Iron and zinc bioavailability from Madhukar × Swarna derived biofortifed rice lines

Raghu Pullakhandam[1,#], Surekha Agarwal[2,#], V. G. N. Tripura Venkata[2], C. V. Ratnavathi[3], K. Madhavan Nair[1] and Sarla Neelamraju[2,*]

[1]ICMR-National Institution of Nutrition, Indian Council of Medical Research, Hyderabad 500 007, India
[2]ICAR-Indian Institute of Rice Research, Hyderabad 500 030, India
[3]ICAR-Indian Institute of Millets Research, Hyderabad 500 030, India

Rice is the prime target of biofortification as it provides calories for about half of the world's population. We assessed the iron (Fe) and zinc (Zn) bioavailability from polished rice grains of three high Fe and Zn recombinant inbred lines (RILs) using simulated *in vitro* digestion/Caco-2 cell model. Ferritin induction and [65]Zn uptake were used as surrogate for Fe and Zn bioavailability respectively. Fe and Zn concentration in both unpolished and polished rice grains of three RILs was higher compared to Swarna, a parent and popular variety used as control. The grain Fe concentration was positively correlated ($r = 0.94$) to Zn concentration. There was a 2-fold induction of ferritin (42.4 ± 3.2 ng/mg protein) in Caco-2 cells only in the presence of ascorbic acid and 3-fold increase in [65]Zn uptake (17.7 ± 2.4 pmol/mg protein) from the RIL 185M compared to Swarna (ferritin: 24.8 ± 4.0 ng/mg protein; [65]Zn uptake: 5.8 ± 0.3 pmol/mg protein). Phytic acid was highest (8.75 mg/g) in 185 M but that did not affect bioavailability of Fe and Zn. Thus, improving the density of Fe and Zn in grains of conventionally bred rice lines has the potential to enhance the bioavailability of Fe and Zn.

Keywords: Ascorbic acid, Caco-2 cells, ferritin induction, phytic acid, recombinant inbred lines.

RICE (polished/white) is a staple food for over 50% of the world's population, but has little bioavailable iron (Fe) and zinc (Zn) to meet human requirements. In general, non-heme Fe, the major form of Fe in plant foods, is much less bioavailable (2% to 10%) than heme-Fe (15–35%) derived from animal foods[1–3]. Both Fe and Zn deficiencies are likely to coexist, as their absorption in human intestines is inhibited by phytic acid, a secondary metabolite in plant foods[4]. Thus, improving both Fe and Zn concentration and their bioavailability in rice grains is expected to reduce the deficiencies of these nutrients among populations primarily dependent on rice-based diet.

Attempts are being made to enhance Fe and Zn levels in rice through agronomic fortification by application of fertilizer or biofortification via conventional plant breeding or genetic engineering[5,6]. Recombinant inbred lines (RILs) with high grain Fe and Zn concentration have been developed in rice using conventional breeding methods[6]. Though increase in Fe and Zn concentration in rice grains has been shown using genetic engineering methods, but field level studies are lacking[5,7]. Very few studies address bioavailability of Fe and Zn biofortified rice grains, which is required for the intended nutritional outcomes[8]. Further, progress is also being made in identifying new plant compounds such as nicotianamine and ascorbic acid, and other compounds such as EDTA that stimulate bioavailability of Fe from food staples[9–11] and also in identifying food synergies[12,13].

In our previous studies, we identified several RILs from the cross Madhukar × Swarna with high Fe and Zn concentration in grains[6]. Candidate genes were identified and analysed for their expression under Fe deficiency to evaluate whether the expression of metal homeostasis genes in seedlings was affected with the higher level of Fe and Zn in seeds[14]. However, retention of Fe and Zn during polishing and their bioavailability relative to that of the control variety Swarna needs to be tested to advance these lines for use in national biofortification program.

The Fe and Zn bioavailability from food grains is influenced by many factors, including food matrix, processing, and composition of the meal apart from nutritional and physiological status of the host[15]. It is known that phytic acid chelates and inhibits intestinal Fe and Zn absorption while ascorbic acid and nicotianamine stimulate iron absorption[4,11]. Phytic acid or inositol hexaphosphate abundantly present in cereal grains is the major limiting factor in mineral bioavailability, including iron and zinc. Polishing of rice grains reduces phytic acid, and thus improves iron absorption[16]. Simple dietary combinations like inclusion of fresh fruits and vegetables stimulates the bioavailability of iron from cereal foods. For instance, consumption of guava, a rich source of vitamin C, induced iron absorption from rice-based meal in adolescents, but fractional Zn absorption remained unaltered[12,13].

Fe and Zn bioavailability/absorption is assessed by measuring the proportion of ingested nutrients from a test food, that is either stable or traced with radioisotopes[12]. But these methods are both cost and labour intensive, and thus are not suitable for routine screening. Simulated Caco-2 cell (colon adenocarcinoma cells) model has been established as an alternate screening method[17–20]. The model entails feeding of differentiated Caco-2 cells with test food after sequential gastric and intestinal *in vitro* digestion. Further, it has been demonstrated that induction of ferritin expression in response to dietary Fe could be used as a surrogate marker of Fe bioavailability[17,21]. It has also been established that radioactive [65]Zinc ([65]Zn) uptake in Caco-2 cells from digests (traced with [65]Zn) parallels the observation in humans[19].

