders from vegetative to reproductive stages was even more pronounced for Fe uptake rates: values were highest in Tsipala and lowest in Ciherang and IR64 (Fig. 3E). Rates of Fe uptake by excluder genotypes increased more than two-fold from vegetative to reproductive stages, indicating that exclusion mechanisms were less effective during reproductive growth. As a group, includers were inconsistent with generally increasing Fe uptake rates in KA-28 and X265 but decreasing rates in Ciherang and IR64. Low Fe uptake rates in these two genotypes was likely due to their very low growth rates (Fig. 3F).

3.4. Partitioning between shoot tissues during reproductive growth

Iron concentration varied greatly between shoot tissues and genotypes during reproductive growth (Figs. 4A and 4C). The stem and old leaves had the highest concentrations (> 1200 and 800 mg kg^{-1} in Yr 1 and 2, respectively), and the flag and young leaves the lowest. In both years, KA-28 had the lowest Fe concentration in flag leaves and Tsipala had the highest. There were corresponding differences in Fe content (Figs. 4B and 4D).

Table 3 shows the ratios of Fe concentrations in different shoot tissues in the different genotypes during reproductive growth. Excluders L-43, Bahia and includer KA-28 consistently had the lowest ratio of Fe in flag to old leaves and in flag leaves to stems. Whereas excluder Tsipala has a similar ratio to other includers, and IR64 did not maintain the Fe compartmentalization away from young leaves evident during vegetative growth (Table 2). This suggests that L-43, Bahia and KA-28 were able to maintain compartmentalization mechanisms into reproductive growth but IR64 was not.

3.5. P and K uptake during vegetative and reproductive growth

Table 4 shows leaf P and K concentrations during vegetative and reproductive growth. In all the genotypes at both growth stages, concentrations of both nutrients were suboptimal and close to or below deficiency levels. In general, P concentrations were greater in the reproductive than the vegetative stage, whereas K concentrations were smaller. The differences between genotypes were not very consistent for either nutrient at either growth stage.

We calculated rates of P and K uptake during vegetative growth (transplanting to booting) and reproductive growth (booting to maturity) from the changes in shoot contents (Fig. 5). Includers Ciherang and X265 had higher P uptake rates than the other genotypes during vegetative growth in both years (Fig. 5A). But the trends were different during reproductive growth (Fig. 5C), with includers Ciherang and IR64 having low rates in both years, and excluders Tsipala and Bahia having the highest rates. The latter were more than double the corresponding vegetative stage rates. Rates of K uptake also differed between genotypes during vegetative growth (Fig. 5B), and, as for P uptake, Ciherang and X265 had the highest rates in both years. Rates of K uptake during reproductive growth also differed between genotypes (Fig. 5D). Includers (except KA-28 in Yr 1) had lower K uptake rates during reproductive growth, whereas excluders tended to maintain their uptake rates.

3.6. Relationships with grain yield

Grain yield was not correlated with Fe uptake rate during vegetative growth (Fig. 6A) but it was correlated with Fe uptake rate during reproductive growth (Fig. 6B). Hence, genotypes with high Fe uptake rate (KA-28, Bahia, L-43, Tsipala, and X265) had high yield, whereas genotypes with low Fe uptake rate (Ciherang, IR64) had low yield.

Greater leaf bronzing was associated large high Fe concentrations in flag leaves at flowering (Fig. 7B) but there was no consistent association between leaf bronzing and grain yield (Fig. 7A). While Ciherang and IR64 with very high bronzing had low yields, Tsipala maintained high yields despite its high canopy bronzing score.

The results of the lasso regression (Tables S2 and S3), which we carried out to allow for co-linearity of variables, were consistent with

Table 2
Ratios of Fe concentrations in different plant parts in Includer genotypes at booting stage. Values are means of 4 replicates. Similar letters in a column indicate no significant difference by HSD-test; lowest values are highlighted in green. YL = youngest leaf, ML = middle leaves, OL = old leaves, ST = stem/leaf sheath.

Genotype	Fe concentration ratio							
	YL/OL		ML/OL		YL/ST		ML/ST	
	Yr 1	Yr 2	Yr 1	Yr 2	Yr 1	Yr 2	Yr 1	Yr 2
Includers								
X265	0.30^{ab}	0.61^{a}	0.60^{a}	1.35^{a}	0.35^{b}	0.47^{b}	0.69^{b}	1.0.5^{c}
Ciherang	0.25^{b}	0.34^{c}	0.43^{b}	0.78^{b}	0.30^{b}	0.57^{b}	0.50^{c}	1.29^{b}
KA-28	0.13^{c}	0.27^{de}	0.31^{b}	0.51^{d}	0.16^{c}	0.29^{c}	0.39^{c}	0.53^{d}
IR64	0.30^{ab}	0.41^{b}	0.71^{a}	0.85^{b}	0.18^{c}	0.30^{c}	0.41^{c}	0.62^{d}
Excluders								
Tsipala	0.34^{a}	0.33 ^{cd}	0.63^{a}	0.61^{c}	0.91^{a}	1.10^{a}	1.68^{a}	2.03^{a}
L-43	0.16^{c}	0.24^{e}	0.41^{b}	0.44^{d}	0.16^{c}	0.26^{c}	0.41^{c}	0.47^{d}
Bahia		0.34^{c}		0.62^{c}		0.55^{b}		1.03^{c}

