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# Seed Mineral Concentrations of 408 Mung Bean (*Vigna radiata*) Accessions Evaluated for Future Use in Biofortification

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## Abstract

Mung bean (*Vigna radiata* (L.) R. Wilczek) is an important leguminous food crop widely cultivated in East, South, and Southeast Asia with an annual production surpassing 5 million tons. Micronutrient malnutrition affects more than half of the world's population, especially women, teenage girls, and preschool children. Legumes including mung bean can be biofortified to serve as nutrient rich sources of many essential macro-nutrients, minerals and trace elements needed for human health. They can also accumulate detrimental heavy metals. In this study, we used a total of 408 accessions of mung bean from the United States Department of Agriculture, originating in 35 countries across nine world regions to evaluate the seed mineral concentration diversity of the species. The grain was nutritionally characterized with ICP analyses for 22 elements divided into trace minerals, and macro or micro-nutrients. Seed color was classified from yellow to green, brown and black. Results included significant variation for all the elements detected; with six affected significantly (Mg, S, Ca, Fe, Ba and V) by region of origin and four (Mn, Ba, Ni and Sr) by seed color. Zinc levels were slightly higher than expected when compared to other legumes but Fe was like in other legumes. The correlation analyses uncovered relationships among the minerals of interest. For example, Zn had weak and positive correlation with some macro (K, P, Mg, S, and Ca), and micro-nutrients (Fe, Mn, Si, Ni, Sr and V). For the other biofortification goal, Fe had stronger and positive correlations with some macro (K, Mg, and S) and micro (Mn, Sr, V, and Cu) nutrients. The implications for nutritional crop breeding of mung beans are discussed.

## Keywords

Biofortification, ICP Elements, Mung Bean Diversity, Iron and Zinc

Nutrition, Other Minerals, Seed Nutrient Correlations

## 1. Introduction

Nutritional studies in plants evaluate chemical compounds and elements which are necessary for plant growth and metabolism [1]. Except for carbon, hydrogen and oxygen which are absorbed from water or air, plants obtain necessary mineral nutrients from the soil, store them in their tissues and cycle them between plant parts and the environment. Mineral nutrients in plants can be categorized into macro- and micronutrients [2]. Plants macronutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulfur (S) and magnesium (Mg) are taken up in large quantities, used in organic molecules such as amino and nucleic acids and then stored in abundance in various plant parts [3]. Meanwhile micronutrients and trace elements such as iron (Fe), barium (Ba), boron (B), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), nickel (Ni), and strontium (Sr) are present in plant tissue in small quantities [4]. Most of these elements are essential for both plant growth and human nutrition. Efficiency of uptake is of special interest to the process of biofortification of micronutrients as they occur in lower concentrations in most soils.

There are other minerals detected in plants which are not nutrients. These include arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb) and Thallium (Tl) which are categorized as toxic not giving any specific benefits to plants and deleterious to humans [5]. Meanwhile Vanadium (V) is found as a nutrient for Rhizobia but not for plants. As these can be detrimental elements to human health, surveillance of these minerals is essential to ensure food safety for human consumption. The presence of these minerals in plants is influenced by many factors including soil origin and anthropogenic activities such as industrial pollution, pesticide use and fertilization. Aluminum (Al) and chlorine (Cl) are found in soils but excluded for the most part from plant uptake.

The determination of the mineral, micronutrient and trace element concentrations in plants and biofortification breeding can be carried out using several analytical instruments namely Atomic Ab-sorption Spectrometry (AAS), Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) or Mass Spectrometer (ICP-MS), depending on the specificity needed for the analysis [6] or the availability of the laboratory instruments. However, analyzing minerals using ICP-OES is popular given the fact that this technique can analyze multiple elements, up to 26 or more, with high precision in single sample runs.

Mung bean, known as green gram (*Vigna radiata*. (L.) R. Wilczek) in some regions, is a crop of South, Southeast and East Asia now cultivated throughout the world, including in defined regions of Australia, some parts of Eastern and Central Africa and in a limited set of South American countries (Chile and Peru), [7]. It is of increasing interest as a new crop in the United States and additional Latin

American countries for its healthy protein-rich grain [8]. High nitrogen fixation gives mung bean a vital role in intercropping with corn or millet in East Asia or for crop rotation with cereals such as rice or wheat in south and southeast Asia [9].

The quality of mung beans is dependent on seed chemical compositions, which in turn determines the benefits of its consumptions for human health and nutrition [10]. Mung bean contain balanced nutrients including protein, starch, dietary fiber along with a significant number of bio-active phytochemicals [11]. Because of high percentage of protein, polyphenols, oligosaccharides and amino acids on mung bean, this legume has high levels of antioxidant, anti-inflammatory, antimicrobial activities and involved in the regulation on lipid metabolism. Mung bean is usually consumed by cooking the dry bean, eating flour-based products from it or as a vegetable bean sprout [12].

Mineral deficiency is a major concern in global health [13]. Estimated over two-thirds of humans experience an inadequate intake of one or more mineral nutrients, with more than half having iron (Fe) deficiency anemia (IDA) and over one third zinc (Zn) deficiency [14]. Human nutrient deficiencies are usually more prevalent in developing countries where a diverse diet is not affordable and staple crops such as cereals and pulses play an important role as a source of nutrients; however, developed countries can also benefit from a better balance of nutrients to calories [15]-[17]. Food legumes usually contain higher concentrations of mineral nutrients and make good candidates for breeding [12] [18] [19]. To date, despite the importance of mung beans in terms of economic and nutritional value, no assessments have been made in the United States for biofortification potential or micronutrient concentration.

