

Is Seed Iron Concentration Predictive of Resistance to Iron Deficiency in Soybean?

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ABSTRACT

Within the last decade, studies involving numerous crops provide strong evidence that seed Fe concentration ([Fe]) is useful for identifying genotypes possessing superior resistance to Fe deficiency. It is our opinion that using seed [Fe] is equivalent or superior to using visual chlorosis score as a measure of Fe efficiency, that seed [Fe] can be used to identify resistance to Fe deficiency in the absence of Fe deficiency or in the presence of Fe deficiency exacerbated by applying high rates of fertilizer N, that conventional plant breeding can be used to increase seed [Fe] as a strategy for improving resistance to Fe deficiency, and that planting seed [Fe] can be used as a predictor of successful (nonchlorotic) seedling establishment. International agricultural agencies, such as HarvestPlus (CIAT, Cali, Columbia), have promoted using plant breeding as an intervention strategy focused on increasing micronutrient concentrations and bioavailabilities in crops consumed by populations with known micronutrient deficiencies. Can conventional plant breeding be used to increase seed [Fe] as a strategy to reduce Fe deficiency in soybean [*Glycine max* (L.) Merr.]? Evidence presented in this paper combined with earlier reports indicates that Fe-efficient and Fe-inefficient varieties appear to have seed [Fe] maxima that are distinctly different and seldom exceeded; that is, soybean plants tend to maintain [Fe] in the grain within predetermined, genetically controlled limits. Seed [Fe] can be regarded as an integrated measure of resistance to Fe deficiency that is manifest at maturity and that involves the coordinate expression of several genes regulating Fe reduction and uptake, transport, and storage.

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Abbreviations: MR, moderately Fe efficient and moderately resistant to Fe deficiency chlorosis; NR, N rates; NR_{Linear} , linear response to N rate; R, Fe efficient and resistant to Fe deficiency chlorosis; Susc, Fe inefficient and susceptible to Fe deficiency; SPAD, relative chlorophyll concentration as measured by a Minolta SPAD-502 leaf chlorophyll meter (Minolta, Ramsey, NJ); C1, the mean of two R varieties vs. the mean of two MR and two Susc varieties; C2, R V1 vs. R V2; C3, the mean of two MR varieties vs. the mean of two Susc varieties; VCS, visual chlorosis score; YR1, 2004 vs. the mean of years 2003 and 2005; YR2, 2003 vs. 2005. Brackets [] indicate concentration; for example, [N] is N concentration.

IRON DEFICIENCY is a complex disorder that occurs in response to multiple soil, environmental, and genetic factors (Kochian, 2000; Hansen et al., 2003). Iron deficiency chlorosis is symptomatic of the disorder and commonly observed on high pH, highly calcareous soils (Wiersma, 2005). Planting Fe deficiency-resistant soybean [*Glycine max* (L.) Merr.] varieties has been promoted as the best strategy to alleviate or avoid Fe deficiency (Fairbanks et al., 1987; Goos and Johnson, 2000; Naeve and Rehm, 2006). However, screening nurseries used to identify more resistant varieties based on visual chlorosis scores (VCSs) do not always provide consistent, reliable results (Fairbanks, 2000; Wiersma, 2011). Therefore, a major obstacle to breeding for Fe chlorosis resistance in soybean has been that Fe deficiency symptoms and resistance scores cannot be replicated consistently among experiments. One hypothesis is that this lack of consistency is probably due to the complex chemical

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and physical criteria in both the plant and soil that must be met for visual chlorosis to occur (Marschner, 1986; Jolley et al., 1996; Fairbanks, 2000; Naeve and Rehm, 2006). An alternative thought is that a classification variable other than VCS should be used along with a measure of resistance that actually involves Fe concentration ([Fe]) or Fe content (Wiersma, 2011). Similarly, the efficiency of breeding for Fe deficiency resistance in soybean could be substantially improved if a relatively simple laboratory test was developed to screen genotypes for resistance (Fehr, 1982).

Although ferric chelate reductases and quantitative determination of Fe reduction (Jolley et al., 1992; Ellsworth et al., 1998; Blair et al., 2010) appear to be reliable indicators of the genetic potential for chlorosis resistance, they can simultaneously compare only a small number of potential varieties (Fairbanks, 2000). Other accurate, consistent, and inexpensive measures of resistance that could be used to screen hundreds of lines in a breeding program have not been developed (Fairbanks, 2000; Wiersma, 2011). For resistance to Fe deficiency, the “measure of choice” for decades (Weiss, 1943; Cianzio, 1979; Froehlich and Fehr, 1981; Fairbanks et al., 1987; Penas et al., 1990; Goos and Johnson, 2000; Helms et al., 2010) has been a subjective, visual estimate of the degree of yellowing (i.e., VCS). The extent of yellowing or VCSs among plots within a nursery often approaches a continuous distribution from green to yellow. Historically, this range of expression has been subdivided into classes before analysis (Fehr, 1982). However, the degree of yellowing is not always related only to Fe or to [Fe] in various tissues (Marschner, 1995). Deficiencies of N, S, Zn, Mn, and Mg can also confer a yellow phenotype (Imsande, 1998; Marschner, 1995) although to a lesser extent than Fe deficiency. Other micronutrient deficiencies, for example Mn and Zn, often are associated with Fe deficiency in plants growing on calcareous soils (Marschner, 1995; Kobayashi et al., 2003; Jolley et al., 2004).

