

Article

Crop Yield, Ferritin and Fe(II) boosted by *Azospirillum brasilense* (HM053) in Corn

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Abstract: An increasing global population of over 4.5 billion people drives increasing demand for calories—30% of which are satisfied by grain crops, such as maize. High-density farming practices have been implemented but tend to deplete the soil of essential elements resulting in lower nutritional value, notably iron, of cultivated crops. Low iron content in staple crops can contribute over time to severe, even fatal, micronutrient deficiencies. Enhancing grain iron content using post-harvest biofortification strategies can be costly. However, field inoculation using biologics like *Azospirillum brasilense* (HM053) can be a cost-effective alternative to improving crop nutritional value. Using ion chromatography with chemiluminescence detection, we have shown that maize seeds harvested from outdoor pot-grown plants possessed a four-fold higher iron content as ferrous iron (Fe⁺²) compared to non-inoculated plants. Seeds from *A. brasilense* HM053-inoculated plants also contained approximately 13 nmol of ferritin per ground dried weight of kernel compared to 3 nmol from non-inoculated plants. In addition, *A. brasilense* HM053 inoculation increased crop yield 30–50% relative to non-inoculated plants.

Keywords: maize; crop biofortification; iron-ferritin; plant growth-promoting rhizobacteria; *Azospirillum brasilense*

1. Introduction

Iron (Fe) is a necessary nutrient for life, helping form red blood cells and facilitate oxygen transfer from the lungs to other tissues. The recommended daily intake of iron for adults is at least 8 mg for men and 18 mg for women [1]. Iron deficiency is a common malnutrition disorder, causing approximately 50% of all diagnosed anemia cases and affecting more than two billion people worldwide [2,3]. Even mild and moderate iron deficiency can result in impaired human functioning [4].

The various forms of iron present in food differ in bioavailability to humans [5]. Nutritional iron is available from animal sources as heme iron or plant sources as non-heme iron. Heme iron, prevalent in meat and seafood, comes from the center of hemoglobin and myoglobin proteins and has the greatest

bioavailability to humans. Heme iron generally contributes approximately 10–15% of daily iron intake. Many global populations do not have access to enough heme iron but instead rely on staple crops such as rice, maize, wheat and soybeans, leaving them susceptible to dietary iron deficiency.

Recently, increasing iron content of staple crops has been a continuing goal of research for improving nutrition worldwide [6–10]. Strategies to mitigate crop iron deficiency such as conventional breeding to create new cultivars, genetic modification, enhanced soil fertilization using iron enriched supplements and crop bio-fortification in post-harvest processing have been examined. Field-based strategies can often result in deleterious side effects such as concentrating toxic levels of other heavy metals in the crop products, or accumulation of iron in non-edible portions of the plant. Furthermore, the unsightly appearance of iron-enriched plants due to discoloration can often trigger public outcry over the safety of crop products from genetically modified plants [11].

While improving iron content in food crop products adds value, improving the bioavailability of that iron when consumed is crucial. Hence, understanding how iron is stored in developing seeds is important. Ferritin is a protein existing in almost all living organisms in a conserved spherical structure [12,13]. The protein coat is formed from 24 structurally equivalent polypeptide subunits. When assembled, these subunits create a hollow protein shell that can accommodate up to 4500 iron atoms. Inside the polypeptide chain, a conservative ferroxidase center is present and its activity determines the ability of ferritin to oxidize iron ions from Fe(II) to less toxic Fe(III). Iron as a ferric oxyhydroxide is bound in the protein core with varying amounts of phosphates and is reduced back to Fe(II) and released upon cell demand for iron [14,15]. While some studies have shown that dietary supplementation or bio-fortification of food products using plant ferritin can reduce symptomatic behavior due to iron deficiency [11,16], in practice, this can have huge impacts on the cost of food.