The specific goal for our research was to explore the diversity of mung bean seed micronutrients in genotypes from the USDA core collection for biofortification of this pulse legume species. We analyzed the correlations between micro- and macronutrients along with other minerals in large collection of over 400 genotypes grown in Southeastern USA as a potential region of production for mung beans. By evaluating these factors, our research aims to develop nutritious, biofortified genotypes as crop standards for summer planting in Tennessee; and to position mung bean as a novel rotation legume that supports sustainable agriculture in the Mid-South states of the country. Our overall goal was further the understanding of nutrients like Fe and Zn in the crop and their relationships with other minerals, as well as the possibility of a nutritionally based breeding program using mung bean to prevent micronutrient deficiency.

## 2. Materials and Methods

This study used the core collection of 408 genotypes of mung bean from the United States Department of Agriculture (USDA), Plant Genetic Resources Unit in Griffin, GA, USA, which were planted in two season at the field station of Tennessee State University, (TSU) in Nashville TN, United States of America, which

is situated at 36˚10'1"N / 86˚49'45"W with an elevation of 54.3 m above sea level. The planting was done over two seasons (summer 2015 and 2016) but only one season was harvestable. A total of 30 seeds per genotype were sown into each plot, and plots were 2.4 m long with l.0 m row centers and 40 genotype blocks were separated by 0.8 m alleys. The experiments were conducted in an open field and only watered once to induce germination. Thereafter, the crop relied upon rainfall for water supply. This region has hot and humid climate conditions (Koppen zone: Cfa), humid subtropical; with prevalence of summer thunderstorm. The soil was a Byler type Silt Loam with 2 to 5 percent slope towards the West. The soil pH averaged 6.0 and no inorganic fertilizer was used as that could have biased macro-nutrient supply or micronutrient accumulation. Water carrying capacity was estimated to be 50 mm of available water per cubic foot. Soil tests with Univ. of Tennessee Plant and Soil lab in Nashville revealed high P and K at 265 and 140 lbs./acre, respectively. Micronutrients Ca, Fe, Mn, and Zn were all sufficient in parts per million (ppm).

The germplasm represented 35 countries and nine major regions around the world, classified Africa (AF), North America (AM), Central Asia (CA), East Asia (EA), Europe (EU), Oceania (OC), South Asia (SA), Southeast Asia (SEA), and West Asia (WA). These were used previously by our group in genotyping [20]. Based on seed coat color, all the accessions were categorized into six groups of black (BL), brown (BR), green (GR), yellow (YL), variable (VR) and mixed (MX). In total, 223 had green seed coat color, followed by brown and yellow with 72 and 28 accessions, respectively. Black and variable seed coat color had equal number of accessions with 24 each, and there were 37 mixed seed types. A total of 358 accessions reached maturity and were harvested for a subsequent planting in second year which was not harvested due to excess rainfall.

Due to the large number of accessions, the genotypes within seasons were not grown in replicates but averaging was used to account for consistency across plants harvested at random locations throughout the plot. Harvest was conducted by hand for clean pods at the top of three plants from each plot. The seeds from these pods were hand threshed, cleaned and dried in envelopes at room temperature. Whole seed from each plant were then ground to a powder providing triplicate biological replicates for complete microwave digestion in the following conditions: a 0.5 g accurately weighted sample of homogenously ground seeds were digested with 10 mL nitric acid HNO3 (65% v/v) using a MARS5 microwave oven (CEM corporation, Matthews.NC, United States).

Digestion conditions had a power setting at 1200 W, and maximum temperature of 200˚C. The overall digest took two hours with 15 min holding times. Venting was done from the CEM oven into a fume hood set with the appropriate air flow and having the correct 110 V plug for the microwave. After digestion the samples were diluted with ultra-pure water into a 100 mL volumetric flask and filtered through Whatman No.41. All samples were then stored in polyethylene tubes at 4˚C until analysis. All chemical reagents and standard solutions were of

analytical grade and ultra-pure water of 18.2 MΩ/cm resistivity was used throughout the experiment. Nitric acid and the standard stock solution 1000 mg/L for the ICP-OES analysis were supplied by Merck (Darmstadt, Germany).

Determination of elemental concentrations was done on an iCAP 7000 inductively coupled plasma (ICP) optical emission spectrophotometry (OES) from Fisher Scientific (Waltham, MA 02451 USA) in the Plant Analysis lab at TSU. Two wavelengths for each element were tested and the instrument was run using axial and radial emission with blank corrections. Calibration curves (Table S1) were obtained by using six concentrations (0.05 mg/L, 0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 5.0 mg/L and 20 mg/L). Values below detection or quantification limits of the calibration curve were marked as not attainable (n/a).

For data analysis, averages for the 22 mineral elements were calculated from the three replicates for each of the genotypes, and population descriptive statistics found: including overall mean, standard deviation (SD), coefficient of variation (CV) with minimum and maximum values. Then, the averages of minerals were calculated based on 9 geographical regions and 6 seed coat color classes. One-way analysis of variance (ANOVA) was conducted to know whether these factors had significant effects on mineral concentration.