Wiersma (2011) compared 72 soybean varieties grown at three locations using five classification variables and three measures of resistance and concluded that classifying varieties on the basis of planting seed [Fe] and then measuring seed [Fe] or Fe per 1000 seeds at harvest provided an accurate and consistent measure of varietal differences in Fe efficiency and agronomic performance. With soybean, Jessen et al. (1988) reported that there was little association between Fe efficiency and grain yield. Concentrations of desirable microelements in several species segregate independently from agronomic factors, including grain yield and resistance to diseases and other pests (Graham et al., 1999; Nestel et al., 2006). Nonetheless, Moraghan (2004) suggested that the within-seed distribution of Fe also should be considered in plant breeding programs because of concerns about both human nutrition and early seedling growth.

Regarding seed [Fe] as an integrated measure of resistance to Fe deficiency that is manifest at maturity and

that involves the coordinate expression of several genes regulating Fe reduction and uptake, transport, and storage strongly suggests that it would be prudent to address several related questions before initiating a breeding program based on seed [Fe]. Nonetheless, during the last decade, large (twofold or higher) increases in seed microelement densities have been reported for several crops (Wang et al., 2003). These researchers suggest that twofold increases in current mean mineral concentrations appear feasible using classical breeding efforts, despite the very large number of genes involved in micronutrient uptake, transport, storage, and seed accumulation (Waters and Grusak, 2008; Waters and Sankaran, 2011).

The stability of genetic differences in seed mineral concentrations across environments (years, locations, and/or agronomic treatments) is crucial to the success of plant breeding programs designed to improve micronutrient densities. In earlier research involving three varieties (Wiersma, 2005), differences in seed [Fe] between Fe-efficient and Fe-inefficient soybean varieties were determined to be large and consistent within and across environments. The expression of seed composition as well as micronutrient-density traits are primarily genetically determined (Cartter and Hopper, 1942; Kleese et al., 1968; Bennie et al., 1982; Beebe et al., 2000; Moraghan et al., 2002; Bouis et al., 2003; Welch and Graham, 2004; White and Broadley, 2005; Nestel et al., 2006; Rotundo and Westgate, 2009; Blair et al., 2010; Bellaloui et al., 2011; and references therein). Iron and Zn concentrations often are positively correlated and independent of yield whereas [Mn] is often negatively correlated with [Fe] and [Zn] (Wiersma, 2005; Moraghan and Helms, 2005; Ghandilyan et al., 2006). Nitrogen alone was reported to be an important, favorable factor determining grain mineral content in rice (*Oryza sativa* L.) (Zhang et al., 2008). With maize (*Zea mays* L.), N fertilization did not reduce grain micronutrient (Fe, Zn, Mn) concentrations; instead, the highest rate of N increased [Fe] and [Zn] (Losak et al., 2011). With dry bean (*Phaseolus vulgaris* L.), seed mineral concentrations and contents are stable across years and locations (Beebe et al., 2000; Gregorio, 2002). Gregorio et al. (2000) also observed similar stability in rice genotypes.

Plant biotechnology (Vasconcelos et al., 2003, 2006) and classical plant breeding (near-isogenic lines [O'Rourke et al., 2007], recombinant inbred lines [Ghandilyan et al., 2006; Sankaran et al., 2009], F₂ lines, F₂-derived lines, or backcross progeny [Waters and Sankaran, 2011]) have been used to study micronutrient efficiency and to incorporate high seed mineral densities into selected varieties. Waters and Sankaran (2011) list several crop species [*Arabidopsis thaliana* (L.) Heynh., model legume (*Medicago truncatula* Gaertn.), bird's-foot trefoil (*Lotus corniculatus* L. [syn. *Lotus japonicus* (Regel) K. Larsen]), common bean (*Phaseolus vulgaris*), canola (*Brassica napus* L.), field mustard (*Brassica rapa* L.), rice

(*Orzya sativa*), and winter or spring wheat (*Triticum aestivum* L.) in which quantitative trait loci (QTL) mapping of seed mineral concentrations has been completed. Soybean was not included in this update. Bellaloui et al. (2011) reported that genotypic background in soybean was especially important in selecting for seed mineral concentrations and that the effects of year, maturity gene, and interactions of year \times maturity gene \times genotypic background were considerably less important. Previously, Kleese et al. (1968) reported that genotypic variance was considerably more important than year or location variances and that concentrations of several mineral elements in soybean were tightly controlled genetically.

Before cultivar development, it is important to obtain estimates of genotypic differences in mineral densities and trait stability across years, locations, and agronomic treatments. What are the magnitudes of genotypic variances vis-à-vis years, N rates, and two- and three-way interactions? Are differences among varieties consistent across years and N treatments? Do relationships between seed [Fe] and other macro- or micronutrient densities prohibit selection for increased seed [Fe]? Are increases in seed [Fe] associated with increases in [Zn] but with decreases in seed [Mn]? Do increases in seed micronutrient concentrations result primarily from decreases in seed weight? This paper addresses a long-term goal of evaluating whether seed [Fe] is predictive of resistance to Fe deficiency and whether increasing [Fe] in seed used for planting improves season long as well as seedling resistance to Fe deficiency.

MATERIALS AND METHODS

To address several of the concerns described above, our approach was to assay elemental concentrations in seed from experiments done at one location on soils where soybean has historically exhibited mild to severe Fe deficiency. Materials and methods for this trial are described in detail in an earlier manuscript (Wiersma, 2010) with the exception of methods used for measuring elemental composition. Briefly, a split-plot arrangement of a randomized complete block design with four replications was established at Crookston, MN (the Northwest Research and Outreach Center), during 2003, 2004, and 2005. Nitrogen rates (NR) (0, 34, 68, 102, 136, and 170 kg N ha⁻¹) were whole plots and varieties (2 Fe efficient, 2 moderately Fe efficient, and 2 Fe inefficient) were subplots. Following harvest, subsamples (1 g) of seed were dry-ashed and analyzed for Fe using the procedure described by Wiersma (2005). Zinc and Mn were determined by atomic absorption spectroscopy and N was determined using standard Kjeldahl procedures. Elemental contents in whole seed were calculated using seed weights and elemental concentrations. Data were analyzed using standard procedures for an analysis of variance of a split-plot arrangement of a randomized complete block design (Gomez and Gomez, 1984). Separate analyses were done for each year, considering NR and varieties as fixed effects, and a combined analysis across years was done using PROC MIXED procedures of SAS (Littell et al., 2006). Blocks and all terms involving blocks were considered random effects. In the combined analyses, years also