An alternate strategy for naturally boosting seed ferritin content during crop development was investigated by application of liquid inoculants of the plant growth-promoting rhizobacteria (PGPR) *Azospirillum brasilense*. Staple grains derived from grass cropping systems are not known for their ability to form conspicuous symbioses with rhizobia as is the case with legumes. Even so, there is a large body of literature attesting to the effects of certain PGPR in grasses [17–20]. The literature indicates PGPR can promote significant increases in crop yield [21–24] and documents that these bacteria can colonize a number of different grass species relevant to both bioenergy and agriculture [25–27]. In some cases, it has been suggested that biological nitrogen (N₂) fixation may contribute to this plant growth enhancement in grass systems [28,29]. However, in most cases, the underlying mechanisms of plant growth promotion are unknown and have been attributed to a variety of sources, including antagonism toward phytopathogens [30] with induction of plant resistance to diseases [31], or phytostimulation through the transfer of phytohormones to their host [32].

Several PGPR have been identified as endophytes of grass species, including *Azoarcus spp.* in Kallar grass (*Leptochloa fusca* (L.) Kunth) and rice (*Oryza sativa*) [33,34], *Herbaspirillum seropedicae* in sugarcane (*Saccharum officinarum*) [20] and sorghum (*Sorghum bicolor*) [35], and *Gluconacetobacter diazotrophicus* in sugarcane [36]. Others have been identified as epiphytes, including *Azospirillum brasilense* and *Azospirillum lipoferum*, which have been commercialized as crop inoculants for maize and wheat [21,22,37,38]. These strains are gaining increased acceptance in agriculture as PGPR inoculants. They are also usually not major components of the soil microflora [20,39]. These N₂-fixing bacteria infect at the emergence of lateral roots and in the zone of elongation and differentiation above the root tip [40]. Typically, very high numbers of PGPR in roots have been reported (i.e., $\leq 10^8$ /gram root dry weight) and with no observable disease symptoms [33].

In the present work, two mutant strains of *A. brasilense* were examined for their influences on host kernel iron assimilation including HM053, a *Nif*⁺ constitutively expressed mutant of the *nif* gene coding for nitrogen fixation enzymes that fixes excess N₂ and excretes large amounts of ammonium to the rhizosphere; and *ipdC*, a mutant strain disrupted in the *ipdC* gene thus impaired in auxin hormone (indole-3-acetic acid, IAA) biosynthesis. We also compared the effects of soil treatment using exogenously applied auxin on crop yield and seed iron content.

2. Materials and Methods

2.1. Plant Growth

For courtyard studies, 3 maize kernels from Elk Mound Seed Co. (Hybrid 8100) were sown into each of 2.7 gallon pots filled with ProMix. After germination, any excess seedlings were removed from each pot leaving a single plant. A capful of fertilizer (~ 1.2 g) containing nitrogen, phosphate and potash (14-14-14, Osmocote™ Smart-Release Plant Food Flower & Vegetable™, The Scotts Company, Marysville, OH) was added to the assigned pots at the time of planting. Fertilizer was reapplied to pots 30 days after germination (DAG) and 60 DAG. Plants were placed on elevated tables outside (Figure 1). During the 2018 growing season, four study regimes included the following: (i) non-inoculated control plants; (ii) plants inoculated with *A. brasilense* HM053 bacterium; (iii) plants inoculated with *A. brasilense* *ipdC* bacterium; and (iv) plants treated with 30 μ M auxin plant hormone (IAA). Plants were administered liquid inoculants at 21, 42 and 63 DAG. Treatments with auxin hormone followed the same schedule. Treatments were randomized across the planting platforms and pots were rotated weekly during the growing season. The study was repeated in the 2019 growing season with the exception that auxin treatments were not applied.



Figure 1. Outdoor potted plant studies. Plants were grown in 2.7 gallon pots in ProMix with Osmocote™ time-release fertilizer. Treatments were randomized during the study initiation and pots were rotated weekly.