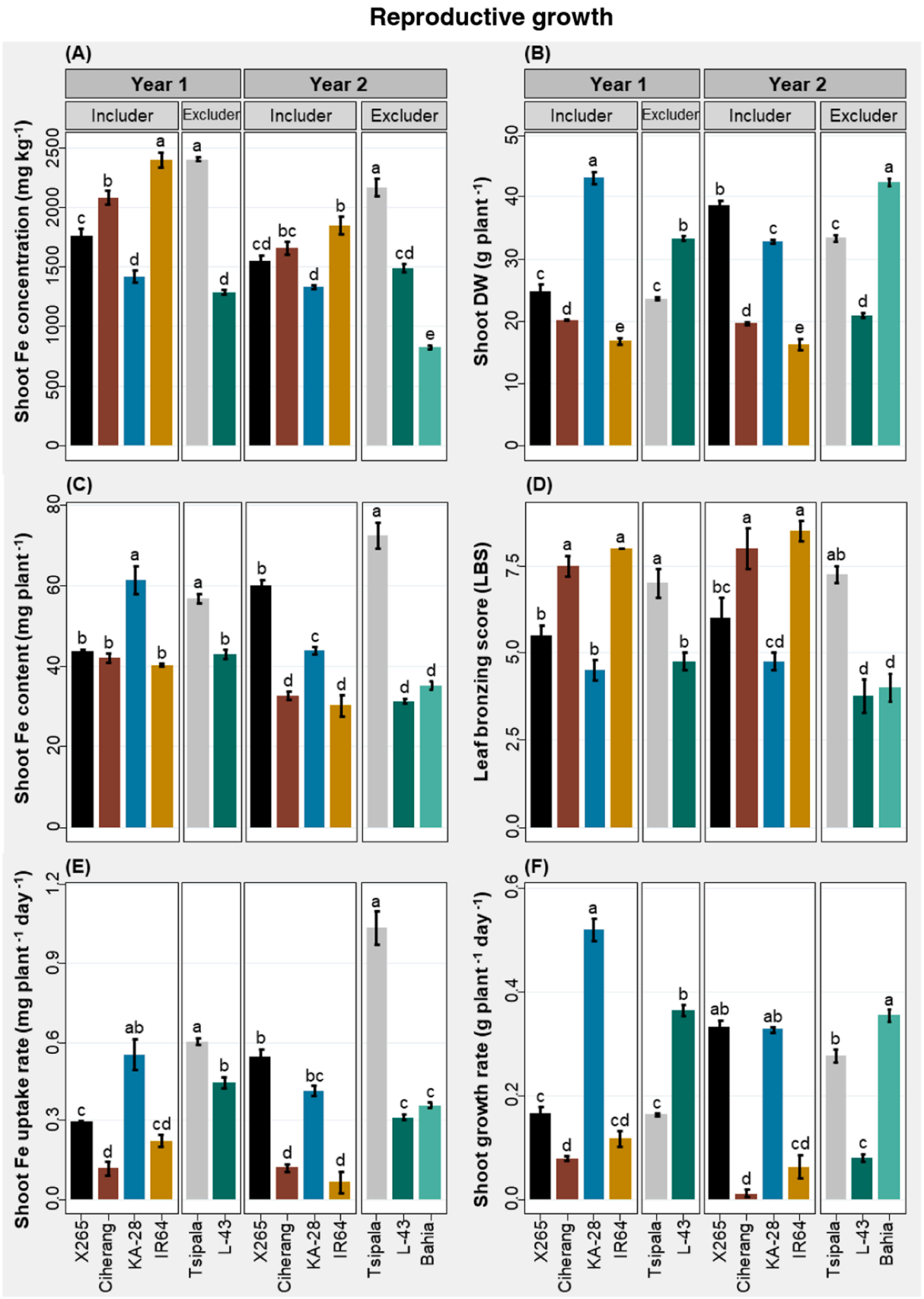

Fig. 3. Reproductive stage data: (A) shoot Fe concentration, (B) shoot DW, (C) shoot Fe content, (D) leaf bronzing score, (E) shoot Fe uptake rate and (F) shoot growth rate. A, B, C are at maturity; D is at flowering; E–F are from booting to maturity. Data are means ± standard errors ($n = 4$). Common letters indicate no significant difference by Tukey's HSD test.