Correlation analyses were then performed to find out the relationship among macro, micro, and heavy metal ions existing in our study's germplasm. After that, a biplot analysis was used to investigate the overall relationship between mineral elements and geo-graphical region and seed coat color. All these analyses were conducted with JMP pro16 from SAS Inc (Cary, NC 27513, USA).

## 3. Results

Data from the ICP analysis was very precise on the Thermo Scientific iCAP 7000. All measurements were carried out using six-point calibration with two wavelengths for each element to ensure the best fit to the standard curves and concentrations were expressed in mg/Kg which is equivalent to parts per million (ppm). The highest R2 and the lowest LOD were used to choose the best working wavelength. Radial view plasma was chosen because we found higher emission intensities compared with the axial view plasma. The wavelength determinations selected along with the plasma viewing of the sample concentration are also shown in Table S1.

Descriptive statistics were calculated for a total of 22 elements for which we had ICP standard curves (Table 1). Among them, K was found to be the highest concentration of any of the elements (14185.66 mg/Kg), followed by P (10195.15 mg/Kg) and S (2596.42 mg/Kg). Mg and Ca concentrations were in the range of 1318.12 - 1782.34 mg/Kg. The average for Zn concentrations was 58.05 ppm, with minimum and maximum of 26.46 and 147.06 found in accessions MB171 (from India) and MB79 (Iran), respectively. The Fe concentration was of interest because it is also a target for biofortification and averaged 50.08 ppm with minimum and maximum of 25.2 and, 84.96 ppm, respectively.

**Table 1.** Mean and standard deviation (SD) in mg/Kg (ppm, parts per million) with the minimum and maximum values for each of 22 elements measured by ICP-OES in the core collection of mung bean (*Vigna radiata*) genotypes.

| Macro-Nutrient | Mean (mg/Kg) | SD | Min | Max | Micro-Nutrient | Mean (ppm) | SD | Min | Max |
|---|---|---|---|---|---|---|---|---|---|
| **Ca** | 1318.12 | 194.90 | 878.22 | 1933.56 | **Zn** | 58.05 | 27.57 | 26.46 | 147.06 |
| **K** | 14185.66 | 4077.78 | 6299.10 | 27530.28 | **Fe** | 50.08 | 10.68 | 25.20 | 84.96 |
| **Mg** | 1782.34 | 527.37 | 800.10 | 2915.64 | **Mn** | 7.37 | 1.89 | 2.34 | 13.68 |
| **P** | 10195.15 | 3955.39 | 4090.86 | 20881.80 | **Ni** | 1.74 | 1.70 | 0.18 | 15.30 |
| **S** | 2596.42 | 312.58 | 1937.34 | 3421.98 | **Cu** | 3.55 | 5.43 | 0.18 | 29.70 |
| | | | | | **B** | 31.53 | 133.98 | 0.18 | 1043.82 |
| **Other Elements** | | | | | | | | | |
| **Al** | 6.78 | 15.23 | 0.00 | 233.64 | **Pb** | 2.46 | 1.50 | 0.18 | 6.84 |
| **As** | 5.31 | 6.75 | 0.54 | 10.08 | **Si** | 72.13 | 552.78 | 0.54 | 8272.26 |
| **Ba** | 1.86 | 1.34 | 0.00 | 10.08 | **Sr** | 0.88 | 0.50 | 0.18 | 2.70 |
| **Cd** | 0.54 | n.a. | 0.54 | 0.54 | **Tl** | 0.45 | 0.36 | 0.18 | 1.80 |
| **Cr** | 20.94 | 18.20 | 1.80 | 44.82 | **V** | 0.78 | 0.51 | 0.18 | 6.12 |
| **Co** | 0.30 | 0.21 | 0.18 | 0.54 | | | | | |

The toxic and other trace elements (As, Ba, Cd, Co, Cr, Pb, Sr, Tl and V) were the lowest concentrations of all elements measured in the seeds of mung bean. The lowest of these were Co (0.30 ppm) and Tl (0.45 ppm) followed by Cd (0.54 ppm), V (0.78 ppm) and Sr (0.88), all barely detectable. Cr was higher at average of 20.94 ppm. Tl was detectable in a total of 44 accessions while Cd was detected only in one accession, MB254 from India. The element As with average seed concentration of 5.31 ppm, was detected in only two accessions (MB387 and MB54). As would be expected, aluminum (Al) was observed in a high number of accessions (304) but was of low average seed concentration (6.78 ppm) ruling out soil contamination in the sample preparation. Mung bean had low Si (average 72.13 ppm) compared to cereals where it is a macronutrient.

In terms of correlations, significant positive and negative associations were observed among the 18 minerals. For the important macro and micro nutrients (Figure 1), weak to moderate level positive correlation existed overall, among the eight macro elements (K, P, Mg, S, Ca, Zn, Fe, and Mn). The highest of these positive correlations were between K and Mg (r = +0.43) and between P and S (+0.63), the first two with bimodal distributions. Only three weak negative correlations were observed: between macro-nutrients K and P (−0.04), P and Ca (−0.11), and S and Ca (−0.08). Zn showed low (K, P, Mg, S, Ca, and Mn) to moderate (Fe) level positive correlation with all the macro-nutrients. Similarly, Zn had positive correlation with all other micro and toxic or non-toxic minerals / elements. For the most part these were weak, except between Zn and Cu with moderate level negative correlation (−0.22). Mn was positively correlated with all macro-nutrients, rang-

ing from r = +0.11 for P to +0.55 for Fe. On the other hand, Fe had moderate level positive correlation with all macro and some micro elements (Sa, Sr, V, and Cu). Most notable for the macro-nutrients, were correlations of Fe with K (+0.32), Mg (+0.42), and S (+0.37). Zn and Fe were weakly correlated (+0.25) as was Fe and Ca (+0.25).