were considered fixed effects and particular attention was given to the linear response to N rate (NR_{Linear}), and occasionally the quadratic response to N rate (NR_{Quadratic}) \times variety contrasts and relationships among mineral elements to seed number, seed weight, and relative chlorophyll concentration (midseason SPAD [relative chlorophyll concentration as measured by a Minolta SPAD-502 leaf chlorophyll meter (Minolta, Ramsey, NJ)] reading) at R3 to R4 (Ritchie et al., 1988).

An estimate of the importance of various sources of variation was determined from analysis of variance by comparing mean squares and ranges. The data were further analyzed using contrast and estimate statements.

Year contrasts were 2004 vs. the mean of years 2003 and 2005 (YR1) and 2003 vs. 2005 (YR2). Variety contrasts were the mean of two Fe efficient and resistant to Fe deficiency chlorosis (R) varieties vs. the mean of two moderately Fe efficient and moderately resistant to Fe deficiency chlorosis (MR) and two Fe inefficient and susceptible to Fe deficiency (Susc) chlorosis varieties (C1), R V1 vs. R V2 (C2), the mean of two MR varieties vs. the mean of two Susc varieties (C3), MR V1 vs. MR V2 (C4), and Susc V1 vs. Susc V2 (C5). Interactions of year \times variety were partitioned using products of year and variety contrasts. Most of the response to NR could be described by linear equations. PROC STEPWISE (Boling et al., 1990) was used to evaluate the importance of elemental concentration vs. seed weight in characterizing seed element content.

RESULTS AND DISCUSSION

Growing Conditions

Environmental and soil stresses often promote Fe inefficiency in soybean (Hansen et al., 2003) and can alter mineral composition of soybean seed (Gibson and Mullen, 2001). Selected properties of soils from each experimental area and growing season temperatures and precipitation data were reported previously (Wiersma, 2010). Despite larger reserves of diethylenetriamine pentaacetate (DTPA)-extractable Fe in 2004, colder, wetter conditions reduced several agronomic measures compared to similar responses in 2003 or 2005 (Wiersma, 2010). Adding excessive fertilizer N (NO₃⁻) increases rhizosphere pH and exacerbates Fe deficiency by decreasing the reducing activity at the root (Brown and Jones, 1962; Aktas and van Egmond, 1979; Terry and Jolley, 1994; Matocha and Coyne, 2007; Zhao and Ling, 2007). Stress that increases or decreases seed size will usually modify elemental concentrations but not necessarily contents.

Genetic and Environmental Variation

Ranges in micronutrient concentrations in numerous crops indicate that there is sufficient genetic variation. to initiate breeding programs aimed at increasing micronutrient concentrations in seed (Pfeiffer and McClafferty, 2007). Common concentrations of Fe, Zn, and Mn in soybean range from 69 to 122, 42 to 91, and 18 to 52 mg kg⁻¹ seed, respectively (Kleese et al., 1968; Parker et al., 1981; Raboy et al., 1984; Gibson and Mullen, 2001). Larger elemental ranges have been reported occasionally for Fe (18–193 mg

Table 1. Ranges of years, N rates (NR), and variety mean values for Fe, Zn, Mn, and N concentration, seed number and weight, and SPAD (relative chlorophyll concentration as measured by a Minolta SPAD-502 leaf chlorophyll meter [Minolta, Ramsey,NJ]) readings at R3 to R4 for six varieties grown at six NR during 2003, 2004, and 2005.

Source of variation	Fe conc.	Zn conc.	Mn conc.	N conc.	Seed number	Seed weight	SPAD
Years (n = 3)	62.6–68.6	30.6–30.7	34.0–42.4	60.7–62.5	1229–1707	154–171	27.2–31.5
NR (n = 6)	59.8–70.0	29.8–31.3	36.5–37.8	60.7–62.1	1322–1593	159–162	27.8–30.7
Varieties (n = 6)	51.4–76.8	28.3–34.6	34.4–42.1	58.8–66.3	961–1735	144–171	22.1–33.8
Mean (n = 108)	65.7	30.6	37.2	61.4	1433	161	29.4

Table 2. Mean squares rounded to the nearest whole number for Fe, Zn, Mn, and N concentration, seed number and weight, and SPAD (relative chlorophyll concentration as measured by a Minolta SPAD-502 leaf chlorophyll meter [Minolta, Ramsey,NJ]) readings at R3 to R4.

Source of variation	Fe conc.	Zn conc.	Mn conc.	N conc.	Seed number	Seed weight	SPAD at R3–R4
Year	1306 NS [†]	<1 NS	2950**	137 × 10 ⁶ **	876 × 10 ⁴ **	117 × 10 ² **	65 × 10 ¹ **
Nitrogen rates (NR)	1164**	26 NS	21 NS	15 × 10 ⁶ NS	74 × 10 ⁴ *	1 × 10 ² NS	9 × 10 ¹ *
Year × NR	241 NS	17 NS	23 NS	3 × 10 ⁶ NS	9 × 10 ⁴ NS	1 × 10 ² NS	1 × 10 ¹ NS
Variety (V)	9547**	327**	481**	644 × 10 ⁶ NS	643 × 10 ⁴ **	67 × 10 ² **	183 × 10 ¹ **
Year × V	1532**	9 NS	42**	12 × 10 ⁶ NS	125 × 10 ⁴ **	6 × 10 ² **	22.4 × 10 ¹ **
NR × V	109**	5 NS	4 NS	6 × 10 ⁶ NS	20 × 10 ⁴ **	2 × 10 ² **	2 × 10 ¹ **
Year × NR × V	64**	6 NS	6 NS	3 × 10 ⁶ NS	4 × 10 ⁴ NS	<1 NS	1 × 10 ¹ NS

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

[†]NS, not significant.