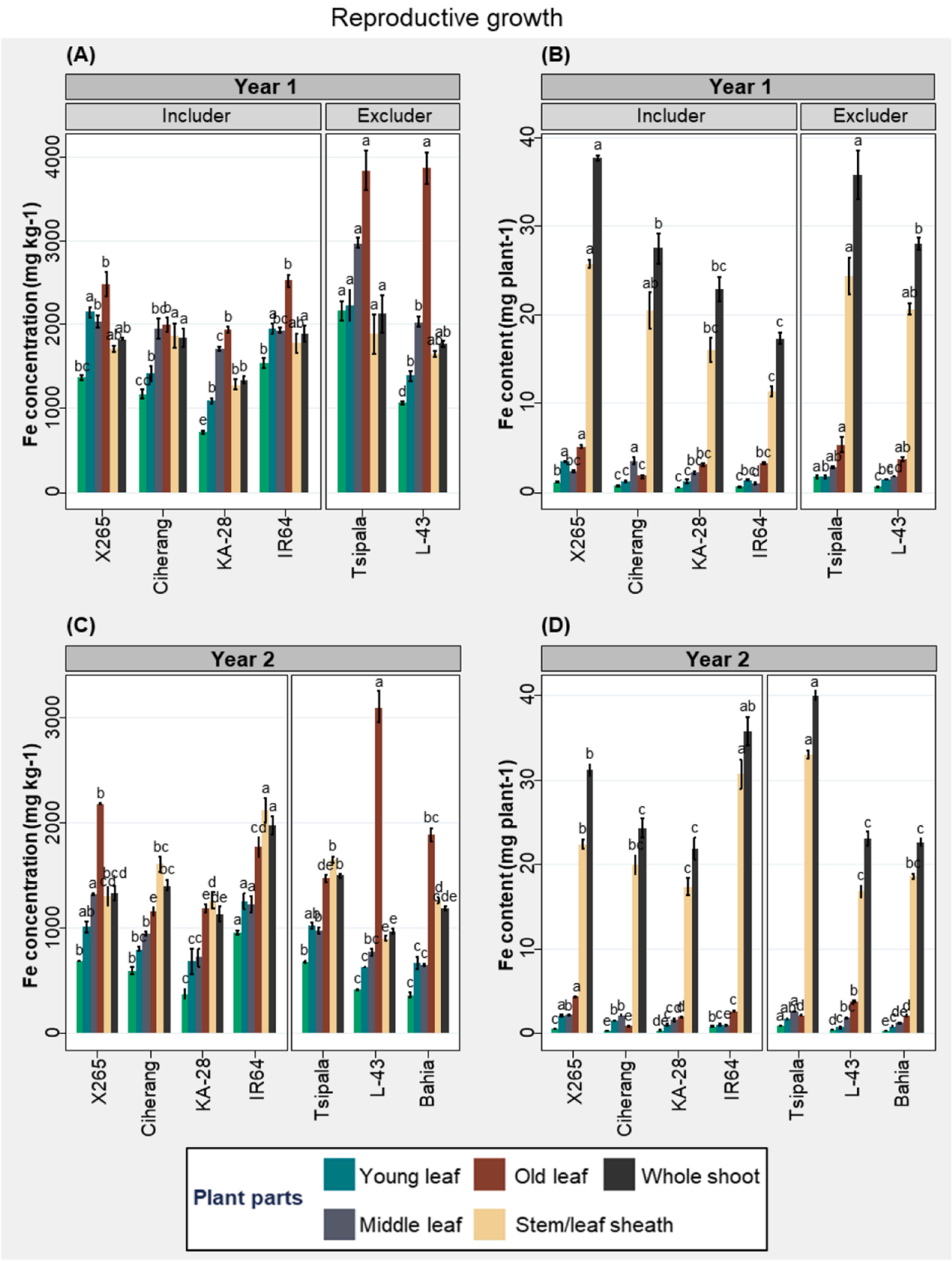

Fig. 4. Iron concentrations and contents in above-ground tissues at flowering stage. Data are means ± standard errors ($n = 4$). Common letters indicate no significant difference between genotypes for a given tissue by Tukey's HSD test.

the absence of positive correlation between grain yield and LBS at flowering stage. The factors that explain grain yield and LBS did not have any overlap, indicating that different physiological mechanisms underlie grain yield and LBS.

4. Discussion

4.1. Exclusion, compartmentalization and other tolerance mechanisms

The results show that multiple tolerance mechanisms were operating in the different genotypes, as summarised in Fig. 8. Firstly, there was a clear separation between Fe includers and excluders during vegetative

Table 3
Ratios of Fe concentrations in different plant parts in includer genotypes at flowering stage. Values are means of 4 replicates. Similar letters in a column indicate no significant difference by HSD-test; lowest values are highlighted in green. FL= Flag leaf, YL = youngest leaf, OL = old leaves, ST = stem/leaf sheath.

Genotype	Fe concentration ratio							
	FL/OL		YL/OL		FL/ST		YL/ST	
	Yr 1	Yr 2	Yr 1	Yr 2	Yr 1	Yr 2	Yr 1	Yr 2
Includers								
X265	0.56^{a}	0.31^{b}	0.88^{a}	0.46^{bc}	0.80^{b}	0.53^{a}	1.25^{a}	0.78^{a}
Ciherang	0.58^{a}	0.50^{a}	0.71^{ab}	0.69^{a}	0.64^{b}	0.37^{bc}	0.77^{c}	0.50^{b}
KA-28	0.37^{b}	0.31^{b}	0.56^{bc}	0.57^{ab}	0.56^{b}	0.29^{c}	0.85^{bc}	0.53^{b}
IR64	0.61^{a}	0.54^{a}	0.77^{ab}	0.71^{a}	0.87^{ab}	0.45^{ab}	1.11^{ab}	0.60^{ab}
Excluders								
Tsipala	0.56^{a}	0.46^{a}	0.58^{bc}	0.69^{a}	1.20^{a}	0.41^{b}	1.22^{a}	0.62^{ab}
L-43	0.27^{b}	0.19^{c}	0.36^{c}	0.20^{d}	0.64^{b}	0.46^{ab}	0.84^{bc}	0.70^{ab}
Bahia		0.13^{c}		0.35 ^{cd}		0.28^{c}		0.53^{b}

Table 4
Concentrations of P and K in youngest leaf (YL) at booting and flag leaf (FL) at flowering. Values are means of 4 replicates. Similar letters in a column indicate no significant difference by HSD-test. For comparison, optimal (deficiency) values are 2–4 (1.0) and 2–3 (1.8) g P kg^{-1} in YL at B and FL at F, respectively, and 18–26 (15) and 15–20 (12) g K kg^{-1} in YL at B and FL at F (Dobermann and Fairhurst, 2000).