In this study, we assessed bioavailability of both Fe and Zn in polished rice grains of selected RILs (designated as 176M, 185M and 196M) using coupled *in vitro* digestion/Caco-2 cell model. These lines have high grain Fe and Zn concentration and the results were compared with that of one parent Swarna, a local popular rice variety.

Caco-2 cells were obtained from National Centre for Cell Sciences, Pune, India. All the reagents and digestive enzymes were procured from Sigma Chemical Co., Bangalore, India, unless otherwise specified. [65]Zn was obtained from Oakridge Radioisotopes, USA.

Rice variety Madhukar grown in deep-water has high Fe and Zn and Swarna (MTU 7029), grown widely in lowlands of India and Bangladesh has low glycemic index. F6 RILs were developed from Madhukar × Swarna cross using single seed descent method[6]. Swarna parent was chosen as control as it is a popular widely grown rainfed lowland cultivar in South and Southeast Asia. It was developed in Maruteru, Andhra Pradesh, India and released in 1982. Three RILs (176 M, 185 M and 196 M) were chosen based on their high Fe and Zn concentration in polished grains. Also, line 176 M was found to have the highest number (11) of quantitative trait loci (QTLs) that improve Fe and Zn concentration identified either from Madhukar or Swarna. 185 M had 6 such QTLs and 196 M had 5 such quantitative trait loci (QTLs)[6].

100 g of moisture-free seed was dehusked manually using metal free palm dehusker to avoid any metal contamination. The dehusked and cleaned brown rice was polished for 95 sec using Fe and Zn free polisher (M/s Krishi International). To ensure consistency in micronutrient analyses, three replicates of seeds were analysed. Polished seeds were analysed for Fe and Zn concentration using energy dispersive X-ray fluorescence (Oxford instruments X-supreme) spectrometry method[22].

Phytic acid concentration of polished rice grains was estimated as per the methods mentioned in Wheeler and Ferral[23].

Caco-2 cells were cultured and differentiated in 6-well plates as described previously[24,25].

The polished rice grains (2 g) were cooked in 3 volumes of water for 30 min and homogenized to fine slurry, the final volume was made up to 9 ml with normal saline solution (0.9% aqueous NaCl) and immediately subjected to simulated *in vitro* digestion as described below.

The rice homogenate was subjected to simulated *in vitro* gastric digestion either in the presence or absence of 1 : 20 molar ratio of Fe to ascorbic acid as described previously for assessing Fe bioavailability[21]. For assessing Zn bioavailability, the homogenates were traced with 5 μCi of [65]Zn prior to the initiation of digestion reaction[19]. The pH of these homogenates was adjusted to 2.0 with HCl followed by addition of 0.5 ml of pepsin (40 mg/ml) and incubated for 1 h at 37°C in a shaking water bath. For simulating intestinal digestion, the pH

was raised with 1 M NaHCO$_3$ to 6.0 and 2.5 ml of pancreatin/bile mixture (0.05 g of pancreatin and 0.3 g of bile in 25 ml 0.1 M NaHCO$_3$) was added. Finally, the pH of all the samples was adjusted to 6.7 with 1 M NaOH, and the final volume adjusted to 15 ml with normal saline (0.9% NaCl) and immediately fed to the differentiated Caco-2 cells as described below. Two mililitre of milli-Q water was subjected to *in vitro* digestion, and used as control. Ferric chloride and ascorbic acid were prepared fresh and mixed in a molar ratio of 1 : 20 just before the experiment and was used as positive control. ZnCl$_2$ (20 μM) traced with 5 μCi of [65]Zn was used as a reference for assessing [65]Zn uptake.

At the end of incubation, the medium was aspirated, and the monolayers were washed 3 times with 10 mM phosphate buffer saline pH 7.2 (PBS). The cells were scraped with a rubber policeman in 500 μl PBS and lysed by sonication. The Micro BCA kit method was used to estimate the protein content of the cell lysates. The ferritin content of cell lysates was estimated by a human ferritin sandwich ELISA method described previously[21]. For estimation of zinc uptake, the cell monolayers were washed thrice with N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer at pH 7.2, containing 1 mmol/l EDTA, to remove non-specifically bound zinc. The cells were scraped in 400 μl of PBS, and the cell-associated [65]Zn radioactivity was measured in a gamma counter (GRS-201 L, PLA electro, Mumbai, India), as described previously[19].

Three replicates were considered in all experiments. The experiments were repeated thrice to generate 9 independent observations. The mean and standard deviation (SD) were computed using Microsoft Excel. One-way ANOVA and least significant difference post hoc test was used in SPSS package version 7 to test statistically significant ($P < 0.05$) differences.

The seeds of four rice lines with and without polishing are shown in Figure 1. The RILs had darker husk than Swarna but all the polished grains were white. The grain Fe, Zn and phytate concentration of three RILs and control, Swarna before and after polishing are given in Table 1. The Fe concentration in both unpolished and polished rice grains of three RILs was higher compared to Swarna. The Fe and Zn concentration was the highest in polished grains of 185 M (14.2 μg/g Fe, 33.2 μg/g Zn) followed by 196 M (8.9 μg/g Fe, 28.2 μg/g Zn) and 176 M (7.7 μg/g Fe, 23.3 μg/g Zn) as compared to that of Swarna. Grain Fe and Zn concentration of the three RILs was found to be positively correlated ($r = 0.94$). However, the phytate concentration was not correlated with either Fe ($r = 0.42$) or Zn ($r = 0.1$) concentration in unpolished rice grains. Phytic acid concentration was the highest in 185 M (8.75 mg/g), followed by 176 M (8.25 mg/g), Swarna (7.8 mg/g) and 196 M (4.0 mg/g).

