Rice grain Fe, Mn and Zn accumulation: How important are flag leaves and seed number?

R.A. Sperotto[1,3], F.K. Ricachenevsky[2], V. de A. Waldow[2], A.L.H. Müller[4], V.L. Dressler[4], J.P. Fett[1,2]

[1]*Department of Botany, Federal University of Rio Grande do Sul, Porto Alegre, Brazil*
[2]*Center for Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil*
[3]*Biologial Sciences and Health Center, Graduate Program in Biotechnology (PPGBiotec), University Center UNIVATES, Lajeado, Brazil*
[4]*Department of Chemistry, Federal University of Santa Maria, Santa Maria, Brazil*

ABSTRACT

Flag leaves play an important role in synthesis and translocation of photoassimilates in the rice plant, affecting grain yield; similarly they were believed to be a major source of remobilized minerals for the seeds. At the same time, the seed's sink strength plays an important role in dry matter accumulation. To investigate the relative contribution of rice flag leaves and seed sink strength to seed mineral accumulation, field experiments were conducted to evaluate the effect of flag leaf or half-seed removal on seed Fe, Mn and Zn concentration and content. Flag leaf or 50% of the seeds were removed at anthesis. Seed Fe, Mn and Zn accumulation were not affected by flag leaf or second leaf removal. Plants with only half of the seeds showed higher Fe and Zn seed concentrations, but similar Mn concentrations. It is concluded that the flag leaf is not necessary for metal remobilization to the seeds and that seed sink strength and seed number have different roles in Fe/Zn and Mn seed concentrations.

Keywords: anthesis; flag leaf; iron; manganese; seed sink strength; zinc

Flag leaves play a major role in synthesis and translocation of photoassimilates to the rice seeds, affecting grain yield. Removal of the rice flag leaf at any stage after panicle emergence was reported to cause significant reduction in grain yield (Singh and Ghosh 1981). Another report showed that flag leaf contributed to 45% of rice grain yield and, when removed, was the major component for yield loss (Abou-Khalifa et al. 2008). According to Mae (1997), 60–90% of total carbon in the panicles at harvest is derived from photosynthesis after heading, while 80% or more of nitrogen (N) in the panicles at harvest is absorbed before heading and remobilized from vegetative organs. In wheat, up to 34.5% grain yield reduction was reported after flag leaf removal at the heading stage (Mahmood and Chowdhry 1997), while Birsin (2005) showed that flag leaf removal resulted in approximately 13, 34, 24% reduction in grain per spike, grain weight per spike and 1000-grain weight, respectively, and 2.8% increase in grain protein contents. Similarly, rice flag leaves are also believed to be a major source of remobilized minerals for the seeds, and recent reports tried to correlate gene expression levels on flag leaves with concentration of mineral nutrients in rice seeds (Narayanan et al. 2007, Sperotto et al. 2009, 2010). However, to the best of our knowledge, no single report has pointed flag leaves as the major source of metals to the rice developing seeds.

Raul A. Sperotto and Felipe K. Ricachenevsky contributed equally to this work.

Seed sink strength has been suggested to control dry matter accumulation on rice seeds. The poor grain filling of *indica-japonica* rice hybrids was suggested to be related to deficient seed sink strength, and dry matter accumulated by rice seeds was linearly related to the increase in endosperm cell number and the activities of starch biosynthesis-related enzymes (Liang et al. 2001, Mohapatra et al. 2009). According to Yang et al. (2002), poor grain filling of *indica-japonica* rice hybrids can also be related to poor translocation and partitioning of assimilates to the grains, resulting in more resources for vegetative growth. Recently, Yang and Zhang (2010) suggested that carbohydrate supply should not be the major problem, because inferior spikelets (which have slow grain filling rate and low grain weight) showed adequate sucrose levels at their initial grain filling stage. Low activities of key enzymes in carbon metabolism may contribute to poor grain filling (Yang and Zhang 2010), as well as high cytokinin levels in rice shoots, which can improve grain filling of inferior spikelets (Zhang et al. 2010). However, there is no previous report indicating a role of seed sink strength, based on seed number and seed metal accumulation. If sink strength plays a major role, the final seed metal content could be highly affected by the total number of seeds in a plant. On the other hand, a fixed content of metals could be remobilized from vegetative tissues and distributed into the existing seeds, independently from seed number.