Meanwhile, for the other soil derived elements, including some considered toxic, there were some positive and negative correlations with macro and micro-nutrients as described below. First, Al had a strong positive correlation with Si (0.90) and B (0.99), whereas weak negative correlation with S, V, and Pb (Data not shown). Element Ba showed moderate and positive correlation with all macro and some micro-elements (Sr, V, and Cu), whereas it had low level of negative correlation with two toxic elements (Pb and Tl). Both Pb and Tl showed low level positive correlation with macro nutrients, whereas moderate (0.40) and strong (0.87) positive correlation was observed for the two elements with B. Fe showed weak negative correlation with Ni and Tl. Zn had positive correlation with Sr and negative correlation with Cu. However, in both cases, these were weak only reaching +0.27 and =0.22, respectively. The highest positive correlations between plant nutrients and other soil elements were for Mn with Ba, Sr, and V. Those comparisons had r-values ranging from +0.43 to +0.60. Finally, low but significant negative correlations were found between Ni and P (−0.23) and Ni and S (−0.28).

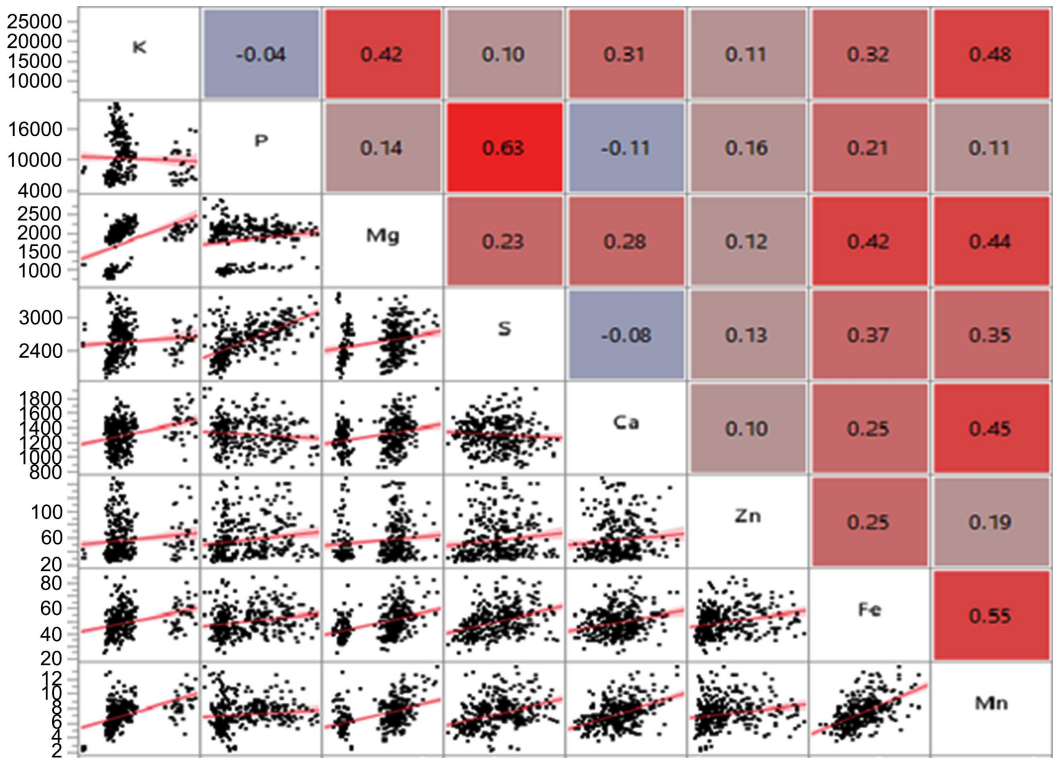


**Figure 1.** Correlations between macro and micro-elements for the core collection of mung bean (*Vigna radiata*) genotypes as measured by ICP-OES.

The observed effect of geographical region of origin on nutrient concentrations

was also analyzed and found to be significant in some cases. The average mineral concentration was calculated based on nine different geographical regions for the mineral elements, including trace ones, and for which five (As, Cd, Cr, Pb, and Tl) were toxic and the remaining were macro and micro-nutrients. Based on one-way ANOVA analysis, the average mineral concentrations of Ba, Ca, Fe, Mg, S, and V were significantly different across the nine geographical locations (Table 2). No significant differences in aluminum concentration were found as would be expected since Al is just a superficial contaminant on seed (data not shown). Some regions had higher average micronutrient seed Fe but not seed Zn. In this case East and Southeast Asia mung beans had more Fe rich seeds than accessions from Africa and Central Asia. Further studies would be needed to confirm a regional effect by evaluating each germplasm group more extensively with planting in a single location as we did in this study.