Genotype	**P concentration (g kg^{-1})**				**K concentration (g kg^{-1})**			
	YL at booting		**FL at flowering**		**YL at booting**		**FL at flowering**	
	Yr 1	**Yr 2**	**Yr 1**	**Yr 2**	**Yr 1**	**Yr 2**	**Yr 1**	**Yr 2**
Includers								
X265	1.41^{a}	1.32^{b}	1.32 ^{cd}	1.84^{a}	10.37^{d}	10.28^{b}	10.88^{ab}	9.91^{e}
Ciherang	1.24^{abc}	1.46^{a}	1.62^{abc}	1.60^{b}	14.09^{a}	12.65^{a}	12.48^{a}	10.50^{de}
KA-28	1.15^{bcd}	1.27^{bc}	1.79^{a}	1.40^{d}	13.35^{ab}	11.71^{ab}	11.12^{ab}	10.54^{cde}
IR64	1.03^{d}	1.55^{a}	1.70^{ab}	1.84^{a}	11.57 ^{cd}	11.90^{ab}	12.05^{a}	11.64^{bcd}
Excluders								
Tsipala	1.04 ^{cd}	1.08^{d}	1.46^{bc}	1.51^{bc}	12.57^{bc}	10.83^{ab}	9.97^{bc}	11.79^{bc}
L-43	1.25^{ab}	1.28^{bc}	1.05^{d}	1.48 ^{cd}	12.00^{bc}	11.77^{ab}	8.65^{c}	12.29^{ab}
Bahia		1.21^{c}		1.51^{bc}		11.34^{ab}		13.45^{a}

growth based on shoot Fe concentrations and contents irrespective of differences in growth. Second, some of the genotypes sequestered excess Fe in older tissues to protect younger leaves. Third, in addition, some of the genotypes had specific tissue tolerance mechanisms given differences in LBS for the same tissue Fe concentration. Fourth, tolerant genotypes were able to sustain their tolerance from vegetative stages into reproductive growth despite having apparently abandoned their Fe exclusion mechanism. Hence, the distinction between excluders and includers based on concentrations and contents did not persist into reproductive growth.

It is informative to compare the sensitive genotype IR64 with the tolerant KA-28. They had very similar growth and Fe concentrations, contents and tissue partitioning during vegetative stages, but they had very different LBS, indicating differences in tissue tolerance. Then, after booting, they diverged with KA-28 growing well and producing high grain yields but IR64 deteriorating and having much poorer growth and yield. What chain of events could explain these differences? Evidently IR64 was so damaged by Fe toxicity by the booting stage that it was unable to recover.

Another interesting genotype is Tsipala which excluded Fe during vegetative growth but lacked tolerance mechanisms at the maturity stage according to our indices. Based on flag leaf Fe concentration and LBS, it should have performed very poorly, but in fact it produced good yields. Possibly Tsipala has tissue tolerance mechanisms that we have not directly measured. Or it may be sufficient to keep the plant healthy during the vegetative stages by Fe exclusion and limited tissue damage, and that grain filling may proceed relatively unimpeded in an otherwise seemingly affected plant. In this regard it would be interesting to compare the effects of Fe toxicity with the loss of photosynthetic capacity occurring naturally during senescence. By contrast, the sensitive genotype Ciherang produced high biomass during vegetative growth, despite high shoot Fe concentrations and LBS, but it did not maintain good growth into the reproductive stages. Evidently Ciherang lacks tissue tolerance or compartmentalization mechanisms and was so damaged by Fe during the earlier stages that it could not recover.

Some of the genotypes stored excess Fe in stem/leaf sheath tissues and/or old leaves, particularly the tolerant includer KA-28 and excluder L-43 at booting, and Bahia at flowering. Presumably smaller Fe concentrations in younger, more active tissues allows greater net photosynthesis. Likewise, Engel et al. (2012) found Fe retention in leaf sheaths/stems was important in includers. Compartmentalization into stems and old leaves may initially work by storage of Fe in vacuoles (Moore et al., 2014) or within ferritin proteins (Stein et al., 2009) but plants may later simply sacrifice old leaves if they are no longer essential source leaves.

The positive relation between grain yield and Fe uptake rates during reproductive growth (Figs. 3 and 6) is seemingly counter-intuitive. If greater Fe uptake in the absence of exclusion mechanisms during the reproductive stages is simply due to greater growth rates and maintaining high growth rates lead to greater grain yield, then a positive correlation would follow. For KA-28 and X265 (in Yr 2) high Fe uptake rates were coupled with greater growth rates (Fig. 3) and this apparently led to a dilution effect.

Genotypes differed in plant maturity dates with Ciherang, L-43 and Bahia having significantly longer growth duration than the others (Table S1). Yields of the longer duration genotypes may have been affected by low temperatures (Fig. S3). Low temperatures are known to impair rice yields in the Central Highlands of Madagascar (Dingkuhn et al., 2015). Hence the tolerant genotypes L-43 and Bahia might otherwise have yielded a little better. This would have established a stronger positive association between grain yield and Fe exclusion during the vegetative stages and Fe sequestration in old leaves during reproductive stages (Table 3) than currently detected.