The aim of this study was to investigate the relative contribution of rice flag leaves and seed sink strength to seed Fe, Mn and Zn accumulation through flag leaf or half-seed removal. We are not aware of previous publications in which such approaches were used to investigate metal remobilization in rice plants, except for nitrogen in wheat plants (Birsin 2005).

MATERIAL AND METHODS

Plant growth conditions. Plants of rice (*Oryza sativa* L. ssp. *japonica*) cv. Nipponbare were grown in soil under flooded conditions in an experimental unit of the Rio-Grandense Rice Institute (IRGA), in Cachoeirinha, RS, Brazil (29°54'58.61''S, 51°10'02.65''W), during the rice growing season (October 2010 to March 2011). Soil characteristics of this site were reported by Stein et al. (2009). Flag leaves, second upper leaves or 50% of the seeds were removed from eight plants with multiple tillers at anthesis, which corresponds to the R4 stage, according to Counce et al. (2000). Removal of 50% of the seeds was performed alternately from the bottom to the top of each panicle. Tissues (entire flag leaf blades or seeds) were collected during R9 (full maturity) stage, according to Counce et al. (2000).

Sample preparation and determination of Fe, Mn and Zn by ICP-OES. Rice tissues samples were previously dried in air circulation drying oven at 60°C during 48 h. Samples were decomposed using a heating block (Model DK 42, Velp Scientifica, Usmate Velate, Italy) with maximum capacity of 42 glass (borosilicate glass) tubes (L = 30 cm and ø = 2 cm). All tubes were closed with polypropylene caps. About 200 mg of sample were weighed and transferred to the glass tubes and 4 mL of 14 mol/L HNO_3 was added. The mixtures were heated according to the following steps: 100°C during 1 h and 150°C during 1 h. Final solutions were diluted with ultra-pure water (18.2 MΩ cm) to 30 mL in polypropylene vessels.

An inductively coupled plasma optical emission spectrometer (ICP-OES), model Spectro Ciros CCD simultaneous spectrometer (Spectro Analytical Instruments, Kleve, Germany) with axial view configuration was used for Fe, Mn and Zn determination. A crossflow nebulizer coupled to a double pass (Scott type) nebulization chamber was used throughout. Operating conditions used for Fe, Mn and Zn determinations were the following: RF power: 1400 W; plasma gas flow rate: 13.00 L/min; auxiliary gas flow rate: 1.00 L/min; nebulizer gas flow rate: 0.95 L/min. The analytical wavelengths for Fe, Mn and Zn determinations were 259.941, 257.611 and 213.856 nm, respectively. Argon of 99.996% purity (White Martins, Praxair, São Paulo, Brazil) was used for plasma generation. Apple leaves (NIST 1515) and pine needles (NIST 1575a) of certified reference materials (CRMs) were used for accuracy evaluation of the method. The CRMs were decomposed using the same conditions used for the samples.

Statistical analyses. When appropriate, data were subjected to analyses of variance (ANOVA) and means were compared by the Tukey HSD (honestly significant differences) test or Student's *t* test ($P \leq 0.05$ and $P \leq 0.01$) using the SPSS Base 12.0 for Windows (SPSS Inc., New York, USA). The Levene's test (for homogeneity of variance) was used prior to ANOVA.

RESULTS AND DISCUSSION

Flag leaf removal does not change Fe and Zn concentration or content in mature seeds. To evaluate the relative importance of flag leaves as sources of remobilized metals to rice seeds, we analyzed the effects of flag leaf or second upper leaf removal. As seen in Figures 1B and C, flag leaf or second upper leaf removal promoted no statistical difference on Fe, Mn and Zn concentrations and contents in rice seeds. Several studies emphasized the importance of remobilization of reserves to supply rice seeds with minerals (Jiang et al. 2007, 2008, Fang et al. 2008, Wu et al. 2010, Yoneyama et al. 2010), but the real contribution of stored minerals to total seed mineral content is unclear. Data from our group shows that mineral remobilization can be severely affected by Fe status. Rice plants watered with a high Fe concentration (200 µmol/L) showed only K and S remobilization from flag leaves. With abundant Fe supply at the root level, continued uptake during seed fill may have reduced the need for remobilization to serve as a source of Fe for seeds. On the other hand, plants watered with low Fe concentration (5 µmol/L) showed the highest Fe remobilization, probably due to reduced uptake during seed fill (Sperotto et al. 2012). Under Zn deficiency, a similar pattern had been previously observed (Jiang et al. 2008). In *Arabidopsis*, continuous uptake and translocation of minerals to source tissues during seed fill is as important, if not more important, than remobilization of previously stored minerals (Waters and Grusak 2008). Our data show that remobilization from flag leaves in rice is not absolutely required for seeds to acquire minerals in plants growing in field conditions. It is known that loss-of-function of vacuolar iron transporters OsVIT1 and OsVIT2, which are highly expressed in flag leaves, leads to increased concentrations of Fe and Zn in grains. Presumably, these increases are due to lower sequestration of metals in the vacuoles of flag leaf cells, which in turn increases availability of Fe and Zn for remobilization to developing seeds (Zhang et al. 2012). Considering that OsVIT1 and OsVIT2 are preferentially expressed in flag leaves, it suggests they are the main site for metal remobilization.

