JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Screening of Iron Bioavailability Patterns in Eight Bean (*Phaseolus vulgaris* L.) Genotypes Using the Caco-2 Cell in Vitro Model

MAGNOLIA ARIZA-NIETO,† MATTHEW W. BLAIR,‡ ROSS M. WELCH,§ AND RAYMOND P. GLAHN*,§

Food Science, Cornell University, and U.S. Plant, Soil and Nutrition Laboratory, U.S. Department of Agriculture, Ithaca, New York 14853, and CIAT, International Center for Tropical Agriculture, Cali, Colombia

The common bean (*Phaseolus vulgaris*) is an important staple plant food in the diets of people of Latin America, East Africa,and other regions of the developing world. It is also a major source of dietary iron. The primary goal of this research was to use an in vitro digestion/Caco-2 model to study iron bioavailability in eight genotypes (three Mesoamerican and five Andean) that represent the diversity of grain types in this crop. Complementing this goal, we measured the distribution of both iron and phytate in different bean grain tissues (cotyledon, seed coats, and embryos). Seed coats were confirmed to be the exclusive tissue containing polyphenols. The removal of the seed coat and associated polyphenols improved Caco-2 iron bioavailability, and significant differences were observed between genotypes. The addition of ascorbate enhanced iron bioavailability and exposed additional differences in Fe availability among the genotypes. These results indicate that iron accumulation and in vitro iron bioavailability vary among bean genotypes and that polyphenols had greater inhibitory effects on Caco-2 iron bioavailability as compared to phytate.

KEYWORDS: Iron bioavailability; iron; phytate; polyphenols; beans (*Phaseolus vulgaris* L); in vitro digestion/Caco-2 model

INTRODUCTION

Common beans (*Phaseolus vulgaris* L.) are an important staple food crop in Latin America and Africa, which provide calories and protein as well as minerals and vitamins. For low-income populations, common beans are an inexpensive supply of macro- and micronutrients (*1*). Genetic variability for grain micronutrient concentration exists in common beans as shown by Welch et al. (*2*), and a biofortification project (i.e., HarvestPlus) to improve the level of iron in common bean has begun (*3*). However, the question of whether higher iron accumulation in the grain is correlated with higher bioavailable iron levels or whether polyphenols and phytate differentially affect bioavailability is still unresolved, although a rat model study reported a positive linear relationship between bean seed concentration and bioavailable iron (*2*) using various genotypes of beans. A definitive study to determine if a higher bean Fe concentration results in greater human Fe uptake has not been done.

There is clearly a need to learn more about potential genotypic differences in bean Fe bioavailability. Because of the high cost of in vivo studies and the need to screen a high number of samples to address this question, the most practical approach to this problem entails initial screening via in vitro methods. A high-throughput in vitro methodology for measuring iron bioavailability has been developed and applied to differentiate the nutritional quality of different vegetative tissues or different genotypes of a given food species either alone or in mixtures with other foods (*4*). This model utilizes simulated gastric and intestinal digestion coupled with the presence of a human intestinal epithelial cell line (Caco-2), which serves as a detector of available Fe. Caco-2 cell Fe uptake serves as the marker for bioavailability and thus gives a distinct advantage over other in vitro methods such as solubility and dialyzability, as Fe can often be soluble yet unavailable for uptake.

It is known that iron bioavailability from common bean grains is affected by components found within the grain such as polyphenols and phytate, the predominant form of which is *myo*-inositol hexakisphosphate (IP6) (*2*). Studies by Beninger et al. (*5*) have shown that polyphenols in beans are exclusively localized in the seed coats; thus, phytate is likely to be the major inhibitor of Fe bioavailability in the cotyledon. IP6 is known to be less likely to occur in the seed coat but is concentrated in zygotic or embryogenic tissues (*6*). To our knowledge, the distribution of iron among different common bean seed tissues has not been reported.

* To whom correspondence should be addressed. Tel: 607-255-2452. Fax: 607-255-1132. E-mail: rpg3@cornell.edu.
† Cornell University.
‡ International Center for Tropical Agriculture.
§ U.S. Department of Agriculture.

10.1021/jf070023y CCC: $37.00 © 2007 American Chemical Society
Published on Web 08/17/2007

The present study is part of the global HarvestPlus biofortification plant-breeding program, which includes the determination of bioavailable iron using the in vitro digestion/Caco-2 cell model in screening staple food crop genotypes for high bioavailable Fe. A series of candidate bean (*P. vulgaris* L.) genotypes from the breeding program at International Center for Tropical Agriculture (CIAT) were screened in an effort to identify those with significantly higher amounts of bioavailable Fe. The genotypes used for the study reflect the diversity of common bean genotypes widely consumed in Latin America and East Africa and represent the major commercial classes preferred by consumers in these regions, namely, the black and small red grain types of Brazil, Mexico, and Central America and the large red and large red-mottled grain types of East Africa and the Andean region of South America. Considering the diversity of components in these genotypes, the tool of choice for screening is the in vitro digestion/Caco-2 cell model, which allows for the high-throughput screening of bean samples and can detect the presence of inhibitors or promoters of iron bioavailability in the bean grain organs from each genotype (*4*).