2.2. Bacteria Growth

Two mutant strains of *A. brasilense* (*ipdC* and HM053) were grown in liquid NFbHP-lactate medium following published procedures [40]. The medium contained 20 mM ammonium chloride as a nitrogen source and streptomycin antibiotic selection (80 μ g mL⁻¹). The bacterial cultures were grown in 25 mL flasks in a shaking incubator set to 30 °C and 130 rpm until OD₆₀₀ = 1.0 (Optical Density at 600 nm, corresponding to 10⁸ cells mL⁻¹) is reached. Bacterial cultures were washed with sterile water and diluted to approximately 10⁶ to 10⁸ colony forming units (CFU). Bacterial culture (10 mL) was injected into the soil at the base of the stem at the specified 3 time points during the growing season. Verification of bacteria presence at harvest was not carried out.

2.3. Plant Growth Measurements

Maize plants were measured with a tape measure from the soil of the pot up to the highest point of the plant in centimeters. They were measured at three time points: 17, 28, and 55 days after germination. Stem diameters were measured at the second internode up from the base of the stem using calipers. Tape was applied at these stem locations after the first time point measurement which served as a visual marker for subsequent measurements.

2.4. Chlorophyll and Leaf Thickness Measurements

Vegetative tissues were separated from roots and placed in pre-weighed and pre-chilled Eppendorf™ tubes. They were flash frozen in liquid nitrogen, ground to a fine powder and weighed. A volume of acetone equivalent to four times the milligram mass of the frozen ground tissue (assuming uniform densities) was added to the centrifuge tube. The content was vortexed (VWR analog vortex mixer; Sigma-Aldrich Corp., St. Louis, MO, USA) then sonicated (Branson Bransonic 32; Sigma-Aldrich Corp. St. Louis, MO, USA) at 0 °C for 2 minutes. Additional vortexing was performed during this period to ensure complete mixing. The Eppendorf™ tubes were then centrifuged for 2 minutes at 15,000 rpm to separate the insoluble and soluble portions. The insoluble portion contained mostly cell-wall polymers and starch while the soluble portion contained small soluble compounds such as sugars. The liquid extract was spotted onto a silica TLC plate in a 5 mm band using 2 µL of sample. The silica plate was developed in a 6:1.6:1:0.4 ratio of petroleum ether: hexane: acetone: methanol solution. Once the plate was fully developed, a razor was used to scrape off bands containing chlorophyll a and chlorophyll b. The shavings were placed in two separate Eppendorf™ tubes. Acetone (1 mL) was added to the tube containing chlorophyll a and the tube containing chlorophyll b. The samples were then vortexed and centrifuged for 4 minutes at 15,000 rpm. The absorbance of the resulting supernatant of each sample was analyzed via ultraviolet–visible spectroscopy (UV–VIS). Chlorophyll a was recorded at a wavelength of 663 nm, and Chlorophyll b was measured at a wavelength of 645 nm. Beer's Law ($A = \epsilon bc$) was then utilized to determine sample concentration of chlorophyll. Precision calipers (Fowler High Precision, Newton, MA, USA) were used to determine leaf thickness.

2.5. Ion Chromatography Measuring Seed Iron

Ferric, ferrous, and total iron were quantified from corn kernels with ion chromatography coupled with chemiluminescence detection [41] following the collection and drying of the kernels in an oven for 3 weeks at 65 °C. Seeds were pulverized between plastic sheets using a wooden mallet and dissolved in 1 mL of 1M HCl. Samples were subjected to ultrasonication for 5 minutes at 100% amplitude (Branson Bransonic 32; Sigma-Aldrich Corp. St. Louis, MO, USA) then centrifuged for 15 minutes at 3000 rpm. The supernatant was removed for sampling and stored in brown glass vials in a refrigerator (2–8 °C).