**Table 2.** Mean mineral concentrations in mg/Kg by geographic regions for the core collection of mung bean (*Vigna radiata*) genotypes as measured by ICP-OES. Low concentration minerals not shown and those with significant difference bolded.

| Mineral | AF (mg/Kg) | AM (mg/Kg) | CA (mg/Kg) | EA (mg/Kg) | OC (mg/Kg) | SA (mg/Kg) | SEA (mg/Kg) | WA (mg/Kg) |
|---|---|---|---|---|---|---|---|---|
| B | 1.98 | 2.79 | 4.21 | 7.32 | 7.08 | 17.17 | 3.75 | 93.83 |
| **Ba*** | 0.99$^{ab}$ | 1.86$^{ab}$ | 1.75$^{ab}$ | 2.05$^{ab}$ | 2.34$^{ab}$ | 2.14$^{a}$ | 1.66$^{ab}$ | 1.49$^{b}$ |
| **Ca*** | 1268.7$^{ab}$ | 1331.5$^{ab}$ | 1296.1$^{b}$ | 1310.7$^{ab}$ | 1415.22$^{ab}$ | 1283.4$^{b}$ | 1269.1$^{b}$ | 1399.4$^{a}$ |
| Cu | n.d. | 2.52 | n.d. | 1.98 | 1.44 | 3.66 | 2.40 | 4.52 |
| **Fe*** | 39.1$^{c}$ | 54.46$^{ab}$ | 47.5$^{bc}$ | 56.84$^{a}$ | 52.51$^{ab}$ | 48.31$^{b}$ | 56.07$^{a}$ | 49.6$^{b}$ |
| K | 16761.5 | 12008.0 | 14040.6 | 15636.1 | 17472.74 | 14133.2 | 14948.7 | 13557.2 |
| **Mg*** | 1648.2$^{bc}$ | 1638.8$^{bc}$ | 1878.20$^{abc}$ | 2213.8$^{a}$ | 2102.6$^{abc}$ | 1714.7$^{c}$ | 1939.5$^{ab}$ | 1737.26$^{bc}$ |
| Mn | 5.85 | 6.88 | 7.53 | 7.82 | 8.49 | 7.57 | 7.40 | 7.04 |
| Ni | 1.38 | 1.31 | 1.02 | 1.21 | 1.44 | 1.57 | 1.56 | 2.31 |
| P | 10434.6 | 9509.5 | 10387.2 | 9488.6 | 8778.47 | 10948.2 | 10481.9 | 9487.3 |
| Pb | 4.59 | 2.16 | 2.16 | 3.31 | 2.76 | 2.10 | 2.92 | 2.54 |
| **S*** | 2587.4$^{abc}$ | 2623.9$^{abc}$ | 2513.6$^{bc}$ | 2717.1$^{a}$ | 2575.54$^{abc}$ | 2662.29$^{a}$ | 2649.7$^{ab}$ | 2480.6$^{c}$ |
| Si | 12.36 | 18.92 | 15.23 | 10.56 | 19.47 | 29.30 | 48.28 | 215.3 |
| **V*** | 0.78$^{ab}$ | 0.70$^{ab}$ | 0.68$^{b}$ | 0.90$^{ab}$ | 1.01$^{ab}$ | 0.69$^{b}$ | 0.73$^{b}$ | 1.02$^{a}$ |
| Zn | 48.3 | 56.4 | 55.6 | 55.4 | 55.1 | 55.7 | 63.1 | 62.3 |

Abbreviations: Africa (AF); Central Asia (CA); East Asia (EA); North America (AM); Oceania (OC); South Asia (SA); Southeast Asia (SEA); West Asia (WA). Europe not shown. N.B: * = statistical significance differences for regions at $\alpha = 0.05$ level.

The observed effect of seed color differences on mineral and nutrient concentrations was also analyzed. Like the analysis by geographic region, the mean mineral concentrations were sometimes significant for mung bean accessions based on six observable seed coat colors ranging from black to yellow. Interestingly, sig-

nificant differences were found for some minerals (Mn, Ba, Ni, and Sr) but not for others (Table 3). No seed color association was the case for Fe and Zn, the micronutrients of interest. However, Mn was higher in black seed coated accessions than green ones. Mixed and variable seed classes were also common but like black, green and yellow mung beans in most mineral concentrations.

Table 3. Mean mineral elements concentration in parts per million (ppm) or mg/Kg for different seed color types found in the core collection of mung bean (*Vigna radiata*) genotypes as measured by ICP-OES.