*Multiply the reported numbers by this to obtain the actual numbers.

kg⁻¹; Kapoor et al., 1975) and Mn (72–79 mg kg⁻¹; Rani et al., 2008). In this research, [Fe], [Zn], and [Mn] ranged from 45 to 78, 29 to 33, and 34 to 41 mg kg⁻¹ seed, respectively (Table 1). These values are considerably lower for Fe and Zn, probably because of the highly calcareous, high pH soils. Values for Mn were about average. Variances associated with each source of variation from analyses of variance for Fe, Zn, Mn, N, seed number and weight, and SPAD at R3 to R4 are given in Table 2. Although two- and three-way interactions often were statistically significant, the variances associated with varieties were always much larger than those associated with these interactions or with NR. These results, combined with those reported in 2005 and 2011 (Wiersma, 2005, 2011), and similar results reported by Kleese et al. (1968) provide substantial evidence that most of the variation in micronutrient concentration in soybean is tightly genetically controlled. Iron deficiency, such as that which occurs with excessive NO₃⁻¹, may induce different changes in gene expression among genotypes (Blair et al., 2010). Although the degree of expression of seed micronutrient concentrations may vary with environments, superior mineral content selected at one location is usually stable across years and other sites or treatments (Slipcevic et al., 1993; Spehar, 1994; Beebe et al., 2000; Welch and Graham, 2004).

Seed Fe

Shen et al. (2002) concluded that high seed Fe levels improved chlorosis resistance in wheat and that genotypic seed Fe levels were an aspect of genotypic differences in resistance to Fe deficiency. In our research, numerous sources of variation were statistically significant for seed

[Fe] (Table 3); however, variety contrasts C1 and C3 and interactions involving C1 and C3 contrasts were considered of primary importance. Resistant varieties had higher seed [Fe], Fe content, and Fe removal values than MR and Susc varieties (C1) and MR varieties had higher seed [Fe], Fe content, and Fe removal than Susc varieties (C3) (Table 3). All three measures of seed Fe declined as NR increased (Fig. 1), especially for Susc varieties. For example, the NR_{Linear} × variety contrasts for seed [Fe] (Table 3) and regression analyses indicated that R varieties declined at a slower rate (–1.08 mg kg⁻¹ Fe per kg N ha⁻¹) than the average of MR and Susc varieties (–2.54 mg kg⁻¹ Fe per kg N ha⁻¹), and MR varieties declined at a slower rate (–1.44 mg kg⁻¹ Fe per kg N ha⁻¹) than Susc varieties (–3.64 mg kg⁻¹ Fe per kg N ha⁻¹). Seed [Fe] of Susc varieties were especially sensitive to added N and presumably to a decrease in the reducing activity in the rhizosphere (Fig. 1). Aktas and van Egmond (1979) also observed more response (less H⁺ and reductant and more OH⁻ excretion) to added NO₃⁻¹ with an Fe-inefficient variety than with an Fe-efficient variety. They concluded that increasing the amount of nitrate supplied to plants growing in calcareous soils worsened the symptoms of chlorosis in Fe-inefficient varieties but increased the growth of Fe-efficient varieties. The present results and those reported by Wallace and Cha (1986) support their conclusions. Similar responses with Fe-efficient and Fe-inefficient genotypes have been reported for tomato (*Solanum lycopersicum* L. [syn. *Lycopersicon esculentum* Mill.]; Zhao and Ling, 2007), spinach (*Spinacia oleracea* L.; Assmakopoulou et al., 2006), and grape (*Vitis vinifera* L.; Jimenez et al., 2007).

Table 3. Summary of analyses of variance across years for seed Fe, Zn, Mn, and N concentration, content, and amount removed at harvest for six soybean varieties grown at six rates of added N in 2003, 2004, and 2005.