The objectives of this research were to determine the concentration and location of iron, phytate, and polyphenols in the major grain tissues of common bean (cotyledon, seed coat, or embryo) and to use the in vitro digestion/Caco-2 cell model to identify the genotypes with the highest concentration of bioavailable iron. This in vitro research is the first step toward understanding the location of inhibitors and possible promoters of iron bioavailability in the bean grain and which bean organ or tissue should be targeted for improving levels of bioavailable iron.

MATERIALS AND METHODS

Plant Materials. Eight genotypes of common beans were obtained from CIAT, which included five Andean genotypes (AND696, CAL96, CAL149, G19833, and Radical Cerinza) and three Mesoamerican genotypes (DOR390, DOR364, and Tio Canela). Tio Canela and Radical Cerinza are small red and large red popular varieties from Central America and highland Colombia, respectively. DOR364 and DOR390 are small-grained, red and black advanced lines from CIAT that were released as varieties in Honduras, El Salvador, Nicaragua, Cuba, and Mexico, while CAL96 is a large-grained, red-mottled advanced line also from CIAT that was released in Uganda. CAL149 and AND696 are similar, red-mottled advanced lines that have not been released as varieties but that are well-adapted to tropical highland production zones. G19833 is a germplasm accession from the CIAT gene bank with yellow and red-mottled seed coloring and is considered to be an important genetic stock along with DOR364 as these varieties have been well-studied for their nutritional characteristics.

Sample Preparation. All bean samples were harvested in May 2005 in Darien, Colombia, and sent to Cornell for processing. Prior to cooking, the dry beans were imbibed in water overnight. The bean parts were separated manually from each bean. To collect a sufficient amount of sample, three separate experiments were done with 20 beans each for each genotype. Once separated, the individual bean tissues (cotyledons, seed coats, and embryos) were cooked separately. After cooking, the samples were lyophilized and ground to a fine powder. The ground samples were used for quantification of iron, phytate, and polyphenols and estimation of bioavailable iron. For nutritional purposes, our study was focused on ready-to-eat food, so the results presented in this research are from cooked samples unless otherwise stated.

Iron Bioavailability and Caco-2 Cell Cultures. The in vitro Caco-2 digestion model followed the procedure of Glahn et al., (*4*) with 250 mg samples analyzed per replicate. In this method, gastric and intestinal digestion of foods were simulated and applied to Caco-2 cell monolayers. Iron uptake by the Caco-2 cell monolayers was measured by cell ferritin formation, a known response of enterocytes to iron uptake. Six replicates of each iron bioavailability measurement were performed. Quality controls to monitor the responsiveness of the Caco-2 cells included a baseline with no iron added, 1 μmol of $FeCl_3$ extrinsic iron, and 1 μmol of $FeCl_3$ with 20 μmol of ascorbic acid as an enhancer of bioavailability.

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 25–33. Cells were grained at a density of 50000 cells/cm^2 in collagen-treated six-well plates (six-well cell culture cluster dishes, Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) with 10% v/v fetal bovine serum (Gibco) and 25 mmol/L HEPES. The cells were maintained at 37 °C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity; the medium was changed every 2 days. The cells were used in the iron uptake experiments at 13 days postgraining. Under these conditions, the amount of cell protein measured in each well was highly consistent from well to well.

Polyphenols. Prior to the polyphenol extraction, 10 g of dry beans from each genotype was imbibed in 30 mL of water until completely saturated. The cotyledons were separated from the seed coats. The seed coats and the remaining water if any were mixed with 30 mL of extraction solution containing 40% ethanol and 1% 1 N HCl. The determination of total polyphenols was done using the colorimetric protocol for Folin–Ciocalteau, using gallic acid for calibration (*7*).

Phytate. A Dionex Liquid Chromatograph system, conductivity detector model ED50, and gradient pump GS50 were used along with an IonPac AG11 Guard Column and IonPac AS11-column to quantify phytate. The PeakNet 6.40 software was used to process chromatographic data. The mobile phases were (A) 200 mM NaOH (carbonated-free) and (B) deionized water, using a flow rate of 1 mL/min. Phytate was extracted from a 250 mg dry cooked, lyophilized sample, in 10 mL of a 1.25% H_2SO_4 solution; the extraction process was 2 h, after which the samples were centrifuged at 3660*g* for 10 min.

Iron and Other Mineral Quantification. Iron and the other minerals were quantified using an inductively coupled argon-plasma emission spectrometer (ICP-ES) (ICAP model 61E Trace Analyzer, Thermo Jarrell Ash Corp., Franklin, MA) after sample digestion using HNO_3 and $HClO_4$. To prevent possible iron contamination from laboratory ware, everything used in the quantification was placed overnight in 10% HCl and rinsed carefully with 18 MΩ deionized water before use.

Statistical Analyses. Data were analyzed using the software package JMP 5.1 and/or GraphPad Prism (GraphPad Software, San Diego, CA). Analysis of variance (ANOVA) with Tukey's post-test was used to compare differences among means. Where appropriate, data were transformed to achieve equal sample variances. All of the experiments included six replicates ($n = 6$) unless otherwise stated. Differences among means were considered significant at $P \leq 0.05$.