| Mineral or Nutrient | BL (mg/Kg) | BR (mg/Kg) | GR (mg/Kg) | MX (mg/Kg) | VR (mg/Kg) | YL (mg/Kg) |
|---|---|---|---|---|---|---|
| *Macronutrients* | | | | | | |
| K | 13986.32 | 14047.15 | 14308.11 | 13360.50 | 13463.15 | 14850.71 |
| P | 11888.58 | 10529.64 | 10247.92 | 8801.77 | 8841.82 | 9641.15 |
| Mg | 1725.89 | 1683.19 | 1842.79 | 1579.97 | 1669.74 | 1848.26 |
| S | 2581.84 | 2635.58 | 2584.95 | 2517.28 | 2477.63 | 2729.22 |
| Ca | 1362.90 | 1348.10 | 1303.74 | 1282.01 | 1327.78 | 1304.99 |
| Micronutrients | | | | | | |
| Zn | 45.50 | 60.18 | 59.22 | 53.69 | 60.21 | 55.20 |
| Fe | 47.68 | 50.33 | 50.02 | 51.55 | 50.78 | 50.71 |
| B | 27.60 | 16.63 | 53.31 | 12.06 | 6.17 | 4.21 |
| Cu | 1.08 | 2.75 | 4.16 | 6.66 | 3.02 | 2.84 |
| **Mn*** | 8.07 $^{a}$ | 7.84 $^{a}$ | 7.19 $^{b}$ | 6.37 $^{b}$ | 7.63 $^{a\,b}$ | 7.05 $^{a\,b}$ |
| Si | 46.38 | 25.16 | 118.19 | 16.38 | 16.91 | 18.28 |
| *Other Elements* | | | | | | |
| Al | 7.76 | 6.15 | 7.71 | 5.31 | 4.40 | 3.89 |
| **Ba*** | 2.39$^{a}$ | 2.27$^{a}$ | 1.68$^{b}$ | 1.90$^{ab}$ | 1.86$^{ab}$ | 1.60$^{b}$ |
| **Ni*** | 1.40$^{b}$ | 1.87$^{b}$ | 1.55$^{b}$ | 2.57$^{ab}$ | 3.72$^{a}$ | 1.20$^{b}$ |
| Pb | 2.00 | 2.49 | 2.30 | 5.13 | 2.81 | 3.29 |
| **Sr*** | 1.15$^{a}$ | 0.98$^{ba}$ | 0.86$^{bc}$ | 0.70$^{bc}$ | 0.78$^{bc}$ | 0.69$^{c}$ |
| Tl | 0.54 | 0.56 | 0.42 | 0.24 | 0.18 | 0.36 |
| V | 0.72 | 0.70 | 0.78 | 0.81 | 1.10 | 0.75 |

Abbreviations: black (BL), brown (BR), green (GR), yellow (YL), variable (VR) and mixed (MX). * = Minerals in bold are statistically significant at $\alpha = 0.05$ level for different seed coat colors.

To summarize all the results, a principal component analysis (PCA) and a multivariate AMMI test with biplotting (Figure 2) were carried out to further evaluate the effects of region and seed coat color on concentration of mineral elements and graphically summarize correlations between micro and macronutrients. The overall diversity of the collection was high, but most genotypes were clustered with

two axes in the PCA (Figure 2(a)). Based on the charting of the genotypes, the first two components explained 24% and 17% of the total variability, respectively. Together the first two axes accounted for 41% of the variance. No obvious pattern was observed in the first two components. Overall, PCA showed tight clustering of all the data, and no specific pattern observed between regions and coat color groups. But overall, 74% of cumulative variance in the PCA was explained by the first five components, indicating existence of other factors in the germplasm besides origin and seed coat color affecting mineral concentrations.

Meanwhile, the AMMI biplot (Figure 2(b)) was more instructive and summarized the clustering of the mineral results, with Zn distinct from a cluster of Fe with Ca, K, Mg, Mn and Ni. Other elements were on separate vectors. Biplot demonstrated two major clusters with two minor groups in each based on eighteen minerals, showing the relationship between samples and variables. Zn and Fe were mapped on the first component along with other ten mineral elements, whereas six minerals were influenced by the second component. The overall biplot also depicted the effects of the region of origin and seed coat color on average mineral concentrations. Black, green, and mixed seed color of accessions from West Asia regions strongly affected the amount of Al, Cu, P, Tl, B, and Si. On the other hand, the concentration of all remaining minerals was affected by green, brown, black, variable, and mixed seed coat color from East Asia, Oceania, West Asia, South Asia, Central Asia, and North America. Most importantly, accessions from Africa, Southeast Asia, and Europe with all kinds of seed coat color had negative relationship with all elements.

The Zn concentration was higher in the accessions with brown or yellow seed coat color from Southeast Asia, Central Asia, South Asia, whereas was less in the variable seed coat color accessions from East Asia.

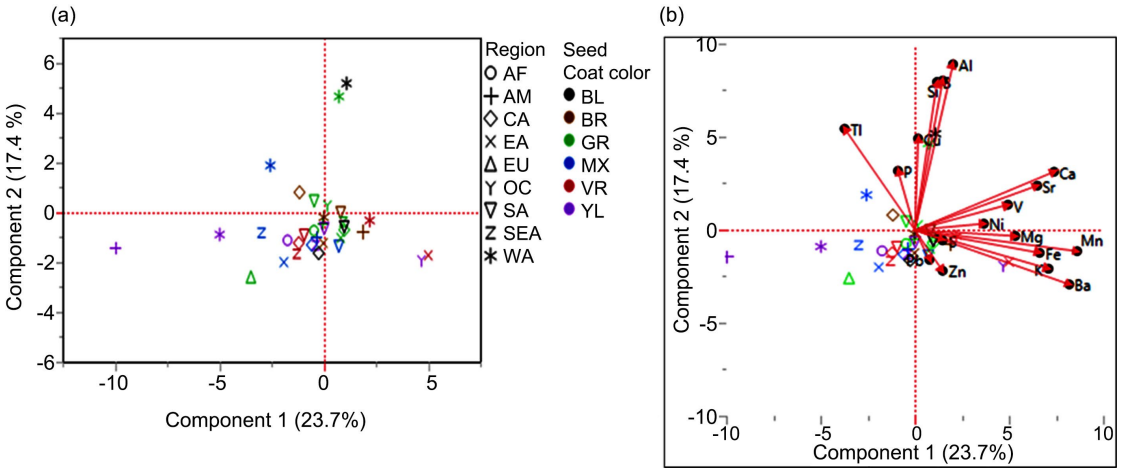


**Figure 2.** Principal component analysis and AMMI biplot for effect of geographical origin by region and seed coat color on 18 mineral elements. (a) The first two principal components depicted overall variation of the mung bean germplasm for geographical region and seed coat color. (b) Biplot between 18 minerals and region and seed coat color, depicting the influence of geographical origin and coat color on the mineral concentration of the studied mung bean germplasm and genotypes as measured by ICP-OES.