Stability studies were completed for the samples, varying time between sample extraction to injection on the ion chromatograph as well as storage conditions of temperature, light, and glass versus plastic storage containers. It was found that plastic Eppendorf tubes were not effective for long term sample storage, and neither was storage at room temperature. Under these circumstances, the oxidation state of iron was observed to change quickly over a short time (< 2 h). Storing samples in brown glass vials placed in the refrigerator allowed sample stability (no change in iron oxidation states) for up to 6 hours.

Iron standards were made in 0.1M HCl. Ferric chloride (FeCl_3 , 1 mg mL⁻¹) was used as a ferric iron standard and ferrous sulfate hexahydrate ($\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, 1 mg mL⁻¹) was used as the ferrous iron standard. The ferric chloride sample was then diluted 50-fold for standard curve generation, while the ferrous sulfate was diluted 10-fold. On average, it was observed that 3% of the ferric standard was reduced to ferrous iron when measured with ion chromatography chemiluminescence likely due to a solvent effect.

The analytical system consisted of a Thermo Scientific Dionex AXP Metal-Free HPLC with a Rheodyne metal-free injector and PEEK tubing 1/20 cm inner diameter. The ion chromatography column was a Thermo Scientific IonPac CS5A (4 × 50 mm guard column and 4 × 250 mm analytical column) designed to separate a broad range of metal complexes by cation and anion chromatography. The outlet of the column was connected to a mixing tee, where the post-column reagents were introduced with the column effluent to elicit the chemiluminescent reaction. Chemiluminescence was measured with the HERM data monitor (HERM LB 500, Berthold Technologies LLC, Oak Ridge, TN, USA) to allow quantification of iron in its oxidation states. Ferric iron had a retention time of approximately 4.3 minutes, while ferrous iron had a retention time of approximately 8.2 minutes.

In the analytical system, sterile water (HyPure™ WFI Quality Water, HyClone Laboratories, Logan, UT, USA) was used in solvent preparation to avoid introduction of additional iron to the samples. The column was rinsed with the WFI Quality Water, followed by flowing 1 M Na₂SO₃ at 0.5 mL min⁻¹ for 45 minutes to remove any dissolved oxygen on the column. During this time, the pre-column eluent (10 mM 2,4-pyridinedicarboxylic acid [PDCA], 80 mM potassium chloride, 37 mM formic acid, and 33 mM potassium hydroxide pH adjusted to 4) was bubbled with argon gas to remove dissolved oxygen. The pre- and post-column reagents were flowed through the system at rates of 1.00 and 0.75 mL min⁻¹, respectively. The post-column reagents consisted of 600 mM hydrogen peroxide and 0.025 mM luminol with 50 mM sodium carbonate pH adjusted to 11.5 [41]. Standard curves were generated daily prior to sample injection and samples were injected in triplicate.

2.6. Size-Exclusion Chromatography used in Seed Ferritin Analysis

A stainless steel High-Performance Liquid Chromatography (HPLC) Sonntek Pump was connected to a Rheodyne injector with a 20 µL PEEK injection loop to separate biological metabolites on an Agilent Zorbax GF-250 4 µm size exclusion column (4.6 × 250 mm). Samples were quantified on an Azura UVD 2.15 Knauer UV detector set to 254 nm [42]. Mobile phase was pumped at 0.5 mL min⁻¹ and consisted of 0.2 M ammonium sulfate with 0.05 M tris-HCl buffer at pH of 7. An antimicrobial was added (0.005% sodium azide) to maintain column integrity throughout the study. Maize kernels were acquired from the courtyard grown maize harvest and analyzed for the amount of ferritin across treatment types previously described. Dried kernels (~0.5 g) were pulverized between plastic sheets using a wooden mallet and placed in 1 mL of 50 mM tris-HCl buffer (pH 7) and ultrasonicated for two minutes at 100% amplitude. The sample was then heat treated at 75 °C for 10 minutes followed by immediate cooling on ice for 15 minutes. Centrifugation 15,000 g for 30 minutes pulled large particulates out of the supernatant which was injected (20 µL) for quantification against a ferritin standard (MyBioSource, Inc., San Diego, CA, USA).