Ferritin induction was similar in cells treated with ferric Fe or saline control. In absence of ascorbic acid,

Table 1. Iron, zinc and phytic acid concentration of rice RILs developed from cross Madhukar × Swarna

Sample	Iron (µg/g)				Zinc (µg/g)				Phytate (mg/g)		% loss during polishing	
	Unpolished	SD	Polished	SD	Unpolished	SD	Polished	SD	Polished	SD	Iron	Zinc
Swarna	17.1	1.069268	5.6	0.360555	27.8	1.053565	14	0.472582	7.8	1.03	67.2	49.6
176 M	18.1	0.585947	7.7	1.021437	28.7	0.404145	23.3	0.602771	8.25	0.000	57.4	18.8
185 M	21.8	0.360555	14.1	0.750555	38.8	0.964365	33.2	0.665833	8.75	0.948	35.3	14.4
196 M	14.3	0.64291	8.9	0.321455	29.7	0.90185	28.2	0.888819	4	0.134	37.7	5.05

Values are the mean of three replicates. SD, Standard deviation.
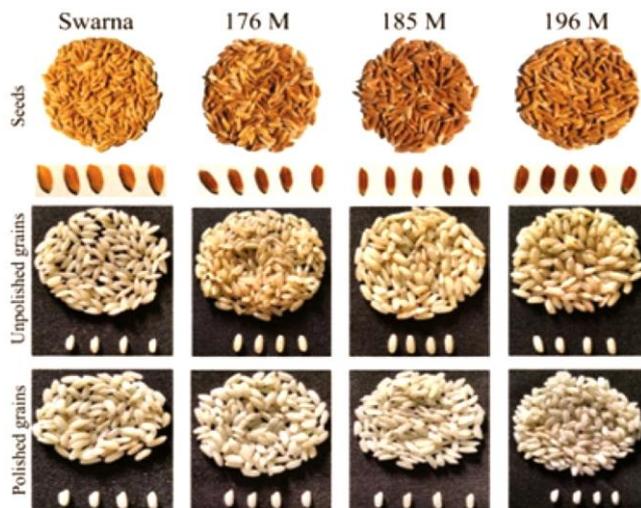

Figure 1. Rice seeds with husk, unpolished and polished grains of recombinant inbred lines and Swarna.

the ferritin concentration (13.6 ± 2.5 ng/mg protein) remained similar in all the rice lines. However, inclusion of ascorbic acid increased the ferritin concentration from all the rice lines significantly, except from that of 176 M (18.2 ± 3.2 ng/mg protein) (Figure 2 a). Further, ferritin concentration from 185 M (42.4 ± 3.2 ng/mg protein) was significantly higher than from Swarna (24.8 ± 4.0 ng/mg protein). The bioavailability of Fe therefore was 2-fold higher from 185 M compared to Swarna in the presence of ascorbic acid and 3-fold higher compared to 185 M without ascorbic acid (Figure 2 b).

^{65}Zn (pmol/mg protein) uptake from all the four rice lines was significantly lower compared to $ZnCl_2$ control. ^{65}Zn uptake in all the three RILs was significantly higher compared to Swarna (5.8 ± 0.3 pmol/mg protein) (Figure 3). Among the three RILs, ^{65}Zn uptake was highest from 185 M (17.7 ± 2.4 pmol/mg protein) followed by 176 M (12.1 ± 0.7 pmol/mg protein) and 196 M (9.1 ± 0.4 pmol/mg protein).

Biofortification allows enrichment of micronutrients such as Fe and Zn in food crops via conventional plant breeding or transgenic approaches to fill the nutritional gaps of the population. However, adequate bioavailability of target nutrients from these foods needs to be ensured for the anticipated benefits of their consumption. We

have previously reported the development of several RILs from the cross Madhukar × Swarna and lines with high grain Fe and Zn concentration[6,14]. We selected three best RILs with high grain Fe/Zn concentration for studying the Fe and Zn retention during polishing and bioavailability using simulated in vitro digestion/Caco-2 cell model. The results demonstrated that significant amount of Fe and Zn were lost during polishing of rice, yet remained higher compared to Swarna (Table 1). Overall, the percent loss of Fe (35.3% to 67.2%) was more than the percent loss of Zn (5.05% to 49.6%) after polishing. Likewise, loss of 65% of the total Fe and 43% of the total Zn was reported after polishing[26]. Most of the Fe and Zn in rice grain is localized in the aleurone layer, which is lost during polishing, leading to reduction in Fe and Zn concentration[26,27]. The Fe concentration of both unpolished and polished rice grains was highly correlated with corresponding Zn concentration. Previous reports also showed a positive correlation between seed Fe and Zn concentration in rice[28] and in both unpolished and polished grains[5]. In field trials also, 185 M [IET23814] showed high mean Zn concentration (20 µg/g in 2013 and 31 µg/g in 2014 using XRF) in polished rice consistently when grown in All India Coordinated Rice Improvement Project (AICRIP) multi-location trials at 12 locations across India in 2013 and 16 locations in 2014. The mean Fe concentration, however, was low (2.8 µg/g in 2013 and 3.3 µg/g in 2014). Also, the grain yield of 185 M was quite low (2453 kg/ha in 2013 and 2725 kg/ha in 2014 AICRIP Biofortification trials). Fe concentration varies with location but Zn values appear to be more consistent[29]. The mean Zn concentration of 185 M over all locations has been the highest, reported in AICRIP biofortification trials from 2013 to 2018. 176 M [IET24764] showed 20.54 ppm Zn and 3243 kg/ha yield in 2014 AICRIP biofortification trials. It is known that environment, genotype and genotype × environment interaction significantly affect Fe concentration in rice grains[30].