RESULTS

Concentration of Iron and Other Minerals in Dry Bean Grain. Differences in mineral accumulation are presented in **Table 1**. The maximum iron concentration was 74 μg/g found in the Mesoamerican genotype G19833. Generally, the Andean genotypes contained more iron than the Mesoamerican ones, with the lowest being Tio Canela that contained 48 μg/g. The Mesoamerican DOR390 showed the highest concentration of zinc (28 μg/g), followed by the Mesoamerican DOR364 and the Andean G19833, both with 25 μg/g Zn. Overall, the Andean genotypes accumulated less Zn with CAL96 having the lowest concentration of 17 μg/g. The overall accumulation of P in the Mesoamerican genotypes was similar to the Andean genotypes. The Andean G19833 had the highest P level in the group, 5175 μg/g P, although the concentration was comparable to the Mesoamerican DOR364 with 5072 μg/g P and Tio Canela with 4556 μg/g P. In the Andean genotypes, the Mn concentration ranged from 11 to 15 μg/g, and in the Mesoamerican genotypes, it ranged from 13 to 15 μg/g Mn. In the Andean genotypes, Cu

Table 1. Seed-Mineral Concentrations in Common Bean Genotypes[a]

		concentration (μg/g)							
	gene pool	K	P	Ca	Mg	Fe	Mn	Cu	Zn
AND696	Andean	11836 a	3804 bc	1547 bcd	1593 cd	63 b	12 b	5 d	18 e
CAL149	Andean	11332 a	3295 c	1616 b	1688 bc	55 c	11 c	5 cd	17 e
CAL96	Andean	12497 a	3760 bc	2015 a	1473 e	54 c	15 a	5 d	21 d
G19833	Andean	11551 a	5175 a	1397 cd	1564 de	74 a	14 a	8 b	25 b
Radical Cerinza	Andean	13813 a	4041 bc	1684 b	1722 b	65 b	15 a	5 c	24 bc
DOR364	Mesoamerican	13701 a	5072 a	1568 bc	1916 a	53 cd	13 b	10 a	25 b
DOR390	Mesoamerican	13509 a	3775 bc	2038 a	1958 a	57 c	14 a	8 b	28 a
Tio Canela	Mesoamerican	13586 a	4556 ab	1360 d	1787 b	48 d	15 a	5 d	23 c
SEM		762	180	42	24.5	1.28	0.23	0.09	0.4

[a] Within each column, mean values with a letter in common are not significantly different from one another. SEM, pooled standard error of mean calculated from error mean square of ANOVA ($n = 9$). Least significant difference (LSD) = 3.133 ($P \leq 0.05$).

Table 2. Proportional Weight (mg per Grain) and Concentrations of Fe (μg g^{-1}) and Phytate (μmol g^{-1}) in Three Bean Seed Tissues (Cotyledon, Embryo, and Seed Coat) of Common Bean Genotypes, and Their Corresponding Percent by Weight in Whole Bean Grains[a]

genotype	cotyledon	embryo	seed coat	whole bean*
		Fe concentration (μg g^{-1})		
AND696	53 ± 0.79 (78.6%)	96 ± 4.4 (1.7%)	154 ± 10.8 (19.7%)	63 ± 6.9
CAL149	64 ± 0.22 (94.3%)	109 ± 0.64 (1.1%)	36 ± 0.63 (4.6%)	55 ± 1.0
CAL96	61 ± 0.46 (94.5%)	123 ± 9.5 (1.8%)	28 ± 0.93 (3.7%)	54 ± 1.7
G19833	83 ± 1.01 (93.6%)	146 ± 14.9 (2.2%)	36 ± 3.6 (4.1%)	74 ± 2.5
Radical Cerinza	71 ± 0.39 (92.8%)	113 ± 24.3 (1.3%)	42 ± 6.9 (5.9%)	65 ± 2.0
DOR364	49 ± 1.18 (77.5%)	136 ± 38.8 (3.9%)	107 ± 1.8 (18.5%)	53 ± 1.8
DOR390	45 ± 0.45 (71.4%)	88 ± 4.9 (2.2%)	156 ± 21.2 (26.4%)	57 ± 6.5
Tio Canela	54 ± 0.07 (90.8%)	103 ± 12.9 (3.6%)	30 ± 3.01 (5.6%)	48 ± 3.3
		phytate concentration (μmol g^{-1})		
AND696	9.35 ± 1.13 (97.5%)	8.02 ± 0.01 (1.0%)	1.66 ± 0.09 (1.5%)	8.7 ± 0.54
CAL149	6.6 ± 0.01 (98.0%)	16.56 ± 0.02 (1.7%)	0.24 ± 0.02 (0.3%)	6.2 ± 0.17
CAL96	8.65 ± 0.01 (97.3%)	19.47 ± 0.01 (2.0%)	0.73 ± 0.11 (0.7%)	8.1 ± 0.06
G19833	12.31 ± 0.87 (97.5%)	6.62 ± 0.03 (0.7%)	2.31 ± 0.83 (1.8%)	11.3 ± 0.6
Radical Cerinza	10.29 ± 0.54 (97.3%)	18.34 ± 0.01 (1.5%)	1.15 ± 0.37 (1.2%)	9.5 ± 0.23
DOR364	12.4 ± 0.50 (94.8%)	5.80 ± 0.01 (0.8%)	5.27 ± 0.33 (4.4%)	11.6 ± 0.80
DOR390	9.57 ± 0.52 (96.9%)	7.75 ± 0.03 (1.2%)	1.74 ± 0.37 (1.9%)	8.8 ± 1.06
Tio Canela	11.02 ± 0.01 (96.6%)	17.22 ± 0.03 (3.1%)	0.29 ± 0.09 (0.3%)	10 ± 1.03
		weight distribution (mg)		
AND696	467.2 ± 5.04 (91.04%)	5.5 ± 0.06 (1.07%)	40.4 ± 0.44 (7.87%)	513 ± 55
CAL149	444.6 ± 46.3 (91.43%)	3.1 ± 0.32 (0.64%)	38.6 ± 4.02 (7.94%)	486 ± 87
CAL96	602.3 ± 57.5 (91.52%)	5.7 ± 0.55 (0.87%)	50.1 ± 4.78 (7.61%)	658 ± 98
G19833	463.0 ± 70.9 (89.71%)	6.3 ± 0.97 (1.22%)	46.8 ± 7.16 (9.07%)	516 ± 57
Radical Cerinza	479.6 ± 5.6 (89.54%)	4.1 ± 0.05 (0.77%)	51.9 ± 0.61 (9.69%)	535 ± 98
DOR364	187.5 ± 33.2 (88.78%)	3.4 ± 0.06 (1.61%)	20.3 ± 3.59 (9.61%)	211 ± 27
DOR390	205.3 ± 11.4 (89.11%)	3.2 ± 0.18 (1.39%)	21.9 ± 1.21 (9.51%)	230 ± 31
Tio Canela	229.0 ± 31.1 (88.49%)	4.7 ± 0.65 (1.82%)	25.1 ± 3.44 (9.70%)	258 ± 35