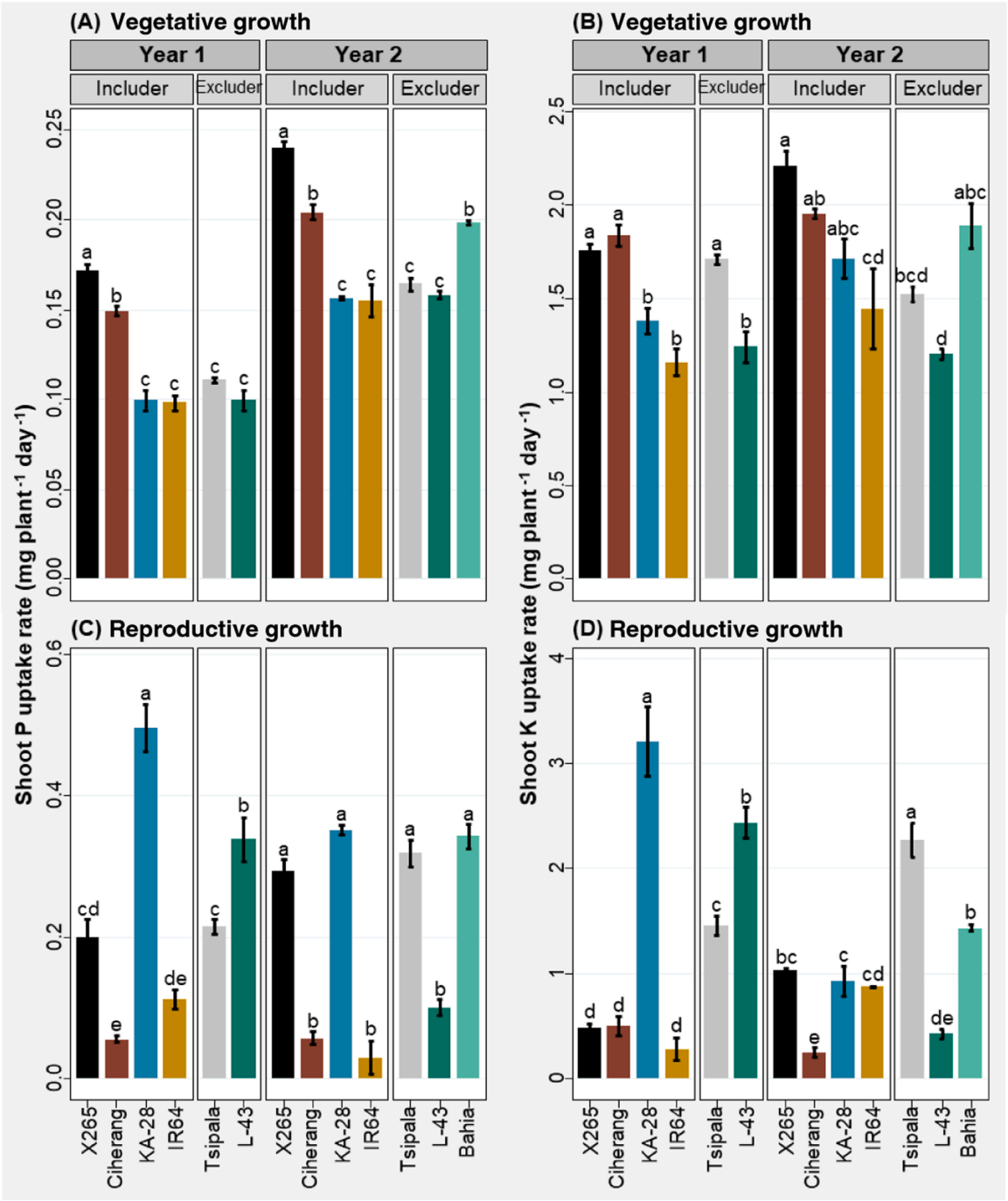

Fig. 5. Rates of P and K uptake into shoots (A) and (B) during vegetative growth (tillering to booting), respectively, and (C) and (D) during reproductive growth (booting to maturity), respectively. Data are means ± standard errors ($n = 4$). Common letters indicate no significant difference by Tukey's HSD test.

4.2. *Changes between growth stages*

Tolerant includers X265 and KA-28 continued to take up Fe during reproductive growth at comparable rates to vegetative growth, and sensitive includers Ciherang and IR64 decreased their uptake. By contrast, the excluders had low uptake rates during vegetative growth but higher rates during reproductive growth. This suggest that, for some reason, exclusion mechanisms were relaxed during reproductive growth, allowing more Fe into roots and shoots. Presumably this was because continuing Fe exclusion during reproductive growth had some deleterious effect or effects that exceeded the value of continuing Fe exclusion.