In our study, phytic acid concentration in polished rice did not correlate with grain Fe or Zn concentration. Liang et al.[31] also found no correlation between Fe and phytic acid levels but found a week correlation between Zn and phytic acid levels among 56 varieties of Chinese rice. Pelig-Ba[32] also reported that there was no correlation
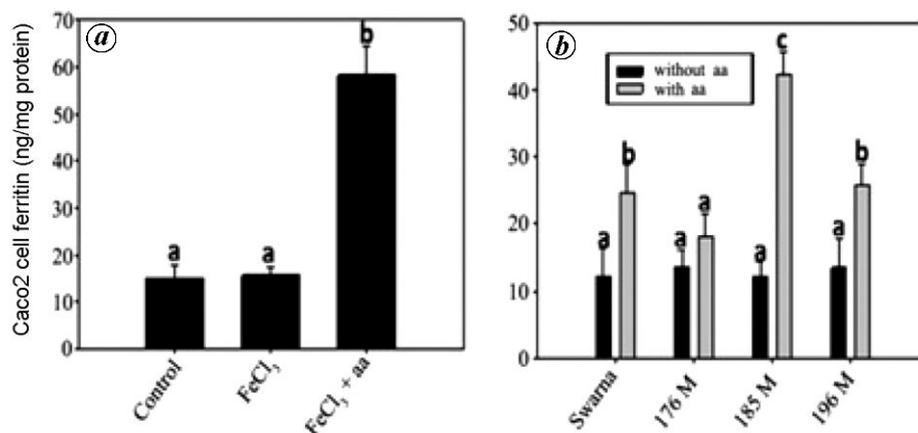
Figure 2. Effect of ferric chloride, ascorbic acid and rice grains in ferritin induction in Caco-2 cells: Saline (control) or ferric chloride (*a*) or 2 g of cooked rice grains (*b*) were subjected to simulated *in vitro* digestion and fed to the differentiated Caco-2 cells as described in methods section. The Caco-2 cell ferritin concentration was estimated by ELISA method. The experiments were done in triplicate and repeated thrice to generate 9 independent observations. The bars with different superscripts differ significantly ($P < 0.05$). aa: ascorbic acid.
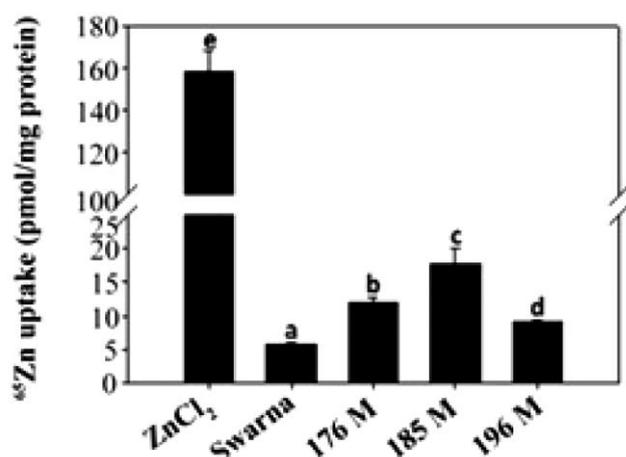

Figure 3. ^{65}Zn uptake from $ZnCl_2$ and cooked rice: Zinc chloride or 2 g of cooked rice grains were traced with ^{65}Zn and subjected to simulated *in vitro* digestion and fed to the differentiated Caco-2 cells as described in methods section. The cell associated ^{65}Zn radioactivity was measured using gamma counter. The experiments were done in triplicate and repeated thrice to generate 9 independent observations. The bars with different superscripts differ significantly ($P < 0.05$).

between phytic acid and Fe and Zn, and that the low phytic acid level has little influence on the level of trace metals in cereals and in human nutrition.