[a] Values are means ± SD; $n = 9$ for Fe, $n = 3$ for phytate, and $n = 10$ for weight.

ranged from 5 to 8 μg/g, and in the Mesoamerican genotypes, it ranged from 5 to 10 μg/g Cu. The Mesoamerican genotypes showed a higher overall average in the accumulation of K with 13598.6 μg/g, and the Andean genotypes had an overall average of 12205.8 μg/g K, although no significant difference was found among the group. Differences in seed-mineral concentrations were analyzed via ANOVA and Tukey's post-test to compare differences among means.

Distribution of Iron in the Bean Grain. The approximate concentration and percentage of iron in the bean parts of three Mesoamerican and five Andean genotypes are presented in **Table 2**. The amount of iron stored in the cotyledons ranged from 71.4 to 94.5%, in the embryo from 1.1 to 3.6%, and in the seed coat from 4.1 to 26.4% of the total iron in the bean. The Andean genotype G19833 had the highest concentration of iron in the whole bean, which also corresponds to the highest concentration of iron in the cotyledon with 83 μg/g and also the highest concentration of iron in the embryo with 146 μg/g. However, G19833 showed one of the lowest concentrations of iron in the seed coat with 36 μg/g. The Mesoamerican DOR364 and DOR390 and the Andean AND696 had the lowest percentage and concentration of iron in the cotyledons and the highest in the seed coat. Aside from the Andean AND696, the rest of the Andean genotypes had a high iron concentration ranging from 61 to 83 μg/g and from 92.8 to 94.5% of the total iron in the cotyledon and a low 28–42 μg/g and 3.7–5.9% in the seed coat. In the Mesoamerican group, Tio Canela had the highest concentration of iron in the cotyledon with 54 μg/g, which corresponds to 90.8% of the total iron, and the lowest concentration of iron in the seed coat with 30 ppm and 5.6% of the total iron.

Distribution of Phytate in the Bean Grain. The highest overall concentration of phytate in the whole bean was found in the Mesoamerican DOR364 with 11.6 μmol g^{-1} and the lowest in the Andean CAL149 with 6.2 μmol g^{-1} (**Table 2**). Overall, the Mesoamerican genotypes had a higher concentration of phytate than the Andean ones. Almost all of the phytate (94–98%) was located in the cotyledons. Phytate concentrations in the seed coats (0.24–5.27 μmol g^{-1}) and embryo (5.8–19.5 μmol g^{-1}) were small, which accounted for low phytate percent in this tissue.

Table 3. Amount of Iron Leached from the Grains (μg g^{-1} ± SD), Percentage of Total Iron that Leached, and Weight Gain during Water Imbibition[a]

	whole beans after soaking		
genotype	Fe leached (μg g^{-1})	Fe leached (%)	weight gained (%)
AND696	4.7 ± 0.86	8	216
CAL149	5.5 ± 0.37	9	220
CAL96	1.9 ± 0.29	3	220
G19833	5.8 ± 0.34	8	224
Radical Cerinza	3.8 ± 0.29	6	231
DOR364	2.8 ± 0.44	5	201
DOR390	3.9 ± 0.56	8	212
Tio Canela	6.5 ± 0.32	13	220

[a] A total of 100 grains from each genotype were imbibed in water, and measurements were taken from six pools ($n = 6$).

Distribution of Weight in the Bean Grain Organs and Tissues. The Mesoamerican genotypes had a significantly smaller weight than the Andean genotypes (**Table 2**). The Andean CAL96, with 658 mg, has the highest seed weight of all of the genotypes. The weight of each seed tissue and individual contribution (as a percent) to the whole bean seed weight was also measured, and across all genotypes, the cotyledon represented 88–91% of the bean weight, the embryo 0.6–1.8%, and the seed coat 7.6–9.7% of total seed weight.