## 4. Discussion

Biofortification is the process of improving the nutritional quality of plant products for human diets based on concentration of the needed nutrients and their bioavailability [15]-[18]. This plant breeding goal is primarily conducted by evaluation of seed mineral content. Among seed bearing crops the majority studied have been cereal grains or legume pulses. Seed mineral concentration studies can also involve consideration of mineral concentration of other plant parts. Pulses that have been biofortified include peas, lentils and beans [19]. Although not as well studied for nutritional traits, mung bean is highly consumed in Asia and is of growing importance in Africa with the potential to be a large export crop in the Americas. In addition, mung beans are often processed into flour or other products [21]-[23]. To understand the nutritional quality of these products it is important to know the potential of mung bean seed to accumulate macro and micronutrients in any given region of production [24] [25]. The focus of our research was concentration of seed Fe and Zn in relationship to other minerals, especially for fighting iron deficiency anemia in the United States and beyond. We also wanted to know the macro-nutrients that may interact with Fe or Zn and if toxic elements to human diets were accumulated. Fe is often correlated with Zn in a positive manner [25] [26] and this was borne out here. However, levels of correlation were lower ($r = 0.25$) than observed in most other studies of legume seeds and germplasm. This could be due to the soil Fe and Zn levels being only 25 and 7 ppm, respectively, at our site.

This de-coupling may be related to the fact that Zn is a less common element in soils compared to Fe which is abundant. Alternatively, Fe uptake is regulated by homeostatic mechanisms that differ from those for Zn. Soil scavenging efficiency for Zn may be more finely tuned than Fe uptake in mung bean. However, the lack of differences between geographic origins for higher Zn may indicate that all mung beans perform similarly, although the opposite might be the case for Fe.

Searching for an association of seed coat color with Zn and Fe concentration, we found no association in either case. Mung bean is unusual amongst pulse crops in having a rather limited range of seed coat colorations [27] [28]. The range of seed color for the crop is from yellow to light green, dark green and then black. The underlying genetics is not understood but may parallel the I gene in garden peas, *Pisum sativum*; where a stay-green protein was cloned for a major gene determining green vs. yellow seed coat [29]. Studies in common bean have shown that darker seeds might have higher tannins that can trap higher amounts of Fe or Zn [30]. The role of the seed coat may be minimized in mung bean as this tissue is thin and easily split off during processing for this species. Therefore, the mung bean's thin seed coat may explain the lack of association, and this may be a selected trait for easy decortication, advantageous for processing of mung bean into flours and starch extraction. An added benefit of thin seed coat and low tannin would be that nutrients would be more bioavailable since they consist of a greater percentage of embryo and cotyledon [31]. The question remains whether seed color

affects bioavailability in mung beans. In other legumes the tannin accumulation traps Fe and Zn into the seed coat where it is at a higher concentration than for the cotyledons which are colorless. Notably, mung beans in South Asia are often sold split or decorticated with no seed which would make them of even higher bioavailability.

In terms of other minerals, mung beans seem to be very good at taking up Ca, Mn and Mg. They have similar levels of K and P as other legumes. Since most P is found as phytates in legumes, the balance of these macronutrients along with nitrogen and protein levels may be important in determining bioavailability. No association was found for seed color and Ca, Mg, K or P; however, Mn was associated with seed color. Geographic region of accession origins was associated with Ca, Fe, Mg and S, with this last element perhaps reflecting the sulfur containing amino acid levels which in turn affect bioavailability. Correlation of Fe and S suggests high protein as the reason for high Fe in mung bean genotypes. Protein levels of sulfur amino acids could be selectable targets for the crop [23].

Of concern would be selection for micronutrients that would inadvertently select for seed loading of an undesirable element such as Cd or Pb for example. However, Zn was not correlated with concentrations of the toxic metals in either regressions or biplot tests. Fe may be more of a concern being correlated with Ba, Cu, Sr and V. Among the six toxic minerals, As and Cd were above threshold of detection for two samples and one sample only, respectively. Tl and Pb were above detectable levels in 44 and 97 samples, respectively, and would be of greater concern. The testing site for our research is a flood-prone area down river from heavy industry in the Cumberland River valley within the city of Nashville and a Zn mining operation in Carthage, Tennessee, and this may have resulted in higher zinc benefiting our soils or lead contamination. A 2010 flood reached part of the field that was used in our experiment. Neighboring industries include a petroleum storage tank facility and gas processing areas with risk of Pb in underground water or in the air.