Ferritin induction in Caco-2 cells treated with ferric form of Fe or digests of the rice grains from 3 RILs remained similar to the control Swarna in the absence of ascorbic acid. Fe bioavailability studies from biofortified lines have been carried out with and without ascorbic acid (vitamin C) previously and helped to rank the rice genotypes for assessing Fe bioavailability[18]. Consistent with their results, we also observed that inclusion of vitamin C markedly increased ferritin induction in Caco-2

cells. Line 185 M with highest Fe induced more ferritin in Caco-2 cells compared to 176 M or 196 M. The bioavailability of Fe was 2-fold more in 185 M compared to Swarna in the presence of ascorbic acid and 3-fold more compared to 185 M without ascorbic acid. Trijatmiko et al.[33] determined the Fe bioavailability in T4 polished grains of transgenic events NASFer-234 and NASFer-274 and untransformed IR64 rice in the absence/presence of ascorbic acid using Caco-2 cells. They also showed 2–3 fold increase in Fe bioavailability for both the transgenic events in the presence of ascorbic acid. Glahn et al.[18], compared Fe bioavailability from unpolished rice of 15 selected Fe-dense and normal genotypes with control variety Nishiki using Caco-2 model. Fe concentration in rice samples ranged from 14 to 39 µg/g. They observed no correlation between Fe uptake and grain-Fe concentration. We observed ferritin induction increases with the grain Fe concentration but only in presence of ascorbic acid. Thus, it would be better to screen and select rice lines for improved Fe bioavailability rather than Fe concentration in order to increase the supply of Fe in target populations.

Increasing the concentration of Fe in rice grains increased the bioavailable Fe in rat models fed with ^{59}Fe labelled grains of six rice genotypes[34]. The Fe concentration in six genotypes ranged from 17.5 to 38.6 µg/g and Fe bioavailable to rats ranged from 12 to 23 µg. Similarly in beans, Fe concentration in 24 genotypes ranged from 51 to 156 µg/g and Fe bioavailable to rats ranged from 32 to 105 µg (ref. 34). Tako et al.[35], developed high iron maize through molecular breeding and showed more absorbable iron from high Fe maize. They reported more ferritin induction from high Fe maize line (22.51 ± 0.9 ng/mg of total protein) than from low Fe maize line (13.40 ± 0.6 ng/mg of total protein) in Caco-2 cells.

However, ascorbic acid was not included in their experiment. The bioavailability of Fe and Zn from a typical rice meal in children aged 13–15 years, was enhanced two-fold by inclusion of 100 g of guava fruit which is rich in ascorbic acid[12]. Therefore, it appears possible that the bioavailability of iron from biofortified rice lines could be improved further by simultaneous consumption with ascorbic acid rich vegetables and fruits. The synergy in consumed foods is thus very important[13].

185 M showed highest ferritin induction despite its phytic acid concentration being twice that of 196 M, suggesting that phytic acid is not the only determinant of Fe absorption from rice grains. Similar to these results, Glahn et al.[18] and Promuthai et al.[36] also reported that phytic acid concentration and Fe absorption in rice varieties was not significantly correlated. However, in wheat, Eagling et al.[37], showed that phytic acid is the main determinant of Fe bioavailability. Recently, Petry et al.[38], showed that Fe absorption in young women increases due to selective reduction of phytate in common bean (Phaseolus vulgaris L.) seeds. A 90% reduction in phytic acid leads to an increase in bioavailable Fe from beans, independent of the polyphenol concentration. They suggested that the low phytic acid mutation could be a key tool for improving Fe bioavailability from beans. Sedef Nehir et al.[39], also found that the phytate : Fe molar ratio was not the major inhibitory factor for Fe bioavailability in cereals for infants. Other possible factors such as type of Fe compound (i.e. $FeSO_4$, $FeCl_3$, etc.) used in fortification and possible interactions of Fe with other ingredients in the diet can affect Fe bioavailability. Culinary practices such as soaking, heating, germination and microbial fermentation have been found to reduce phytates and polyphenol levels. The in vitro bioavailability of Fe and Zn from three white sorghum varieties was significantly improved as a result of soaking (Fe: 14.62–20.75 and Zn: 9.07–10.72%) and germination (Fe: 16.67–20.63 and Zn: 12.06–18.30%) treatments as compared to raw sorghum (Fe: 8.02–13.60 and Zn: 7.35–9.73%)[40].

Recently, nicotianamine, a chelator of Fe, was shown to stimulate Fe absorption in Caco-2 cells with higher activity compared to vitamin C (ascorbic acid) or other plant metal chelators[37]. Zheng et al.[11], showed two fold increase in Fe bioavailability from rice grains due to overexpression of nicotianamine synthase (NAS) gene compared to respective control rice in Caco-2 cell. Ascorbic acid was not used in their study. In our study a similar fold increase in Fe bioavailability was observed in 185 M but only in the presence of ascorbic acid. The level of NA in 185 M is not known but it is possible that 185 M has high level of NA. An indirect evidence for this is the observation of 1000-fold high expression of gene OsNAS2 involved in synthesis of NA in 185 M shoots when grown under Fe deficient condition[14]. Estimation of NA in these lines should provide direct evidence of a link between NA and bioavailability of Fe and Zn.

Similar to the results of Fe bioavailability, ^{65}Zn uptake was also found to be significantly higher from 185 M, which had higher Zn concentration in grains compared to the other two RILs. The bioavailability of Zn was significantly higher from all the three RILs compared to Swarna. Among the three RILs the zinc bioavailability was highest (3-fold) in 185 M. Interestingly, ^{65}Zn uptake from the RIL 196 M was lower than that of 176 M, despite its high grain Zn concentration. Also, Fe bioavailability was lowest in 176 M, while Zn bioavailability was lowest in 196 M. It is to be noted that phytic acid concentration of 196 M was only half that of 176 M. Therefore, it appears that as in the case of Fe, phytic acid is not the major determinant of Zn absorption. Lee et al.[7], showed increased Zn concentration in the endosperm of rice when OsNAS2 was over-expressed. They fed Zn depleted mice with OsNAS2-D1 seeds and wild type seeds and showed that body weight of mice fed with OsNAS2-D1 seeds recovered within 6 day, whereas it took 13 days with wild type seeds. Thus, they demonstrated that the level of bioavailable zinc in rice grains can be enhanced significantly by activation tagging of OsNAS2.