Dry Beans and Moisture Gain Prior to Cooking. As dry bean seeds are often soaked before cooking, we evaluated the moisture content of seeds. When genotypes were imbibed in water, they gained an average of 218% of their weight in water (**Table 3**). During the soaking period, the Mesoamerican genotypes gained between 201 and 220% and the Andean genotypes between 216 and 231% of their weight. The amount of iron lost or released into the imbibing solution during the process of soaking was also measured (**Table 3**). On average, 7% of the iron was leached from the Andean genotypes and 9% from the Mesoamerican genotypes. The highest amount of iron leached was 13% during imbibition of the Mesoamerican genotype Tio Canela.

Bioavailable Iron as Determined by in Vitro Digestion. The Caco-2 cell iron uptake from the bean genotypes was studied under three conditions: whole cooked beans, cooked cotyledons (beans without seed coats), and cooked cotyledons with extrinsic ascorbate added (1:20, Fe:Ascobate) (**Figure 1**). The baseline cell ferritin was 3.6 ng ferritin/mg protein. Caco-2 cell ferritin formation for the treatments of whole cooked beans that contained seed coats was found to be very low ranging from 1.1 to 11.5 ng ferritin/mg protein. Only two genotypes, AND696 (11.5 ng ferritin/mg protein) and Radical Cerinza (4.6 ng ferritin/mg protein), presented iron uptake above the baseline. Multivariate analysis revealed a low correlation value of 0.0374 between the phytate/iron molar ratio and the bioavailable iron as determined by Caco-2 Cell Ferritin formation.

Bioavailable iron (i.e., Caco-2 cell ferritin formation) was higher for the cooked cotyledons and ranged from 10.8 to 31.9 ng ferritin/mg proteins; they were significantly above the baseline levels. The highest uptake for cooked cotyledons was found with genotype AND696 (32.2 ng ferritin/mg protein), and the lowest uptake was found for genotype CAL96 (10.75 ng ferritin/mg protein). In the cooked cotyledons plus ascorbate, the highest uptake was found with the Andean genotype G19833 (147.9 ng ferritin/mg protein), and the lowest was found with genotype CAL96 (67.8 ng ferritin/mg protein). A statistical significant difference was found between Cal 96 and G19833. Differences in bioavailable Fe were conducted with ANOVA and Tukey's post-test to compare differences among means ($n = 6$ and $P \leq 0.05$). The concentration of phytate was not correlated with iron bioavailability (**Table 4** and **Figure 1**).

Phytate:Iron Molar Ratios. The effect of phytate on iron bioavailability has been related to the phytate:Fe molar ratios in plant foods; some studies indicate that ratios above 10 lead to reduced iron bioavailability to humans (*8*). Thus, the concentration and distribution of bean-iron, -phytate, and -seed weight (**Table 2**) were used to calculate the phytate to iron molar ratios (**Table 4**). The ratios of phytate:Fe were between 5.73 and 14.26 for cooked cotyledons and between 6.29 and 12.18 for whole cooked beans.

Polyphenols in Dry and Cooked Beans. After imbibing the seeds prior to cooking in water, the aqueous phase was found to contain significant amounts of extractable pigments detectable in spectrophotometric scans between 200 and 600 nm (**Figure 2**). Total polyphenols on both the raw and the cooked samples were assayed, but because of instability and very low levels on the cooked processed grains, data are not reported; only results on raw bean grain are shown on (**Table 5**). The small red and black-grained Mesoamerican genotypes DOR364 and DOR390 and the small red Tio Canela released a higher concentration of pigments than the Andean genotypes; they were also found to contain anthocyanin (Glahn's unpublished information). The five Andean genotypes did not have anthocyanins, and the spectophotometric scans suggest close similarities (**Table 2**).

DISCUSSION

Common beans (*P. vulgaris*) are recognized by consumers as a rich source of minerals including iron. Iron bioavailability depends on the interaction between the iron and the other dietary components in the food matrix, and this condition is genotype-dependent. In this study, a set of eight genotypes was screened using the Caco-2 in vitro digestion model as a tool to identify differences in iron bioavailability. Those differences can be due to promoters and/or inhibitors present in the food matrix. The primary factors affecting iron solubility in beans have been reported to be pH and complexation with polyphenols, proteins, and organic acids (*8*). The concentration, solubility, and strength of the Fe complexes have a major role in iron bioavailability, since these factors influence the degree to which iron can interact with the enterocytes transporters in the intestinal lumen. Most iron absorption is thought to occur in the duodenum and upper jejunum (*9*).

Phytate is known to inhibit iron uptake when in molar excess relative to the mineral (*8*). Low-phytate varieties or high seed-phytase activity have been developed through traditional breeding or transgenics (*10, 11*); however, phytate and polyphenols play important roles in plant metabolism and when consumed are linked to lowering chronic disease rates in humans (*10*). Therefore, eliminating or lowering the levels of these compounds must be done with caution (*12*). In addition, animal studies with rats, dogs, and humans have reported that monoferric phytate is a highly soluble and stable complex and can be a highly bioavailable source of iron (*13, 14*). Human studies comparing whole vs dephytinized wheat brand showed a positive iron balance in men when monoferric phytate in the whole-wheat bran was consumed (*15*). Overall, the inhibitory effect of phytate may be no worse than that of other compounds that complex iron such as citric acid (*16*), and it appears that it is the abundance of phytate in plant foods that makes it a major factor of iron bioavailability.