Why should Fe exclusion mechanisms break down during reproductive growth? Possibly, the energy costs of maintaining exclusion are increasingly prohibitive during reproductive growth and grain filling. In general, there is a logarithmic relation between root biomass and total plant biomass such that progressively less photosynthate is allocated to roots as the plant grows (Yoshida, 1981). Further, Fe exclusion at the root surface depends on the development of aerenchyma in the root cortex and suberization of epidermal layers in the basal zones to form a barrier to O_2 loss, allowing a greater length of root to be aerated and O_2 release into the rhizosphere in apical zones and lateral roots (Yamauchi et al., 2018). These processes are metabolic and consume energy (Tadano, 1975; Yamauchi et al., 2018) in competition with other

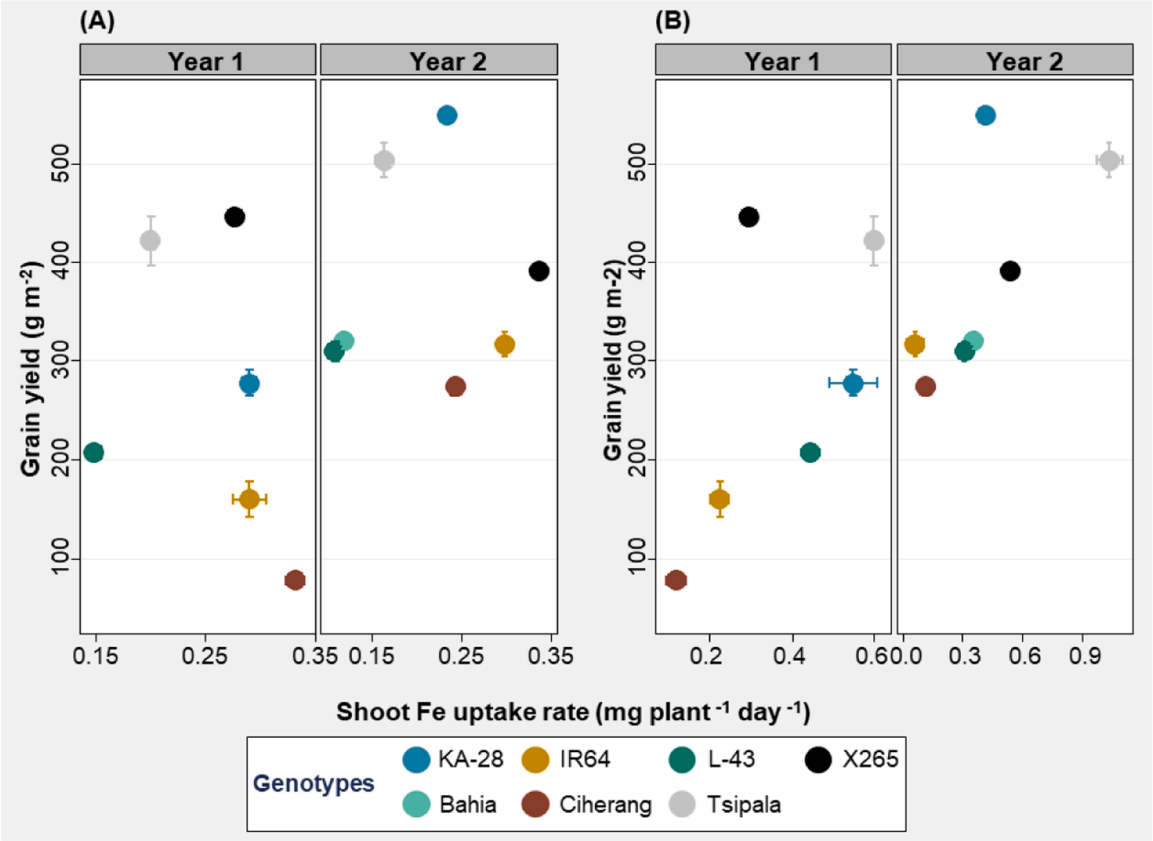

Fig. 6. Relationship between grain yield and Fe uptake rate during (A) vegetative and (B) reproductive stages. Data are means ± standard errors ($n = 4$).

processes. We hypothesize that the benefits of devoting energy to exclusion mechanisms outweigh their cost during the vegetative stages, whereas later on, they are in direct competition with grain filling and given that the sink-strength of roots decreases over time, insufficient energy is allocated to maintain these mechanisms after the vegetative stages. A further possibility is that Fe exclusion interferes with the uptake of other essential mineral nutrients.

4.3. Interactions with mineral nutrition

The unfertilized soil is highly deficient in P, K and other nutrients (Rakotoson et al., 2019), which is typical of highly weathered, Fe toxic soils in the inland valleys of sub-Saharan Africa (Kirk et al., 2022). The plant P and K concentrations were close to or below deficiency limits during both vegetative and reproductive growth, and there were differences in uptake rates between genotypes. However, the concentration differences were much smaller than the differences in growth rates, so growth rates evidently drove the differences in uptake rates. This suggests genotypic responses to Fe toxicity were the more important constraints to growth. Better growth in tolerant genotypes may dilute tissue P and K concentrations. Nonetheless, exclusion of Fe(II) from roots by oxidizing it in the rhizosphere may impede P and K uptake, as we now discuss, and excluder genotypes may therefore make things worse for themselves.