2.7. Statistical Analysis

Data was subjected to the Shapiro–Wilk Normality Test, which was used to identify outliers so that all data groups represented a normal distribution. Data was then analyzed using the Student t-test comparing individual treatments to non-inoculated plants as the control. A level of statistical significance was set at $p < 0.05$.

3. Results and Discussion

An outdoor study using potted plants enabled assessment of crop fitness to maturity and nutritional iron content in the kernel as a function of *A. brasilense* and chemical auxin treatments. Crop fitness during vegetative growth was measured in the 2019 growth season by changes in plant height and stem thickness (Figure 2). Treatments appeared not to affect rates of plant growth as measured by plant height, but differences were seen in the thickness of the plant stems when measured at the second internode. Measurements taken on days 28 and 55 after germination showed a statistically significant increase in thickness from *A. brasilense* HM053-inoculated maize relative to non-inoculated control while other treatments had no effect. In support of improved plant growth from *A. brasilense*

HM053 inoculation, we observed that chlorophyll content when measured in 3-week-old lab grown seedlings (Figure 3A) was significantly elevated from bacterial treatment relative to non-inoculated control plants while plants inoculated with *A. brasilense ipdC* showed a reduced leaf chlorophyll content. Though chlorophyll content was not measured in the outdoor potted plant studies, we do note that leaf thicknesses measured at the end of the 2019 growing season (Figure 3B) were significantly greater with *A. brasilense* HM053 inoculation than non-inoculated controls. Our data suggests that the effects of *A. brasilense* HM053 inoculation on leaf chlorophyll in the early stages of plant growth must persist throughout the growing season since we observed improved plant growth reflected by increased stem and leaf thickness.