Polyphenolics in beans are thought to play a significant inhibitory role in bean Fe availability; however, relatively little

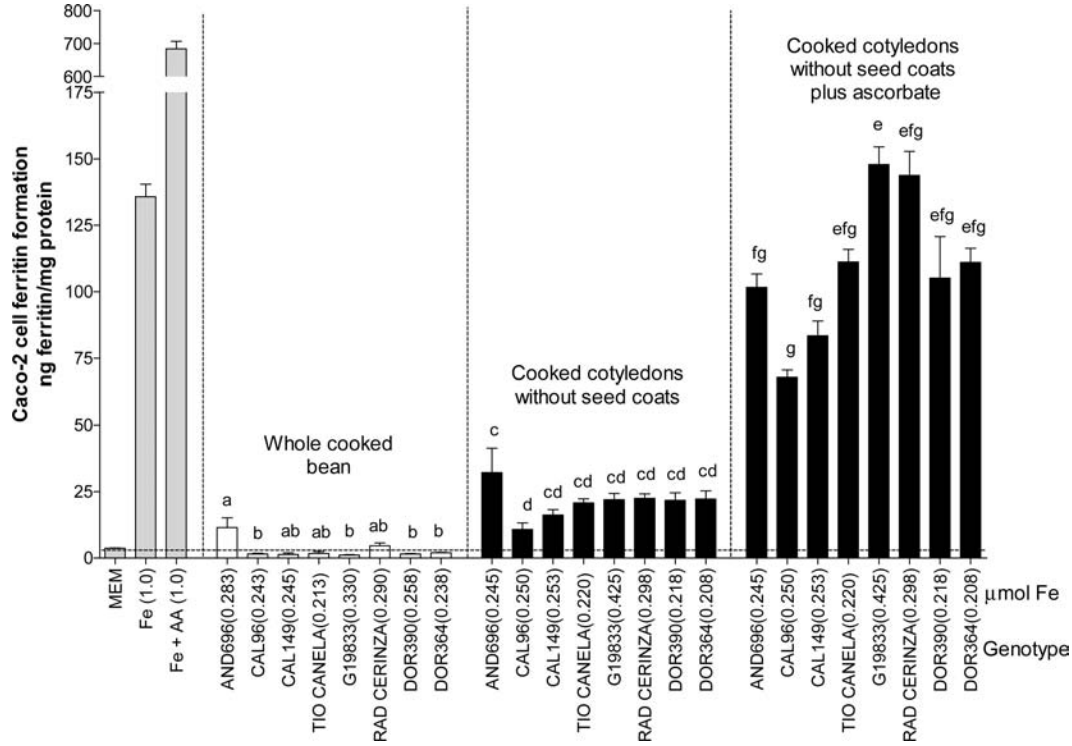

Figure 1. Iron bioavailability as measured by Caco-2 cell iron absorption in eight bean genotypes under three conditions: whole cooked beans containing polyphenols, cotyledons without polyphenols, and cotyledons without polyphenols plus ascorbate added (1:20, Fe:ascobate molar ratio). The dashed line corresponds to the cell ferritin baseline and represents a control with no iron added to the digest solutions. Values indicated in parentheses are SEM ($n = 3$) of the iron level in the 15 mL digests. Bar values with no letters in common are significantly different; LSD among treatments = 3.196 ($P \leq 0.05$). Values are means + SEM ($n = 6$).

Table 4. Phytate/Fe Molar Ratios and Bioavailable Iron as Determined by Caco-2 Cell Ferritin Formation for Cotyledons and Whole Beans of Eight Genotypes[a]

	cooked[b] cotyledons		cooked[b] whole beans	
genotype	ratio[a] (IP6)/Fe	Caco-2 ferritin/mg protein	ratio[a] (IP6)/Fe	Caco-2 Ferritin/mg protein
AND696	9.82	32.21	7.71	11.47
CAL149	5.73	16.19	6.29	3.66
CAL96	7.97	10.75	8.43	1.57
G19833	8.28	21.98	8.61	1.11
Radical Cerinza	8.07	22.48	8.17	4.62
DOR364	14.26	22.20	12.18	1.99
DOR390	11.89	21.71	8.58	1.56
Tio Canela	11.42	20.71	11.83	3.69

[a] Ratios calculated with data presented in **Table 2** and **Figure 1**, with $n = 9$ for Fe, $n = 3$ for phytate, and $n = 10$ for weight. [b] Cooked cotyledons and whole beans grains showed either no or very low levels of polyphenols.

is known as to which polyphenolics inhibit Fe availability. This is mostly due to the fact that these compounds can rapidly oxidize and simple colorimetric assays do not distinguish between the compounds that inhibit Fe availability and those that have no effect (*14*). Recently, it was shown that certain flavonoids such as kaempferol and quercitin are highly prevalent in bean seed coats and inhibit Fe availability to Caco-2 cells (*17, 18*). These studies indicate that certain parts of the flavonoid ring are responsible for the inhibitory effect and, therefore, indicate that high-performance liquid chromatography profiling of the polyphenols may be the best approach to characterize the polyphenolics effect on Fe availability.

Despite the relatively high iron concentrations in the bean genotypes studied, the amount of bioavailable iron was low in all of the whole bean samples tested. To determine the location of iron bioavailability inhibitors (i.e., polyphenols and phytate), the seeds were separated into several organs. Polyphenolics were observed to be concentrated in the seed coats but not in the cotyledons. The seed coats of the different genotypes represented 8–10% of the seed biomass and contained from 4 to 26% of the iron found in the seed coats.