The results revealed that improving the grain iron and zinc concentration using conventional plant breeding can also improve the absorption of these minerals in intestinal cells. The RIL 185 M which has high grain Fe also shows high bioavailability of Fe but only when supplemented with ascorbic acid. Also, intestinal cell zinc absorption from 185 M was highest among the three lines consistent with the highest Zn concentration in its grains. Though phytic acid is a potent inhibitor of Fe and Zn absorption, the extent of Fe and Zn absorption from the three rice RILs does not appear to be solely dependent on phytic acid content. Since conventionally bred rice line 185 M has both higher concentration and bioavailability of Fe and Zn, it is an elite line for further nutritional intervention trials in animal models and human subjects. It is also an ideal line to dissect the genetic, physiological or molecular link between high grain Zn concentration and low grain yield. Highly significant negative correlation has been reported between yield and Zn and yield and Fe in rice[41,42]. New genetic resources such as mutants and chromosome segment substitution lines derived from wild germplasm can also be evaluated for high Fe and Zn in polished rice for immediate use in biofortification programs. Any rice line with consistently high zinc in polished grains needs to be fast tracked for seed multiplication and use to prevent zinc deficiency and subsequent morbidity and mortality in rice eating populations.

Conflict of interest: The authors declare no conflict of interest.

1. Monsen, E. R., Iron nutrition and absorption: dietary factors which impact iron bioavailability. J. Am. Diet Assoc., 1988, 88(7), 786–790.

2. Welch, R. M., Breeding strategies for biofortified staple plant foods to reduce micronutrient malnutrition globally. *J. Nutr.*, 2002, **132**, 495S–499S.

3. Zimmermann, M. B. and Hurrell, R. F., Nutritional iron deficiency. *Lancet.*, 2007, **370**, 511–520.

4. Hunt, J. R., Dietary and physiological factors that affect the absorption and bioavailability of iron. *Int. J. Vitam. Nutr. Res.*, 2005, **75**, 375–384.

5. Johnson, A. A. T., Kyriacou, B., Callahan, D. L., Carruthers, L. and Stangoulis, J., Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS ONE,* 2011, **6**(9), e24476.

6. Anuradha, K., Agarwal, S., Venkateswara Rao, Y., Rao, K. V., Viraktamath, B. C. and Sarla, N., Mapping QTLs and candidate genes for iron and zinc concentrations in unpolished rice of Madhukar × Swarna RILs. *Gene*, 2012, **508**, 233–240.

7. Lee, S. *et al.*, Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. *Plant Biotechnol. J.*, 2011, **9**, 865–873.

8. Hoppler, M., Schonbachler, A., Meile, L., Hurrell, R. F. and Walczyk, T., Ferritin-iron is released during boiling and *in vitro* gastric digestion. *J. Nutr.*, 2008, **138**, 878–884.

9. Candela, E., Camacho, M. V., Martinez-Torres, C., Perdomo, J., Mazzarri, G., Acurero, G. and Layrisse, M., Iron absorption by humans and swine from Fe(III)-EDTA. Further studies. *J. Nutr.*, 1984, **114**, 2204–2211.

10. Lynch, S. R. and Stoltzfus, R. J., Iron and ascorbic acid: proposed fortification levels and recommended iron compounds. *J. Nutr.,* 2003, **133**, 2978S–2984S.

11. Zheng, L. *et al.*, Nicotianamine, a novel enhancer of rice iron bioavailability to humans. *PLoS ONE*, 2010, **5**(4), e10190.

12. Nair, K. M. *et al.*, Inclusion of guava enhances non-heme iron bioavailability but not fractional zinc absorption from a rice-based meal in adolescents. *J. Nutr.*, 2013, **143**(6), 852–858.

13. Nair, K. M. and Augustine, L. F., Food synergies for improving bioavailability of micronutrients from plant foods. *Food Chem.*, 2016; doi:10.1016/j.foodchem.2016.09.115.

14. Agarwal, S., Tripura Venkata, V. G. N., Kotla, A., Mangrauthia, S. K. and Neelamraju, S., Expression patterns of QTL based and other candidate genes in Madhukar × Swarna RILs with contrasting levels of iron and zinc in unpolished rice grains. *Gene*, 2014, **546**, 430–436.

15. Hurrell, R. F. *et al.*, Enhancing the absorption of fortification iron. A SUSTAIN Task Force report. *Int. J. Vitam. Nutr. Res.*, 2004, **74**(6), 387–401.

16. Olivares, M., Pizarro, F. and Ruz, M., Zinc inhibits nonheme iron bioavailability in humans. *Biol. Trace Elem. Res.*, 2007, **117**, 7–14.

17. Glahn, R. P., Lee, O. A., Yeung, A., Goldman, M. I. and Miller, D. D., Caco-2 cell ferritin formation predicts non-radiolabeled food iron availability in an *in vitro* digestion/Caco-2 cell culture model. *J. Nutr.*, 1998, **28**, 1555–1561.