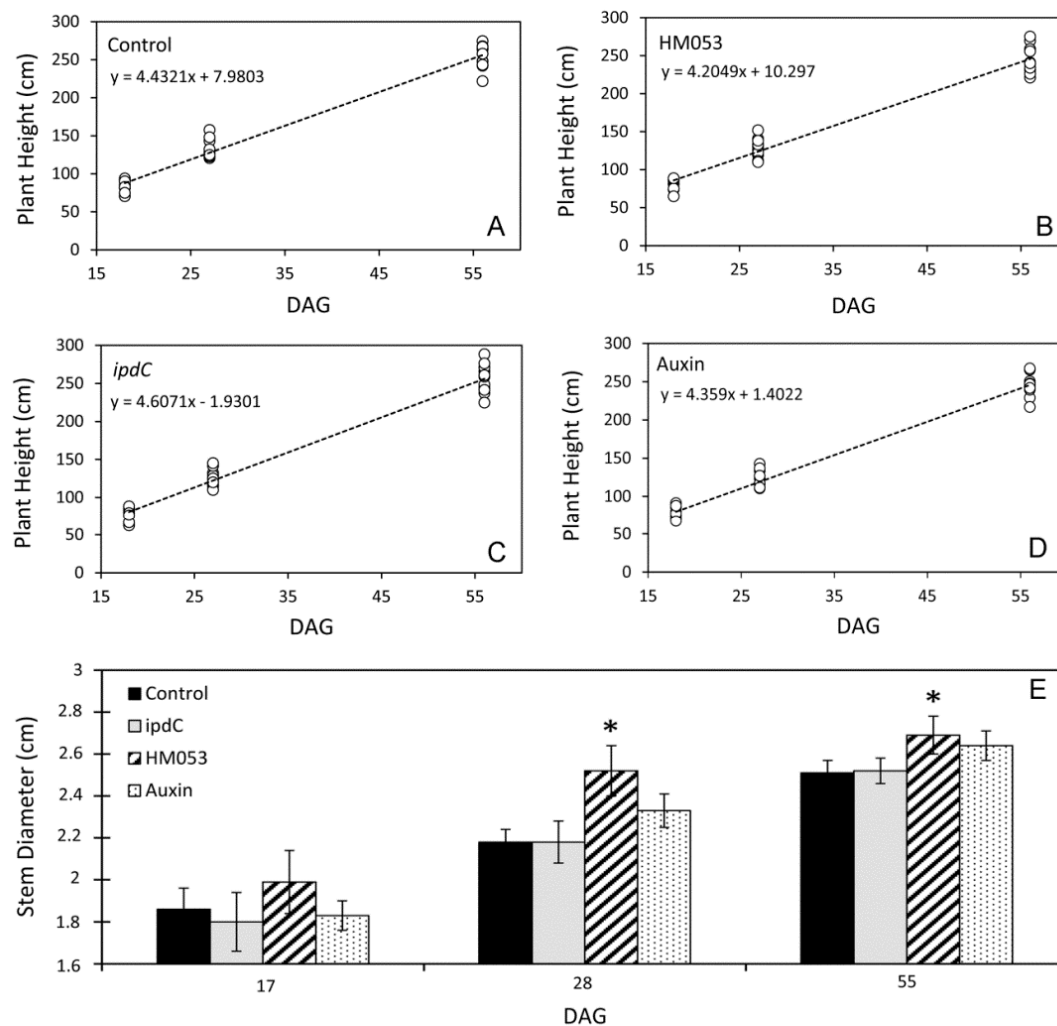


Figure 2. Plant growth rates measured as plant height and stem diameter over time. Plant heights were measured from top of soil to tallest point on shoots during the 2019 growing season spanning 17–55 days after germination (DAG). No significant difference was observed in growth rates across the treatment types. Panels (A–D): based on a comparison of growth rate slopes derived from linear regression analysis. Stem diameters were measured in centimeters at the second internode from the base of the stem. A significant increase in stem thickness was recorded for *A. brasilense* HM053 relative to non-inoculated control maize. Data (\pm SE) reflects $N = 12$ biological replicates. Asterisks indicate significant differences ($*p < 0.05$) using Student paired t-test comparing treated to non-treated control plant stems when measured at the second internode, in which, at 28 and 55 days after germination, *A. brasilense* HM053-inoculated maize exhibited a significantly increased stem thickness relative to non-inoculated control, suggesting the biologic improved plant growth.