The mature seed coat is composed of more than 12 layers of the inner seed coat parenchyma cells squeezed together during development, resulting in a compressed mat of cell wall material (*19, 20*), and the polyphenols are cross-linked with cell wall components in plant cells (*21, 22*). In dicotyledonous plants, primary cell walls are formed mainly from nondigestible carbohydrates, pectin, cellulose, and hemicellulose including pentosans, fructans, chitin, and polysaccharides (*21*). Micrographs produced by scanning electron microscopy have shown that undamaged almond seed coats contained high levels of ferulic acid even after in vivo digestion in the human gut (*23*). Similarly, separation of bean seed coat from cotyledons may occur in the digestive track having implications on iron bioavailability and its interaction with polyphenols. Our results show increasing iron bioavailability resulting from ascorbate

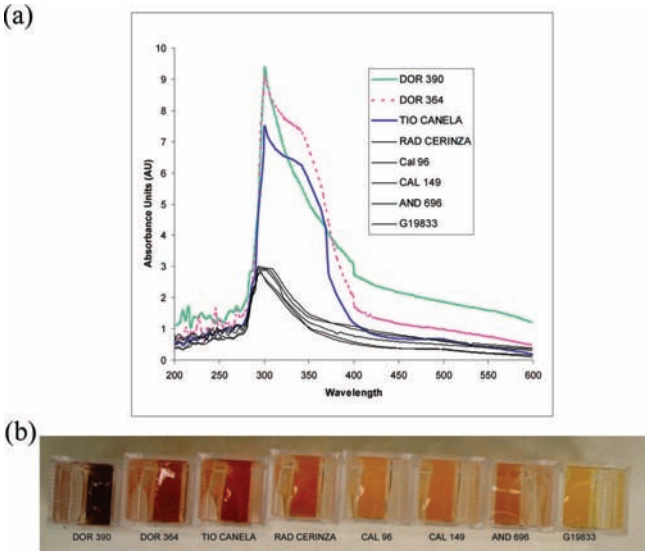

Figure 2. During seed imbibition, iron and colored substances leachate from beans into the water. (**a**) Spectrophotometric scans of leachates from beans imbibed in water. (**b**) Pictures of the bean leachates containing color compounds. Grains were imbibed for 20 h at 18 °C.

Table 5. Total Polyphenols as Gallic Acid Equivalents on Raw Whole Bean Grains[a]

genotype	GAE/100 g sample	SD
AND696	1340 abc	151
CAL96	1311 abc	90
CAL149	1350 abc	98
Tio Canela	1165 c	95
G19833	1382 ab	98
Radical Cerinza	1430 a	126
DOR390	1469 a	121
DOR364	1182 bc	68

[a] Mean values with a letter in common are not significantly different from one another. Values are means ± SD ($n = 9$).

added to the cotyledons using the Caco-2 model, which may reflect dietary interactions of beans with other foods.

Relative to humans, the in vitro digestion results suggest that the Caco-2 model might be oversensitive to the inhibitory effects of the phenolic components in the seed coat (*24*). Clearly, it is important to define these limitations to make proper use of in vitro models; however, although current evidence suggests that such oversensitivity may exist, it is only based on one single study (*24*). Given the limitations of that study, namely, the extrinsic labeling of the beans, further comparisons need to be pursued. Several possibilities exist to explain this difference in the in vitro vs the in vivo comparisons. First, the in vitro model may mimic the upper intestine Fe availability quite well, and it may be that factors in the lower intestine, such as the microflora, are able to modify the Fe availability. Second, the use of extrinsic labeling in the human trial may be slightly in error. In a recent study of the same meals used in the Beseigel et al. (*24*) study, we observed that the extrinsic Fe equilibrated poorly with the intrinsic Fe of the pinto bean meal and equilibrated reasonably well with the white bean (*25*). In either case, the present study demonstrates that the in vitro digestion/Caco-2 model has the potential to screen bean samples for improved Fe bioavailability. This can be done with or without the seed coats. Only further research in direct comparisons of the in vitro vs in vivo will determine how well this model predicts the Fe availability from beans. It is important that an in vitro model be validated for predicting human Fe availability from beans and other foods. In terms of biofortification, this will provide a useful tool that plant breeders are in desperate need of to develop and ultimately monitor their crops for nutritional quality once they are released to the farmer and subject to the multitude of factors present in the environment. In summary, these results indicate that iron accumulation and in vitro iron bioavailability vary among bean genotypes and that polyphenols had greater inhibitory effects on Caco-2 iron bioavailability as compared to phytate.

ACKNOWLEDGMENT

We thank Larry I. Heller for his contributions to phytate analysis.

LITERATURE CITED

(1) Broughton, W. J.; Hernandez, G.; Blair, M. W.; Gepts, P.; Vanderleyden, J. The Phaseomics International Consortium. *Plant Physiol.* **2003**, *131*, 860–862.

(2) Welch, R. M.; House, W. A.; Beebe, S.; Cheng, Z. Genetic selection for enhanced bioavailable levels of iron in bean (*Phaseolus vulgaris* L.) grains. *J. Agric. Food Chem.* **2000**, *48*, 3576–3580.