Source of variation†	df	Seed Fe			Seed Zn			Seed Mn			Seed N		
		Conc.‡	Content§	Removal¶	Conc.	Content	Removal	Conc.	Content	Removal	Conc.	Content	Removal
Year (Y)	2	NS#	*	**	NS	NS	**	**	**	**	*	**	**
YR1#	1	NS	NS	*	NS	NS	**	**	**	**	NS	**	**
YR2††	1	NS	*	*	NS	NS	**	**	**	NS	**	*	**
Nitrogen rates (NR)†	5	**	**	**	NS	*	NS	NS	NS	*	NS	NS	NS
NR _{Linear}	1	**	**	**	NS	**	*	NS	NS	*	**	*	*
NR _{Quadratic}	1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Year × NR	10	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
YR1 × NR _{Linear}	1	NS	NS	NS	*	**	**	NS	NS	NS	NS	NS	NS
YR2 × NR _{Linear}	1	*	**	NS	NS	NS	NS	*	**	NS	NS	NS	NS
Variety	5	**	**	**	**	**	**	**	**	**	**	**	**
C1‡‡	1	**	**	**	NS	**	**	**	**	**	**	**	**
C2	1	NS	**	**	*	**	**	**	**	**	**	**	**
C3	1	**	**	**	**	**	**	**	**	**	**	**	**
C4	1	**	**	**	**	**	**	**	**	**	**	**	NS
C5	1	**	**	**	NS	*	**	NS	NS	**	NS	NS	**
Year × variety	10	**	**	**	NS	**	**	**	**	**	**	**	**
YR1 × C1	1	**	**	**	NS	NS	**	NS	NS	**	**	NS	**
YR1 × C2	1	NS	**	NS	NS	*	NS	NS	**	NS	NS	**	*
YR1 × C3	1	**	**	**	NS	NS	**	**	*	**	NS	NS	**
YR1 × C4	1	*	**	**	**	**	**	NS	**	NS	NS	**	*
YR1 × C5	1	**	**	**	NS	NS	**	NS	NS	**	NS	NS	**
YR2 × C1	1	**	**	**	NS	NS	**	**	**	**	NS	*	**
YR2 × C2	1	*	**	**	NS	NS	**	**	NS	NS	NS	**	**
YR2 × C3	1	**	**	**	NS	NS	**	**	*	**	NS	NS	**
YR2 × C4	1	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	**	NS
YR2 × C5	1	**	**	**	NS	NS	**	NS	NS	**	NS	NS	**
NR × variety	25	**	**	**	NS	NS	**	NS	NS	*	**	**	*
NR _{Linear} × C1	1	**	**	**	NS	NS	**	NS	NS	**	*	**	**
NR _{Linear} × C2	1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
NR _{Linear} × C3	1	**	**	**	NS	NS	**	NS	NS	**	**	**	**
NR _{Linear} × C4	1	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	*	NS
NR _{Linear} × C5	1	NS	NS	*	NS	NS	NS	NS	NS	*	NS	NS	*
Mean		65.7	10.5	15.9	30.6	4.9	7.0	37.2	6.0	8.5	61.4††	9.9	14.1
CV, %		24.1	25.2	45.9	12.5	15.6	39.1	14.6	17.6	40.5	5.7	11.8	37.1

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

†NR_{Linear} linear response to N rate; NR_{Quadratic} quadratic response to N rate; YR1, 2004 vs. the mean of years 2003 and 2005; YR2, 2003 vs. 2005; C1, the mean of two Fe efficient and resistant to Fe deficiency chlorosis (R) varieties vs. the mean of two moderately Fe efficient and moderately resistant to Fe deficiency chlorosis (MR) and two Fe inefficient and susceptible to Fe deficiency (Susc) chlorosis varieties; C2, R V1 vs. R V2; C3, the mean of two MR varieties vs. the mean of two Susc varieties; C4, MR V1 vs. MR V2; C5, Susc V1 vs. Susc V2.

‡Concentration, mg kg⁻¹.

§Content, µg seed⁻¹.

¶Element removed in harvested seed at maturity, µg m⁻².

#NS, not significant.

††Nitrogen values are given in mg.

Differences among varieties in this report ranked the same regardless of NR, and, similar to earlier research (Wiersma, 2005, 2007, 2011), Fe-efficient and Fe-inefficient varieties appeared to have seed [Fe] maxima that are distinctly different and seldom exceeded. This indicates that improvements in seed [Fe] should be possible using conventional plant breeding methodologies. It also seems logical that varieties having a difficult time acquiring Fe (susceptible to Fe deficiency) are not likely

to have higher seed [Fe]. Even when susceptible plants were provided high amounts of Fe chelate—enough to match yields of R varieties—they still had lower seed [Fe] (Wiersma, 2005). Rengel et al. (1999) also remarked that, in general, plants tend to maintain nutrient concentrations in the grain within “predetermined limits.”

The rate-limiting step in Fe uptake and metabolism has long been known to be ferric chelate reductase activity at the root (Brown and Jones, 1962). When Blair et al. (2010)