18. Glahn, R. P., Cheng, Z., Welch, R. and Gregorio, G. B., Comparison of iron bioavailability from 15 rice genotypes: studies using an *in vitro* digestion/Caco-2 cell culture model. *J. Agric. Food Chem.*, 2002, **50**, 3586–3591.

19. Kilari, S., Raghu, P. and Nair, K. M., Zinc inhibits oxidative stress induced iron signaling and apoptosis in Caco-2 cells. *Free Radic-Biol. Med.*, 2010, **48**, 961–968.

20. Nemirovsky, Y., Zavaleta, N., Villanueva, M. E., Armah, S. M., Iman, S. A. and Reddy, M. B., Negative effect of camu-camu (*Myrciariadubia*) despite high vitamin C content on iron bioavailability, using a Caco-2 cell model. *Pol. J. Food Nutr. Sci.*, 2014, **64**, 45–48.

21. Satyanarayana, B., Raghu, P., Ravinder, P. and Nair, K. M., Gastric digestion of pea ferritin and modulation of its iron bioavailability by ascorbic and phytic acids in Caco-2 cells. *World J. Gastroenterol.*, 2007, **13**, 2083–2088.

22. Paltridge, N. G., Palmer, L. J., Milham, P. J., Guild, G. E. and Stangoulis, J. C. R., Energy-dispersive X-ray fluorescence analysis of zinc and iron concentration in rice and pearl millet grains. *Plant Soil*, 2012, **361**, 251–260.

23. Wheeler, E. L. and Ferral, R. E., A method for phytic acid determination in wheat and wheat fractions. *Cereal Chem.*, 1971, **48**, 312–320.

24. Palika, R., Mashurabad, P. C., Kilari, S., Kasula, S., Nair, K. M. and Pullakhandam, R., Citric acid mediates the iron absorption from low molecular weight human milk fractions. *J. Agric. Food Chem.*, 2013, **61**, 11151–11157.

25. Raghu, P., Nair, K. M., Pamini, H. and Punjal, R., Bioavailability of iron and zinc from multiple micronutrient fortified beverage premixes in Caco-2 cell model. *J. Food Sci.*, 2011, **76**(2), H38–H42.

26. Lu, L., Tian, S., Liao, H., Zhang, J., Yang, X., Labavitch, J. M. and Chen, W., Analysis of metal element distributions in rice (*Oryza sativa* L.) seeds and relocation during germination based on X-ray fluorescence imaging of Zn, Fe, K, Ca, and Mn. *PLoS ONE*, 2013, **8**(2), e57360.

27. Gregorio, G. B., Senadhira, D. H., Tut, H. and Graham, R. D., Breeding for trace mineral density in rice. *Food Nutr. Bull.*, 2009, **21**, 382–386.

28. Sperotto, R. A., Ricachenevsky, F. K., Duarte, G. L., Boff, T., Lopes, K. L. and Sperb, E. R., Identification of up-regulated genes in flag leaves during rice grain filling and characterization of *OsNAC5*, a new ABA-dependent transcription factor. *Planta.*, 2009, **230**, 985–1002.

29. Pandian, S. S., Robin, S., Vinod, K. K., Rajeswari, S., Manonmani, S., Subramanian, K. S., Saraswathi, R. and Kirubhakaran, A. P. M., Influence of intrinsic soil factors on genotype-by-environment interactions governing micronutrient content of milled rice grains. *Aust. J. Crop. Sci.*, 2011, **5**(13), 1737–1744.

30. Suwarto, N., Genotype × environment interaction for iron concentration of rice in central Java of Indonesia. *Rice Sci.*, 2011, **18**(1), 75–78.

31. Liang, J., Han, Bei, Z., Han, L., Robert, Nout, M. J. and Hamer, R. J., Iron, zinc and phytic acid concentration of selected rice varieties from China. *J. Sci. Food Agric.*, 2007, **87**, 504–510.

32. Pelig-Ba, K. B., Assessment of phytic acid levels in some local cereal grains in two districts in the upper east region of Ghana. *Pakistan J. Nutr.*, 2009, **8**(10), 1540–1547.

33. Trijatmiko, K. R. *et al.*, *Biofortified indica* rice attains iron and zinc nutrition dietary targets in the field. *Sci. Rep.*, 2016, **6**, doi:10.1038/srep19792.

34. Welch, R. M., William, A. H., Steven, B., Dharmawansa, S., Glenn, B. G. and Cheng, Z., Testing iron and zinc bioavailability in genetically enriched beans (*Phaseolus vulgaris* L.) and rice (*Oryza sativa* L.) in a rat model. *Food Nutr. Bull.*, 2000, **21**, 428–433.

35. Tako, E., Hoekenga, O. A., Kochian, L. V., Glahn, R. P., High bioavailablilty iron maize (*Zea mays* L.) developed through molecular breeding provides more absorbable iron *in vitro* (Caco-2 model) and *in vivo* (*Gallus gallus*). *Nutr. J.*, 2013, **12**, 3.

36. Promuthai, C., Huang, L., Glahn, R., Welch, R. M., Fukai, S. and Rerkasem, B., Iron (Fe) bioavailability and the distribution of anti-Fe nutrition biochemicals in the unpolished, polished grain and bran fraction of five rice genotypes. *J. Sci. Food Agric.*, 2006, **86**, 1209–1215.