In terms of technical accomplishment of this research, we were innovative in using entire seed digests in nitric acid for our sample preparation. This was of high effectiveness as no grinding was needed for the work, and we could conduct a. faster evaluation of the many core collection genotypes (almost 400) used in this study. A single site / year combination was used to grow the plants due to it being a large collection but correlations between years was shown in our Genome Wide Association study with 95 genotypes [10]. Regional differences should be further explored outside the core collection of mung beans. In our study, we evaluated mung beans from nine regions and a total of 35 countries. However, of these, the highest number of accessions (150) were from India, followed by Iran (96), and Afghanistan (17) and the Philippines (17) with few from China, Japan or other East Asian nations. In a final observation about the mung beans used, the genotypes tended to have sub-tropical rather than temperate adaptation because they were selected for growth ability in the hot summer season in Nashville and some

that flowered late had to be discarded. In future studies we could evaluate a smaller subset across soil types and years to see the associations with different locations or weather patterns. However, a single site was fine for accomplishing our main goal to distinguish mung bean seed mineral concentrations based on geographic regions of origin and seed color classifications. The USDA core collection provided many representational genotypes for analysis in each of these categories.

In conclusion, Mung bean Biofortification is a promising research activity and crop improvement strategy. The average zinc concentration was around 50 ppm which is twice the average in common bean, perhaps because the small seed of mung bean concentrates this element in the embryo, endosperm and sometimes seed coat [31] [32]. In contrast, the average iron concentration was lower than for the highest iron common beans, being around 50 ppm as well. Fortunately, multiple sources of breeding for either micro-nutrient were found in the large, four hundred strong, germplasm collection we used. This means that breeding of highly nutritious mung bean in any of the popular seed colors or with multiple adapted source genotypes would be possible [33]. Unlike common bean there is no need to use wild relatives or landraces as the sources for biofortification crosses [34] [35].

In addition, the interaction of Zn with other minerals was not very notable in our study. Zinc is of low concentration in soils around the world, but varieties can be variable in uptake [36]. Therefore, the association of Zn uptake with soil types in different geographic regions should be of interest in future studies with the caveat that mineral concentration alone does not establish dietary bioavailability; phytate, polyphenols, and processing, should measured when doing so. On a practical level, microwave digestion as a technique for sample preparation was suitable for use in mineral and micronutrients determination of mung bean. This analysis could also be used for other bean/legume/pulses samples which share many of the common characteristics with mung beans.

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## Author Contributions

Study conceived by MWB. Data organization and graph preparation by ASMFI, NL and MWB. Field work was done by ASMFI and MWB. Mineral Analysis by NL and ASMFI. Paper written by MWB, NL and ASMFI. Editing of paper by

MWB. Overall funding obtained by MWB. Borlaug Fellowship from USDA-FAS for NL. TSU's central graduate research assistantship for ASMFI.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## Supplemental Tables

**Table S1.** Calibration for ICP-OES sample determination in mung bean (*Vigna radiata*) genotypes.

| Element | Atomic no | Wavelength (nm) Radial | LOD ppm (mg/L) | Calibration range | $R^2$ | BEC (mg/L) |
|---|---|---|---|---|---|---|
| Al | 13 | 396.152 | na | 0.05 - 20.0 | 0.9911 | −0.281 |
| As | 33 | 189.042 | 0.00071 | 0.05 - 20.0 | 0.9999 | 0.034 |
| B | 5 | 249.678 | 0.022 | 0.05 - 20.0 | 0.9992 | 0.321 |
| Ba | 56 | 493.409 | na | 0.05 - 20.0 | 1 | 0.032 |
| Ca | 20 | 315.887 | 0.0157 | 0.05 - 20.0 | 0.9986 | 0.242 |
| Cd | 48 | 228.802 | 0.0002 | 0.05 - 20.0 | 0.9989 | 0.047 |
| Cr | 24 | 284.325 | 0.0111 | 0.05 - 20.0 | 0.9989 | 0.148 |
| Co | 27 | 228.616 | 0.0016 | 0.05 - 20.0 | 0.9989 | 0.047 |
| Cu | 29 | 327.396 | 0.0332 | 0.05 - 20.0 | 0.9989 | 0.0332 |
| Fe | 26 | 259.94 | 0.0044 | 0.05 - 20.0 | 0.9991 | 0.113 |
| K | 19 | 769.896 | 0.0993 | 0.05 - 20.0 | 0.9987 | 1.496 |
| Mg | 12 | 280.27 | na | 0.05 - 20.0 | 0.9994 | 0.093 |
| Mn | 25 | 259.373 | na | 0.05 - 20.0 | 0.9994 | −0.079 |
| Ni | 28 | 231.604 | 0.0015 | 0.05 - 20.0 | 0.9998 | 0.125 |
| P | 15 | 178.284 | 0.0569 | 0.05 - 20.0 | 1 | −0.035 |
| Pb | 82 | 216.999 | 0.0097 | 0.05 - 20.0 | 0.9999 | 0.039 |
| S | 16 | 182.034 | 0.0051 | 0.05 - 20.0 | 1 | 0.053 |
| Si | 14 | 212.412 | 0.0106 | 0.05 - 20.0 | 0.9999 | 0.155 |
| Tl | 81 | 190.856 | 0.0091 | 0.05 - 20.0 | 0.9999 | 0.043 |
| Sr | 38 | 421.552 | na | 0.05 - 20.0 | 0.9997 | 0.052 |
| Zn | 30 | 213.856 | 0.0001 | 0.05 - 20.0 | 0.9997 | 0.222 |
| V | 23 | 309.311 | na | 0.05 - 20.0 | 0.9996 | −0.11 |

Abbreviations: LOD = limit of detection, $R^2$ = Correlation coefficient, BEC = Background equivalent concentration.