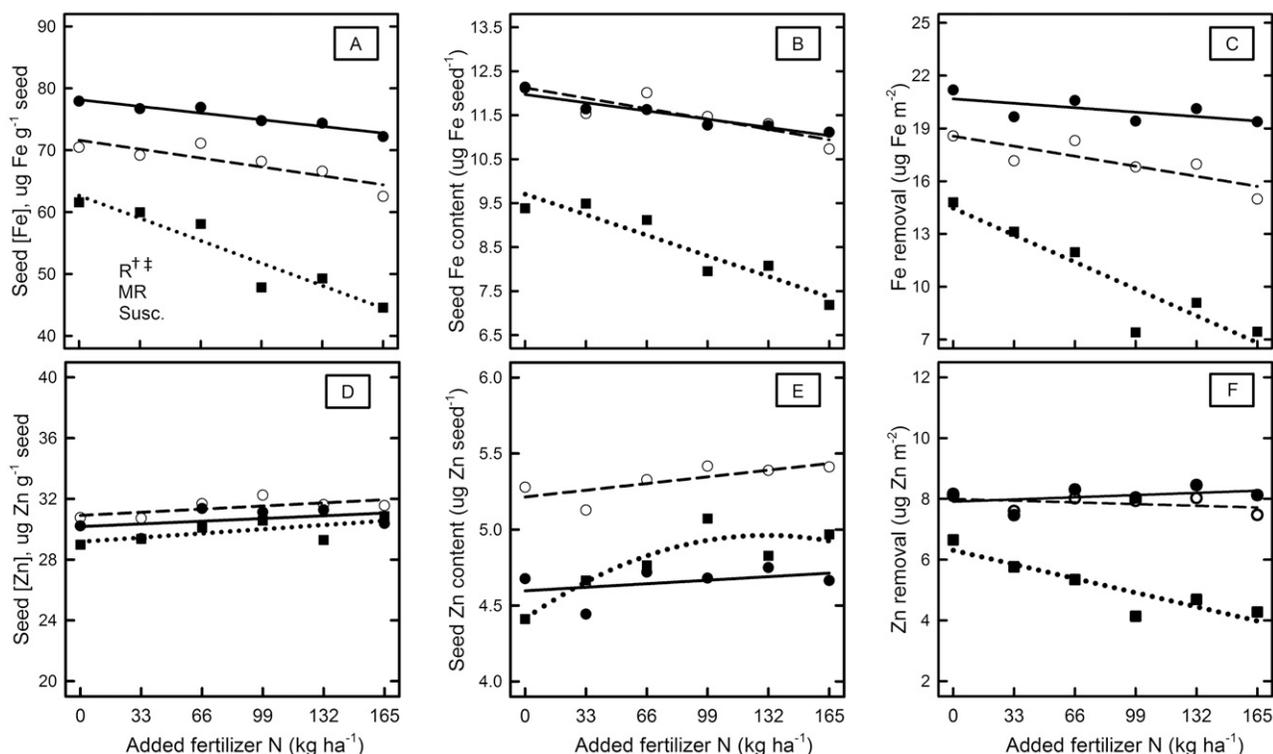


Figure 1. Changes in seed Fe concentration ([Fe]) and Zn concentration ([Zn]), content, and removal in response to added fertilizer N in soybean varieties that are resistant, moderately resistant, and susceptible to Fe deficiency. R (closed circles), Fe efficient and resistant to Fe deficiency chlorosis; MR (open circles), moderately Fe efficient and moderately resistant to Fe deficiency chlorosis; Susc (closed squares), Fe inefficient and susceptible to Fe deficiency.

addressed genotypic variability in ferric chelate reductase activity in dry bean, they selected varieties known to differ in seed [Fe]. Ferric chelate reductase activity was indeed higher in genotypes with high seed [Fe]. As with Fe values, linear decreases in element removal ($\mu\text{g m}^{-2}$) also were observed for Zn, Mn, and N (Fig. 1 and 2). These similarities may be related to an indirect effect of increased Fe deficiency on seed number (Fig. 2A; Wiersma, 2010). In contrast to element removal, changes in element concentration and content in response to NR often varied (Fig. 1 and 2).

Seed Zn

Symptoms of Zn deficiency in barley (*Hordeum vulgare* L.) can be reduced substantially by planting seed having higher Zn levels; that is, seed Zn is predictive of resistance to Zn deficiency (Genc et al., 2000). Gelin et al. (2007) reported similar results with dry bean and noted that using seed Zn content as a measure of resistance to Zn deficiency was particularly useful because genotypes could be evaluated in the absence of Zn deficiency. Moraghan and Helms (2005) reported that harvest seed [Zn] of 31 to 33 mg kg^{-1} seed indicated that soybean seed yield was not affected by Zn deficiency. In this study, [Zn] ranged from 29 to 33 mg kg^{-1} seed, and although differences were small, nearly all of the variation in [Zn] and Zn content was associated with varieties (Tables 1 and 2). In contrast to [Fe] and Fe content, [Zn] and Zn content

increased slightly in response to added N (Fig. 1). The $\text{NR}_{\text{Linear}} \times \text{variety}$ contrasts were not significant for either seed [Zn] or Zn content (Table 3). Small differences in micronutrient concentrations in response to added N also have been reported for maize (Losak et al., 2011). On the other hand, when several plant species were grown in the same environment, [Zn] increased as seed [N] increased (Cakmak et al., 2010). Rankings of varieties for seed Zn content were distinctly different from rankings for seed Fe content (Fig. 1), and whereas seed Fe content of Susc varieties declined almost 22% with increasing NR, seed Zn content increased nearly 14%. Even small [Zn] are known to interfere with Fe uptake and translocation (Lingle et al., 1963). Measures of Fe and Zn in dry bean also indicated that genetic variation for Fe was larger than that for Zn, although both micronutrients were primarily controlled by genotype (Blair et al., 2010). With rice grown on Zn-deficient soils, adding Zn greatly improved grain yield but had almost no influence (6%) on grain [Zn] and rankings of genotypes remained largely unchanged from Zn deficient to high Zn soils (Wissuwa et al., 2008).

Seed Mn

A critical level of Mn in soybean seed is about 20 mg kg^{-1} seed (Reuter and Robinson, 1997), which suggests that the observed [Mn] (34–41 mg kg^{-1} seed) were not yield limiting. With other legumes, primarily lupine (*Lupinus angustifolius*