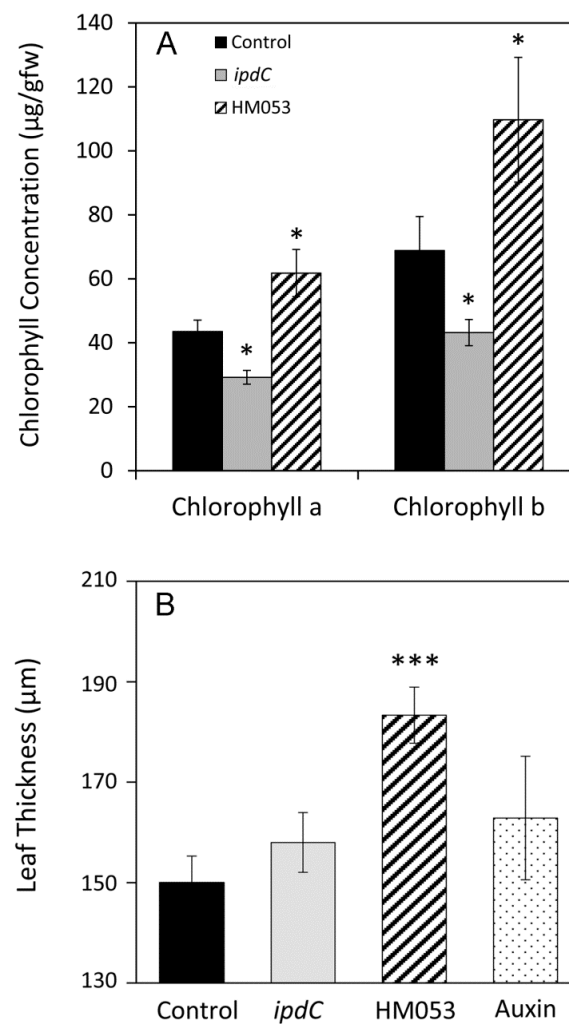


Figure 3. Leaf chlorophyll content and leaf thickness as a function of treatment type. **Panel A:** The 3-week-old lab plants inoculated with *Azospirillum brasilense* HM053 showed significant increases in leaf chlorophyll content compared to non-inoculated control plants, while *A. brasilense ipdC* inoculated plants showed significantly reduced chlorophyll content relative to non-inoculated control plants. **Panel B:** Leaf thickness measurements carried out at the end of the 2019 growing season showed that plants inoculated with *A. brasilense* HM053 had significantly thicker leaves compared to control plants. Data reflects $N = 12$ biological replicates in both panels for each treatment, with error bars indicating \pm SE. Paired comparisons were made using the Student t -test between single treatments and non-inoculated control plants. Asterisks indicate significant differences ($*p < 0.05$, $***p < 0.001$) in these comparisons.

Finally, crop seed yield was measured as the total kernels of corn per cob (Figure 4). It was found that maize associated with *A. brasilense* HM053 had significantly higher crop yields compared to non-inoculated control plants (34% and 53% increases for *A. brasilense* HM053-inoculated plants from the 2018 and 2019 growing seasons). There was also a 26% increase in crop yield from auxin treatment in the 2018 growing season, while *A. brasilense ipdC*-associated maize exhibited a 26% lower yield than non-treated plants, though not significant, during the 2018 growing season and no difference from non-treated plants in the 2019 growing season (Figure 4). The differences in yield between the 2018 and 2019 growing seasons may be attributed to differences in the outdoor environmental conditions. While plants were irrigated on an alternating day cycle, there was increased rainfall during the early portions of the 2019 growing season which could have contributed to the yield improvement.

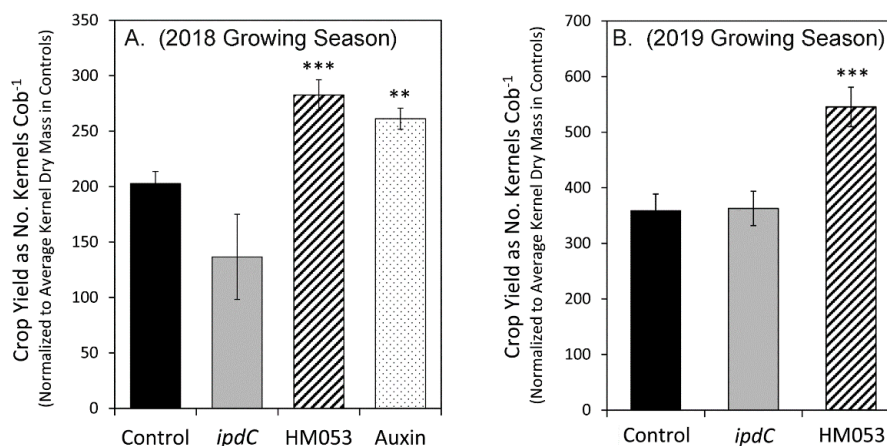


Figure 4. Effect of treatment on corn seed yield. Seed yield was quantified as number of kernels per cob. In both the 2018 and 2019 seasons, plants inoculated with *A. brasilense* HM053 bacteria showed significantly enhanced kernel yields compared to control treatments. Chemical auxin treatment showed significantly enhanced kernel yield as well, while *A. brasilense ipdC* and control maize were not significantly different in yield from one another. Data was normalized to the average dry kernel mass in controls and reflect $N = 12$ biological replicates for each treatment. **Panel A:** data from the 2018 growing season; **Panel B:** data from the 2019 growing season). Error bars indicate \pm SE. Paired comparisons were made using the Student t-tests between single treatments and non-inoculated control plants. Asterisks indicate significant differences (** $p < 0.01$, *** $p < 0.001$) in these comparisons.