Three processes in the rice rhizosphere in Fe toxic soils affect the solubilities and hence plant-availabilities of nutrient ions (Begg et al., 1994; Kirk et al., 2019). First, oxidation of Fe(II) by O_2 diffusing down through aerenchyma and released from roots results in accumulation of insoluble ferric hydroxide on and near root surfaces and generation of acidity: $4Fe^{2+} + O_2 + 10H_2O = 4Fe(OH)_3 + 8H^+$. Second, release of acidity (H^+ ions) from roots to balance excess intake of nutrient cations (especially NH_4^+ in the anoxic flooded soil) over anions tends to further decrease the rhizosphere pH. Third, uptake of dissolved CO_2 into roots and its venting through the aerenchyma decreases the concentration of the acid H_2CO_3 near the root and to some extent offsets the acidity generated in the other two processes. Phosphate anions and K^+ cations may both be immobilized on freshly precipitated $Fe(OH)_3$ in the rhizosphere, depending on the pH (Jianguo and Shuman, 1991; Saleque and Kirk, 1995). Further, removal of exchangeable Fe^{2+} as it is oxidized will mean a greater proportion of surface exchange sites is occupied by other cations such as K^+, so decreasing K^+ solubility and mobility. The cation exchange capacity will tend to decrease as the pH decreases. But if the pH decreases below about 6.0, the concentration of bicarbonate anions (formed from dissolved CO_2) balancing cations in solution will decrease. So overall K^+ mobility and uptake will decrease with Fe oxidation and acidification. However, given the above complexities, a range of responses should be expected in different soils (Kirk et al., 2022).

Hence relaxation of Fe exclusion during reproductive growth might allow greater P and K uptake to meet increased demand during grain filling. The plant P supply is most important during early rice growth stages to promote tillering, root growth and flowering, but K uptake needs to be maintained through reproductive growth (Dobermann and Fairhurst, 2000). Phosphorus deficiency may delay phenological development by up to a month (Dobermann and Fairhurst, 2000; Vandamme et al., 2018). Under chronic low-level exposure to Fe toxicity, slower growth might mean less Fe is accumulated in the plants, and they have longer to acclimatize to it. On the other hand, delayed phenology may expose the plants to other problems later in the season, such as low temperature.

4.4. Implications for rice breeding

This study identified genotypes possessing complementary tolerance mechanisms, with L-43 being a potential donor for Fe exclusion

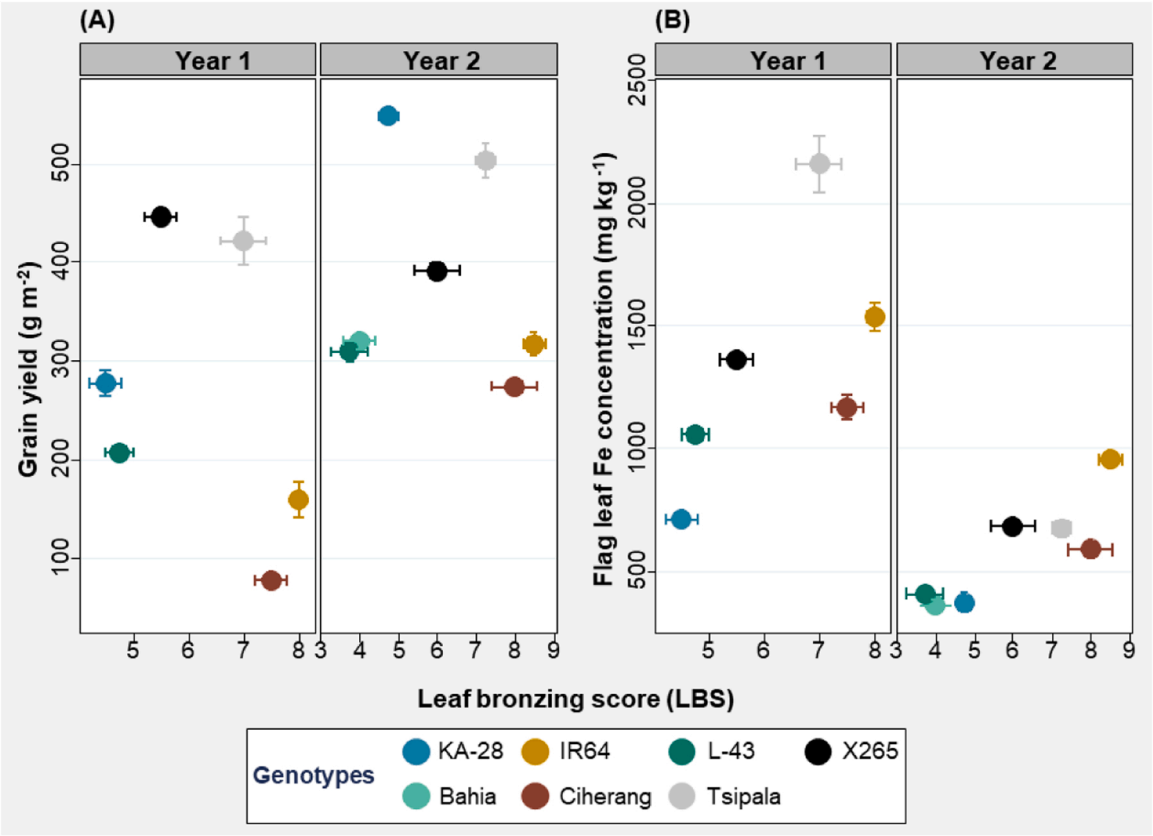

Fig. 7. Relationships between (A) grain yield and (B) flag leaf Fe concentration and leaf bronzing score (LBS) of the canopy at flowering stage for the 6 genotypes in Year 1 and 7 genotypes in Year 2. Data are means ± standard errors ($n = 4$).