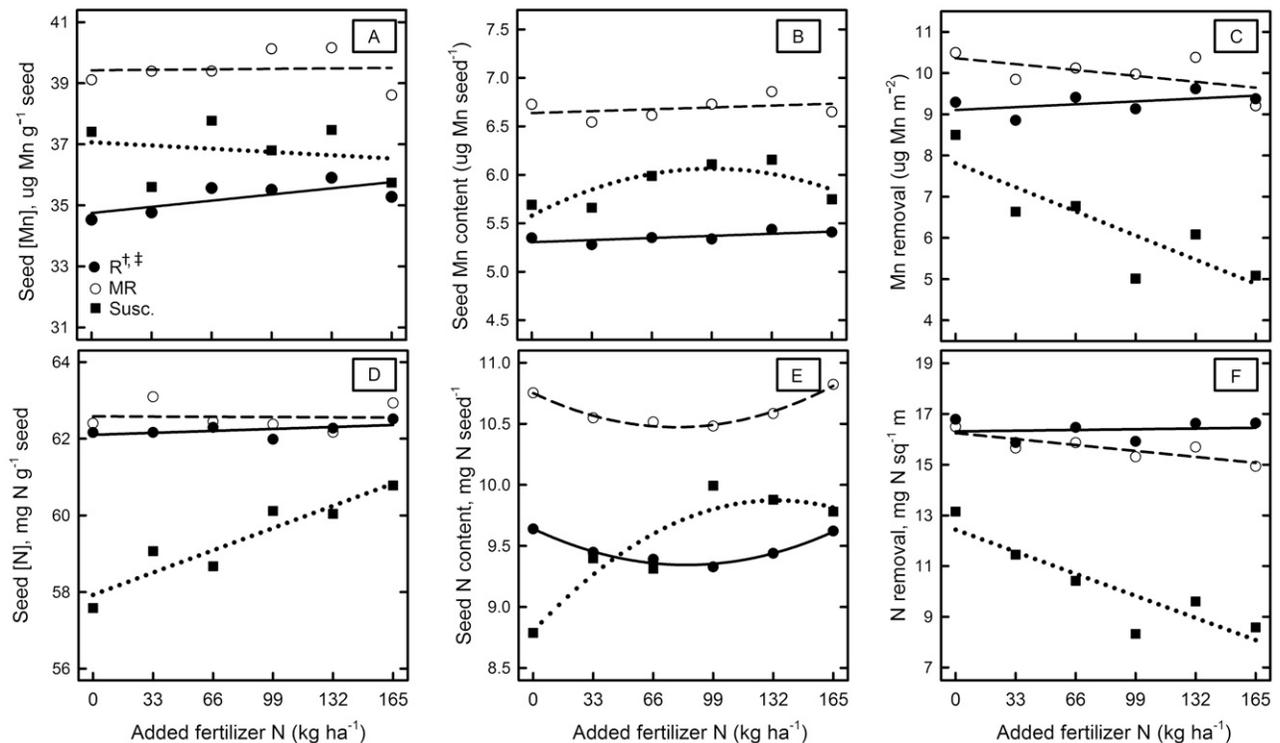


Figure 2. Changes in seed Mn concentration ([Mn]) and N concentration ([N]), content, and removal in response to added fertilizer N in soybean varieties that are resistant, moderately resistant, and susceptible to Fe deficiency. R (closed circles), Fe efficient and resistant to Fe deficiency chlorosis; MR (open circles), moderately Fe efficient and moderately resistant to Fe deficiency chlorosis; Susc (closed squares), Fe inefficient and susceptible to Fe deficiency.

Table 4. Correlations among concentrations, contents, and removal of Fe, Zn, Mn, and N determined using N rate × variety means ($n = 36$).

	Fe] [†]	[Zn]	[Mn]	[N]	SDFE [‡]	SDZN	SDMN	SDN	FESQM [§]	ZNSQM	MNSQM	NSQM	SDWT [¶]	SD No.	SPAD [#]
[Fe]	1	0.48 ^{††}	-0.35	0.64	0.94	0.18	-0.35	0.18	0.93	0.86	0.53	0.82	-0.28	0.77	0.90
[Zn]		1	-0.36	0.85	0.56	0.82	-0.14	0.60	0.25	0.31	-0.12	0.16	0.14	-0.08	0.32
[Mn]			1	-0.36	-0.14	0.12	0.93	0.25	-0.12	0.06	0.50	0.10	0.67	0.00	-0.10
[N]				1	0.75	0.74	-0.11	0.72	0.47	0.50	0.10	0.43	0.20	0.15	0.55
SDFE					1	0.45	-0.04	0.42	0.89	0.88	0.60	0.84	0.07	0.67	0.88
SDZN						1	0.41	0.92	0.06	0.22	0.01	0.11	0.68	-0.25	0.14
SDMN							1	0.56	-0.17	0.03	0.40	0.07	0.90	-0.16	-0.12
SDN								1	0.13	0.28	0.18	0.24	0.82	-0.15	0.22
FESQM									1	0.96	0.77	0.96	-0.19	0.92	0.94
ZNSQM										1	0.85	0.98	0.00	0.89	0.93
MNSQM											1	0.90	0.19	0.84	0.74
NSQM												1	0.01	0.92	0.93
SDWT													1	-0.32	-0.13
SD No.														1	0.86
SPAD															1

[†]Brackets [] indicate concentration.

[‡]Abbreviations beginning with SD refer to elemental content per seed.

[§]Abbreviations ending in SQM are element amounts removed with harvested seed per square meter.

[¶]WT, weight (g) per 1000 seeds.

[#]SPAD, relative chlorophyll concentration as measured by a Minolta SPAD-502 leaf chlorophyll meter (Minolta, Ramsey, NJ).

^{††}Significance levels of correlation coefficients: if $|r| < 0.32$, then $p > 0.05$; if $0.32 < |r| < 0.45$, then $p < 0.05$; and if $0.45 < |r| < 1.00$, then $p < 0.01$.

L.), seed [Mn] is predictive of resistance to Mn deficiency (Longnecker et al., 1996). Variances associated with years were much larger than any other source of variation (Table 2) and ranges across years for [Mn] were nearly identical to those for ranges across varieties (Table 1). As with seed

[Zn] and Zn content, $NR_{Linear} \times$ variety contrasts were not statistically significant for either seed [Mn] or Mn content (Table 3). With [Mn] and Mn content as well as [Zn] and Zn content ranking of varieties was substantially different than that observed for [Fe] and Fe content. Moderately

resistant varieties had concentrations and contents of Zn and Mn that were larger than those determined for R and Susc varieties. Although quite small, these differences were statistically significant (Table 3; Fig. 1 and 2).

Numerous interactions among micronutrients exist in soybean and other crops (Moosavi and Ronaghi, 2010; Roomizadeh and Karimian, 1996). Micronutrient imbalances include the antagonistic iron–manganese relationship, in which uptake and accumulation of Fe has a negative effect on uptake and transport of Mn (Heenan and Campbell, 1983; Moraghan, 1985; Izaguirre–Mayoral and Sinclair, 2005). On the other hand, in tobacco (*Nicotiana tabacum* L.) a deficiency of Fe alone increased the accumulation of competitive metals, for example Mn and Zn (Kobayashi et al., 2003).