37. Eagling, T., Wawer, A. A., Shewry, P. R., Zhao, F. J. and Fairweather-Tait, S. J., Iron bioavailability in two commercial cultivars of wheat: comparison between wholegrain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. *J. Agric. Food Chem.*, 2014, **62**(42), 10320–10325.

38. Petry, N., Egli, I., Campion, B., Nielsen, E. and Hurrell, R., Genetic reduction of phytate in common bean (*Phaseolus vulgaris* L.) seeds increases iron absorption in young women. *Nutrition*, 2013, **143**(8), 1219–1224.

39. Sedef Nehir, E. I., Sibel, K. and Şebnem, S., Effect of phytic acid on iron bioavailability in fortified infant cereals. *Nutrition Food Sci.*, 2010, **40**(5), 485–493.

40. Afify, A. M., El-Beltagi, H. S., El-Salam, S. M. and Omran, A. A., Bioavailability of iron, zinc, phytate and phytase activity during soaking and germination of white sorghum varieties. *PLoS ONE*, 2011, **6**(10), e25512; doi:10.1371/journal.pone.0025512.

41. Swamy, B. P. M. *et al.*, Identification of genomic regions associated with agronomic and biofortification traits in DH populations of rice. *PLoS ONE*, 2018; https://doi.org/10.1371/journal.pone.0201756.

42. Inabangan-Asilo, M. A. *et al.*, Stability and G × E analysis of zinc-biofortified rice genotypes evaluated in diverse environments. *Euphytica*, 2019, **215**, 61; doi.org/10.1007/s10681-019-2384-7.

A significant shift in particulate organic matter characteristics during flooding of River Krishna, eastern peninsular India

C. Prakash Babu[1,*], V. Ramaswamy[2], P. S. Rao[3], V. V. S. S. Sarma[4] and P. Praveen Kumar[5]

[1]CSIR-National Institute of Oceanography, Dona Paula, Goa 403 004, India
[2]A-6, Oceanis Society, Bambolim, Goa 403 202, India
[3]Flat No. 502, First Block, Kamat Riviera, Caranzalem, Goa 403 002, India
[4]CSIR-National Institute of Oceanography, Regional Centre, Visakhapatnam 530 017, India
[5]National Institute of Animal Biotechnology, Hyderabad 500 049, India

Extremely heavy rainfall over a small, semi-arid section of the Indian Peninsula in October 2009, together with release of water from dams resulted in very severe flooding in River Krishna. The sources and type of organic matter during and after the floods were studied by analysing suspended particulate matter (SPM) for organic carbon (C), total nitrogen (N) and isotopic composition of C ($\delta^{13}C_{org}$). The $\delta^{13}C_{org}$ varied from –21.4‰ during the initial heavy flood phase to –27.1‰ in the final receding phase. Discharge of terrestrial carbon (–21.4‰ to –23.5‰) from mixed sources with high C/N ratios (14–19) during the initial phase of the flood originated from the semi-arid section of the river. The light carbon (–25.5‰ to –27.1‰) with low C/N ratios (7.2–9.5) in the receding phase of the flood was from local C3-rich organic debris from the deltaic regions along with phytoplankton from aquatic sources. Since the average suspended sediment discharge of River Krishna has decreased from 68 mt to less than 0.1 mt due to construction of dams and barrages, it appears that sediments and organic matter presently being delivered to the oceans are mainly during flood events, and the type of organic matter delivered depends on the nature of the soil where high rainfall is received.

RIVERS play an important role in transporting sediments and organic matter from land to sea. Fluvial carbon is the biggest source of organic carbon in continental margin sediments. The total global fluvial carbon flux is 0.80–1.33 Pg C/year (ref. 1). During the last century, dams were built across most rivers of the world, including peninsular Indian rivers, resulting in significant reduction in water, sediment and organic carbon delivery to the oceans[2]. River Krishna is an extreme example of this, as more than 650 small and large dams have been constructed across this river during the last 60 years. Since 2002, the holding capacity of the dams by River Krishna nearly equals or exceeds the annual run-off[3–5]. Understanding the amount and nature of carbon in such river basins is important as reduction in carbon supply affects the estuarine and near-coastal environment.

Soil carbon, aquatic plants, agriculture, vascular plants, bacteria, etc. are the main sources of terrestrial organic carbon[6–8]. The soil type, agriculture, vegetation, precipitation, temperature and weathering rates dictate the nature of organic matter in fluvial environment. The C/N (organic carbon/nitrogen) ratio and $\delta^{13}C_{org}$ (isotopic composition of organic carbon) are commonly adopted to characterize the organic carbon sources[9–12]. Relatively lower $\delta^{13}C_{org}$ ranging from –23‰ to –33‰, with an average of –27‰ characterizes the C3 organic matter[13–15], while plants employing C4 pathway are distinguished by $\delta^{13}C_{org}$ enrichment ranging from –9‰ to –17‰ (refs 13, 14, 16–18). Freshwater phytoplankton has $\delta^{13}C_{org}$ values between –25‰ and –30‰ and relatively low C/N ratios (5–10)[18,19]. On the other hand, soil organic matter (SOM) has high C/N ratios (>12)[20].