It is possible that flag leaves are preferential but are not essential as a source of metals, and flag leaf removal can probably be compensated by other leaves and stem/sheath remobilization and/or continuous uptake by roots.

Senescence in cereals is regulated at the individual leaf level, with older leaves mobilizing nutrients to younger leaves and eventually to the flag leaf (Gregersen et al. 2008). Thus, it is conceivable that the absence of flag leaves would yield similar Fe and Zn concentrations, as during senescence a plant without flag leaves could remobilize nutrient pools from other sources. Testing the effect of flag leaf removal at later time points, when senescence has already started, and comparing wild-type plants to senescence-delayed lines such as the NAM-B1 RNAi in wheat (Uauy et al. 2006), could add information to the relative importance of flag leaves as a pool for Fe and Zn remobilization.

Differential role of seeds and seed number in Fe/Zn or Mn seed concentrations. To test

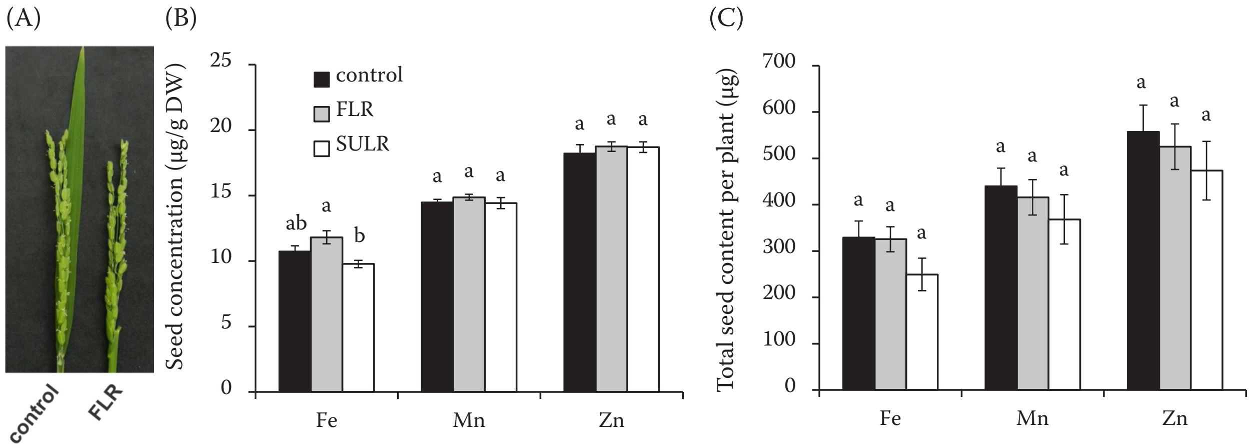

Figure 1. Intact (control) and flag leaf removed (FLR) rice plants (A) at R4 stage. Fe, Mn and Zn concentrations (B) and contents (C) in seeds of intact (control), flag leaf removed (FLR) and second upper leaf removed (SULR) rice plants. Seeds were collected at R9 (full maturity) stage. Values are the averages of eight samples ± SE. Different letters indicate that the means are different by the Tukey HSD test ($P \leq 0.05$). DW – dry weight