		Tolerant		Sensitive
		Excluders (Tsipala, L-43, Bahia)	**Includers (KA-28, X265)**	**Includers (IR64, Ciherang)**
Vegetative growth 	**Characteristics**	• Low shoot Fe conc. • Low LBS • High biomass	• High shoot Fe conc. • Low LBS • High biomass	• High shoot Fe conc. • High LBS • Low/high biomass
	Tolerance mechanisms	• Exclusion	• Tissue tolerance • Compartmentalization	
Reproductive growth 	**Characteristics**	• Higher shoot Fe concentration • Higher LBS • High Biomass		• High shoot Fe conc. • High LBS • Low biomass
	Tolerance mechanisms	• Tissue tolerance • Compartmentalization		
		High grain yield		**Low grain yield**

Fig. 8. Summary of tolerance mechanisms in the different genotypes. LBS = leaf bronzing score.

mechanisms and KA-28 for Fe compartmentalization and tissue tolerance. Further physiological and genetic studies are needed to understand the underlying mechanisms. To date only minor loci have been identified for each mechanism (Matthus et al., 2015; Melandri et al., 2021; Wairich et al., 2021). This may indicate multiple genes control each trait. However, it is also likely that improving phenotyping protocols to specifically screen for a single mechanism without the confounding effects of other mechanisms would result in the detection of QTL with strong enough effects to be applied in marker assisted selection (MAS). Until this is achieved crosses with donors such as L-43 and KA-28, ideally with local varieties already possessing the complementary tolerance mechanism, would be a suitable short-term strategy.

The example of local varieties Tsipala and X265 furthermore indicate that relatively high yields can be achieved despite considerable visible leaf damage. This poses the question to what extent the visual leaf damage reduces photosynthetic rates during grain filling and what degree of damage would be permissible without incurring a yield penalty. As far as we are aware, this has yet to be investigated directly. Presumably the answer will depend on the expected yield level, and it is likely that a target yield of 4–5 t ha^{-1}, which is around 25–40% above the national average of many African countries, can be achieved with plants that exhibit strong symptoms of Fe toxicity towards maturity. To achieve target yields near the potential yield of rice in the humid tropics (i.e., 7–8 t ha^{-1} during the wet season) may require a far-more healthy plant with highly productive source leaves. To what extent plant defences against Fe toxicity need to be maintained during the reproductive stage to maintain such highly productive source leaves and whether the competition for resources between upholding defences and filling a large sink is prohibitive should be investigated further.

5. Conclusions

1. Higher grain yields of tolerant genotypes compared to sensitive ones could be attributed mainly to the plants being kept healthy during the vegetative stages. This was achieved either by reducing Fe uptake (exclusion) or by minimizing the effect of excess Fe uptake through compartmentalization of Fe in older leaves and stems and through higher tissue tolerance.
2. Exclusion mechanisms were relaxed during reproductive growth, leading to increased Fe accumulation in shoots, even in excluder genotypes. But tolerant genotypes were nonetheless able to grow well, and we attribute this to a combination of Fe compartmentalization and tissue tolerance, so that grain filling could proceed relatively unimpeded by increasingly high tissue Fe concentrations.
3. There was no relation between grain yield and visual symptoms, and some genotypes produced high yields despite having strong visual symptoms. Selection for Fe toxicity tolerance based on visual symptoms, particularly at the seedling stage, is therefore overly simplistic and only suitable to identify highly sensitive genotypes, but not for the selection of most tolerant genotypes. The presence or absence of visual symptoms in different tissues may nevertheless be of interest in the detailed study of specific tolerance mechanisms involving compartmentalization or true tissue tolerance.
4. To identify useful donors and markers for breeding it is important to develop screening protocols that capture the individual tolerance mechanisms, keeping in mind that plant growth stages appear to have a strong effect on their relative importance, expression and possible interaction. It is therefore important to allow for changes between growth stages, as well as interactions with mineral nutrition which may change over time.
5. The intensity and dynamics of Fe toxicity are highly site specific and highly variable between years depending on the onset and intensity of rains. Varieties that combine multiple stress tolerance mechanisms will be more resilient than those with single mechanisms. We have identified candidate donors for efficient Fe compartmentalization and tissue tolerance (KA-28), for Fe exclusion (L-43) and for tolerance despite appearing sensitive (Tsipala). Further physiological and genetic studies should investigate underlying causes and genetic factors in these with a view to identifying markers and genes for pyramiding in broadly tolerant genotypes.

CRediT authorship contribution statement

Guy J.D. Kirk and Matthias Wissuwa conceived the project and obtained funding. Toavintsoa Rajonandraina, Tovohery Rakotoson, Matthias Wissuwa and Guy J.D. Kirk designed the experiment. Toavintsoa Rajonandraina and Tovohery Rakotoson performed the bulk of the experimental work. Toavintsoa Rajonandraina analysed the data. Toavintsoa Rajonandraina, Tovohery Rakotoson, Matthias Wissuwa, Yoshiaki Ueda and Guy J.D. Kirk interpreted the results. Toavintsoa Rajonandraina and Guy J.D. Kirk wrote the draft manuscript. All authors edited and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary information

Fig. S1. Grain yield and LBS of 40 genotypes from the initial screening.
Fig. S2. Leaf bronzing symptoms and plant appearance.
Fig. S3. Weather data.
Fig. S4. AGNES dendrogram.
Fig. S5. Iron concentrations in different tissues.
Fig. S6. Iron concentrations in the soil solution.
Table S1. Durations of vegetative and reproductive stages.
Table S2. Relations between grain yield and other variables obtained using lasso regression.
Table S3. Relations between LBS and other variables obtained using lasso regression.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fcr.2023.108953.

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