Seed N

As NR increased, seed [N] in R and MR varieties changed very little whereas in Susc varieties seed [N] increased about 7% (Fig. 2). The small increase in seed [N] ($\mu\text{g g}^{-1}$ seed) was approx. fourfold less than the decrease (–27%) in seed [Fe]. Changes in Zn, Mn, and N contents in response to added nitrate were similar to each other and to changes in seed weight (Fig. 1 and 2; Wiersma, 2010). The $\text{NR}_{\text{Linear}} \times \text{variety}$ contrasts were not significant for either Zn or Mn. On the other hand, seed [Fe] declined and seed [N] increased as NR increased (Fig. 1 and 2). The significance of contrasts involving $\text{NR}_{\text{Linear}} \times \text{C1}$ and $\text{NR}_{\text{Linear}} \times \text{C3}$ was nearly identical for seed [Fe] and for seed [N]; however, the directions of change were opposite: Fe decreased and N increased. A slight increase in seed weight (Fig. 2; Wiersma, 2010) was associated with a reduction (–33%) in seed Fe accumulation and a small increase in N accumulation. In other crops, for example durum wheat (*Triticum turgidum* L.), N nutrition was a critical factor in both the acquisition and grain allocation of Zn and Fe, wherein Zn and Fe uptake per plant were increased up to fourfold by high N supply (Kutman et al., 2011). In rice, seed [Fe] increased about 15% with the addition of N at applications between 0 and 135 kg ha^{-1} (Gregorio et al., 2000).

Correlations

In many legumes, seed [Fe] and [Zn] are positively correlated (White and Broadley, 2005) and often both are positively correlated with seed [N] (Raboy et al., 1984). High correlations between [N] and several micronutrient concentrations are considered to represent co-transport of N (chelators, transport proteins, and/or amino acids) and micronutrients to the seed (Waters and Sankaran, 2011). Positive correlations may indicate common pathways or common transporters (such as nicotianamine) and suggest that improvement in one micronutrient may simultaneously improve the concentrations of other micronutrients (Sankaran et al., 2009). Relationships among micronutrients varied as did individual micronutrient correlations with seed number, seed weight, and measures of

relative chlorophyll concentration (Table 4). Correlation coefficients given in Table 4 display positive relationships between [Fe], [Zn], and [N] whereas all of these elements were negatively correlated with [Mn]. The three correlation coefficients (Fe–Mn, Zn–Mn, and N–Mn) were nearly identical ($r = -0.35$).

Using soybean explants, Nooden and Mauk (1987) demonstrated that the accumulation of different elements may be regulated separately, which would suggest that concentrations of certain elements may be negatively correlated. Although we measured only four elements, the negative correlations involving [Mn] seem to suggest that Mn accumulation was hindered by competition with other microelements. PROC STEPWISE (Boling et al., 1990) results (not shown) indicated that seed Mn content was correlated with neither [Mn] nor seed weight whereas seed Fe, Zn, and N content were all primarily associated with element concentration rather than seed weight. Other authors working with different varieties, soils, and environments have reported that seed [Fe] was not correlated with seed [Mn] whereas seed Fe content was primarily related to seed weight and not [Fe] as in this report (Moraghan and Helms, 2005). Considering relative chlorophyll concentrations as estimates of Fe efficiency, Fe efficiency (midseason SPAD measures) increased as [Fe] and seed Fe content as well as [N] and seed number increased (Table 4). Increases in seed number likely were a consequence of improvements in Fe efficiency. Removal ($\mu\text{g m}^{-2}$) of all micronutrients (Fe, Zn, and Mn) as well as N increased as Fe efficiency (SPAD measures) increased, indicating that healthier plants accumulated and removed more Fe, Zn, Mn, and N than plants with lesser Fe efficiencies.

CONCLUSIONS

Iron-efficient and Fe-inefficient varieties had seed [Fe] maxima that were distinctly different and seldom exceeded, suggesting that soybean plants tend to maintain [Fe] in the grain within predetermined, genetically controlled limits. Differences among varieties in seed [Fe] ranked the same regardless of NR, as earlier research has shown for seeding densities, Fe–chelate rates, years, environments, and a much larger set of varieties (Wiersma, 2005, 2007, 2010, 2011). Other responses to increasing NR differed among micronutrients. Changes in seed weight were not correlated with changes in seed [Fe], [Zn], or [N] and therefore, changes in seed weight did not modify elemental concentrations with the exception of [Mn]. The antagonistic iron–manganese relationship commonly reported was observed in this research as well. Conceivably, large increases in seed [Fe] could result in Mn deficiencies. Small, although statistically significant, differences among varieties in [Zn] and [Mn] and Zn and Mn content were essentially the same across NR. These small differences were associated with very small differences in elemental content and

may require further verification. Linear responses to NR among varieties for seed [Fe] and Fe content were negative and highly significant whereas linear responses to NR among varieties for seed [N] were also highly significant but positive. Iron efficiency (midseason SPAD measures) increased as [Fe] and seed Fe content increased providing additional evidence that seed [Fe] is predictive of resistance to Fe deficiency. Chlorosis screening nurseries may benefit from using fairly high rates of N (170 kg N ha⁻¹) to increase Fe deficiency, to provide more uniformity in chlorosis response across experimental areas, and to separate resistant and susceptible varieties with added confidence. It should also be possible to measure numerous samples of seed from a chlorosis nursery as a reasonable method of characterizing seed [Fe] and genotypic resistance to Fe deficiency. Nonetheless, there is little information reported for soybean that could be used to specifically outline a suitable procedure of breeding for resistance to Fe deficiency using seed [Fe] as a selection criterion.

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