(3) Bouis, H. E. Micronutrient fortification of plants through plant breeding: Can it improve nutrition in man at low cost? *Proc. Nutr. Soc.* **2003**, *62*, 403–411.

(4) Glahn, R. P.; Lee, O. A.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555–1561.

(5) Beninger, C. W.; Gu, L.; Prior, R. L.; Junk, D. C.; Vandenberg, A.; Bett, K. E. Changes in polyphenols of the seed coat during the after-darkening process in pinto beans (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **2005**, *53*, 7777–7782.

(6) Nunes, A. C.; Vianna, G. R.; Cuneo, F.; Amaya-Farfan, J.; de Capdeville, G.; Rech, E. L.; Aragao, F. J. RNAi-mediated silencing of the myo-inositol-1-phosphate synthase gene (Gm-MIPS1) in transgenic soybean inhibited grain development and reduced phytate content. *Plant* **2006**, 1–8.

(7) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. *Methods Enzymol.* **1999**, *299*, 152–178.

(8) Engle-Stone, R.; Yeung, A.; Welch, R.; Glahn, R. Meat and ascorbic acid can promote Fe iron availability from Fe-phytate but not from Fe-tannic acid complexes. *J. Agric. Food Chem.* **2005**, *53*, 10276–10284.

(9) Conrad, M. E. J.; Crosby, W. H. Intestinal mucosal mechanism controlling iron absorption. *Blood* **1963**, *22*, 406–415.

(10) Brinch-Pedersen, H.; Borg, S.; Tauris, B.; Holm, P. B. Molecular genetic approaches to increasing mineral availability and vitamin content of cereals. *J. Cereal Sci.* **2007**. Epub ahead of print.

(11) Raboy, V.; Gerbasi, P. F.; Young, K. A.; Stoneberg, S. D.; Pickett, S. D.; Bauman, A. T.; Murthy, P. P. N.; Sheridan, W. F.; Ertl, D. S. Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. *Plant Physiol.* **2000**, *124*, 355–368.

(12) Welch, R. M.; Graham, R. D. Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* **2004**, *55*, 353–364.

(13) Lipschitz, D. A.; Simpson, K. M.; Cook, J. D.; Morris, E. R. Absorption of monoferric phytate by dogs. *J. Nutr.* **1979**, *109*, 1154–1160.

(14) Morris, E. R.; Ellis, R. Isolation of monoferric phytate from wheat bran and its biological value as an iron source to the rat. *J. Nutr.* **1976**, *106*, 753–760.

(15) Morris, E. R.; Ellis, R.; Steele, P.; and Moser, P. B. Mineral balance of adult men consuming whole or dephytinized wheat bran. *Nutr. Res.* **1988**, *8*, 445–458.

(16) Glahn, R. P.; Lai, C.; Hsu, J.; Thompson, J. F.; Guo, M.; Van Campen, D. R. Decreased citrate improves iron availability from infant formula: Application of an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 257–264.

(17) Hu, Y.; Cheng, Z.; Heller, L. I.; Krasnoff, S. B.; Glahn, R. P.; Welch, R. M. Kaempferol in red and pinto bean seed (*Phaseolus vulgaris* L.) coats inhibits iron bioavailability using an in vitro digestion/human Caco-2 cell model. *J. Agric. Food Chem.* **2006**, *54*, 9254–9261.

(18) Hu, Y.; Cheng, Z.; Heller, L. I.; Glahn, R. P.; Welch, R. M. Kaempferol and quercitrin effect on iron bioavailability in white and colored bean seeds (*Phaseolus vulgaris* L.) using an in vitro digestion/human Caco-2 cell model. *FASEB J.* **2006**, *20*, A197.

(19) Van Dongen, J. T.; Ammerlaan, A. M. H.; Wouterlood, M.; van Aelst, A. C.; Borstlap, A. C. Structure of the developing pea seed coat and the post-phloem transport pathway of nutrients. *Ann. Bot. (London)* **2003**, *91*, 729–737.

(20) Wang, H. L.; Grusak, M. A. Structure and development of Medicago truncatula pod wall and seed coat. *Ann. Bot. (London)* **2005**, *95*, 737–747.

(21) Voragen, A. G. J. Technological aspects of functional food-related carbohydrates. *Trends Food Sci.* **1998**, *9*, 325–335.

(22) Jung, H. G.; Allen, M. S. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. *J. Anim. Sci.* **1995**, *73*, 2774–2790.

(23) Ellis, P. R.; Kendall, C. W.; Ren, Y.; Parker, C.; Pacy, J. F.; Waldron, K. W.; Jenkins, D. J. Role of cell walls in the bioaccessibility of lipids in almond grains. *Am. J. Clin. Nutr.* **2004**, *80*, 604–613.

(24) Beiseigel, J. M.; Glahn, R. P.; Welch, R. M.; Menkir, A.; Maziya-Dixon, B. B. A Caco-2 cell model predicts relative iron absorption from tropical maize by women. *FASEB J.* **2006**, *20*, A624.

(25) Jin, F.; Rutzke, M.; Welch, R. M.; Glahn, R. P. Is extrinsic isotope labeling of plant foods reliable for studies of iron absorption? *FASEB J.* **2007**, *21*, 858.10.

Received for review January 3, 2007. Revised manuscript received July 12, 2007. Accepted July 18, 2007. The U.S. Department of Agriculture, HarvestPlus, CIAT, and Cornell University supported this research. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

JF070023Y