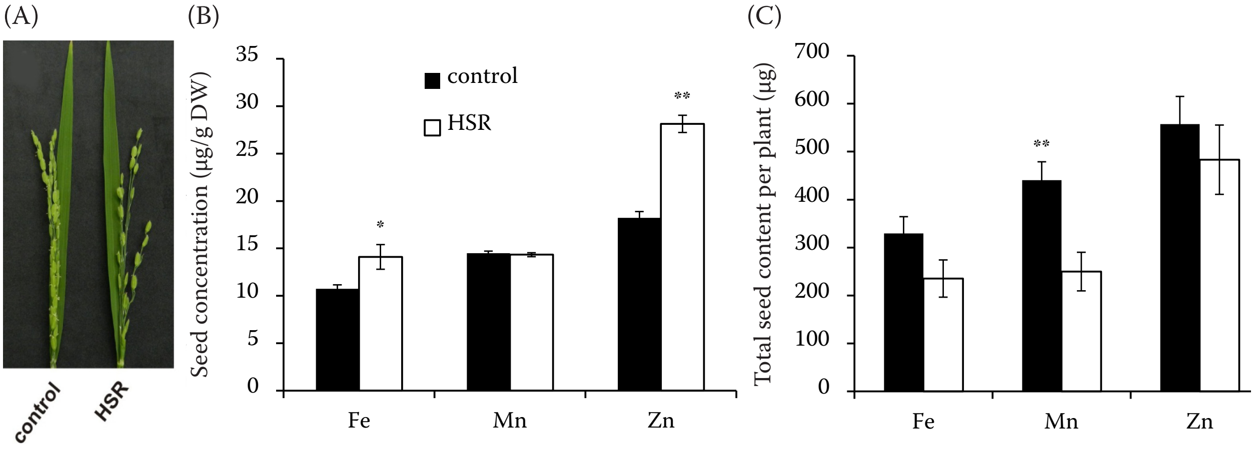

Figure 2. Intact (control) and half-seed removed (HSR) rice plants (A) at R4 stage. Fe, Mn and Zn concentrations (B) and contents (C) in seeds of intact (control) and half-seed removed (HSR) rice plants. Seeds were collected at R9 (full maturity) stage. Values are the averages of eight samples ± SE. **P* ≤ 0.05; ***P* ≤ 0.01 (*t*-test); DW – dry weight

the hypothesis that a fixed amount of minerals is translocated to rice panicles independently of seed number, we removed 50% of the seeds at anthesis. Individual seed weight was not affected by the treatments (data not shown). As seen in Figure 2B, Fe and Zn concentrations were statistically higher in the remaining seeds when compared to seeds of intact plants, indicating that fixed amounts of both metals were translocated to panicles regardless of seed number. However, the observed increase (approximately 31% and 54%, respectively) is lower than 100%, which would be expected if the amount of remobilized Fe and Zn was not dependent on seed number. At the same time, no statistical differences were found in total Fe and Zn contents of intact and half-seed plants. Lower than expected increase in Fe and Zn concentrations indicates that seed number is also an important trait that may help to control the seed sink strength for Fe and Zn translocation to seeds. Taken together, these data suggest that the amount of Fe and Zn translocated to the rice seeds is not completely controlled by the seeds, but also by other factors from the source tissues. The dry matter accumulation in rice grains is also determined by more than one factor, namely endosperm cell number, activity of starch biosynthesis-related enzymes and cytokinin levels (Liang et al. 2001, Zhang et al. 2010).

Surprisingly, half-seed removal did not change Mn concentration of remaining rice seeds, and Mn content in seeds was about 2-fold higher on the seeds of intact plants (Figure 2C). These results suggest that, contrary to Fe and Zn, Mn translocation to panicles may be controlled at the individual seed level. One rice YS1-like gene, OsYSL2, encodes a rice metal-NA transporter that may be responsible for the phloem transport of Fe and Mn and the translocation of Fe and Mn into the grain (Koike et al. 2004). It seems very likely that additional transport proteins are involved in these processes, which would explain the distinct effects observed for Fe and Mn.

Concluding remarks. In summary, this study suggests that flag leaves are not necessarily the major source of Fe, Mn and Zn for rice developing seeds. Probably, a small portion of minerals from flag leaves is incorporated into panicles and seeds. However, considering that flag leaves have extremely low mineral content compared to other vegetative tissues (Sperotto et al. 2012), the maximum possible contribution of flag leaves to panicle mineral content is really low, different of what was found for photoassimilates. This study also suggests that seed number per panicle has differential roles for Fe/Zn or Mn. Mn concentrations in rice seeds are the same regardless of seed number in a panicle, while Fe and Zn concentrations are determined by both seed number and factors in source tissues.

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Corresponding author:

Janette Palma Fett, Ph.D., Federal University of Rio Grande do Sul, Center for Biotechnology and Department of Botany, Avenida Bento Gonçalves, 9500, Porto Alegre, RS, 91501-970, Brazil
phone: + 55 51 3308 7643, e-mail: jpfett@cbiot.ufrgs.br