An increase in maize crop yield was noted under *A. brasilense* HM053 inoculation and auxin treatments; however, the main purpose of leveraging bacterial inoculants in this study was to demonstrate a cost-effective means to increase iron nutrition in the crop product consumed by humans. Thus, it was most relevant to quantify the iron content of seeds as a function of treatment, as well as quantify the bioavailability of that iron bound in the ferritin protein. Ion chromatography was used to quantify total seed iron content as well as distinguish Fe^{2+} and Fe^{3+} oxidation states (Figure 5). The results revealed a strong positive correlation between seed Fe^{2+} accumulation and total seed iron content, with *A. brasilense* HM053 generating the highest iron values. This suggests inoculation in the early stage of plant development can result in a lasting effect throughout the life cycle of the plant. Furthermore, Fe^{3+} showed no change as a function of inoculation. Both *A. brasilense* HM053 and *ipdC* data was loosely clustered at opposite ends of iron values measured in seeds (30–130 nmols iron), whereas non-inoculated control data spanned the entire range of values. Recent studies in tomato indicate early treatments with inoculums can reshape the diversity of the soil microbiome favoring dominance of the inoculum whereas non-inoculated plants can exhibit a broad spectrum of microbiome diversity [43].

All kernels associated with *A. brasilense* HM053 or *ipdC*, as well as those treated with auxin, showed significant increases in kernel ferritin content (Figure 6). This corroborates the assertion that PGPR association improves iron integration to seed, ultimately increasing its nutritional value. The auxin treatment increasing ferritin content alludes to the importance of the auxin production capabilities of the bacteria in influencing this effect, which requires further investigation. However, this does not explain the observed increase in seed ferritin content from inoculation with the auxin deficient-mutant, *A. brasilense ipdC*. Past perceptions have assumed that ferritin biosynthesis is tightly regulated by cellular iron homeostasis [14]. Even so, the present results suggest that its regulation is even more complex.

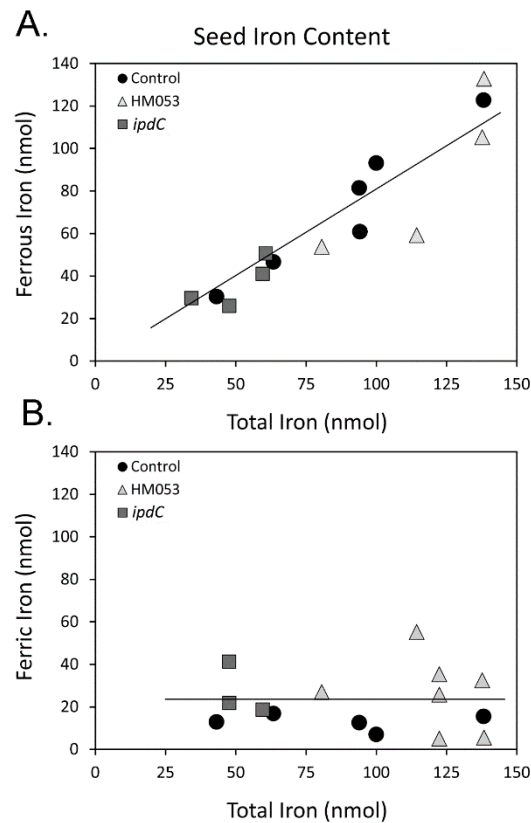


Figure 5. Seed iron content as total iron, ferric iron, and ferrous iron as a function of treatment type. **Panel A:** data on seed ferrous (Fe^{2+}) concentration relative to total iron from the 2018 harvest. There was a positive correlation across all treatment types between ferrous iron and total iron content in maize seed. **Panel B:** data on seed ferric iron (Fe^{3+}) concentration relative to the total iron. There is no strong correlation between changing total iron content and ferric iron in maize seed kernels.

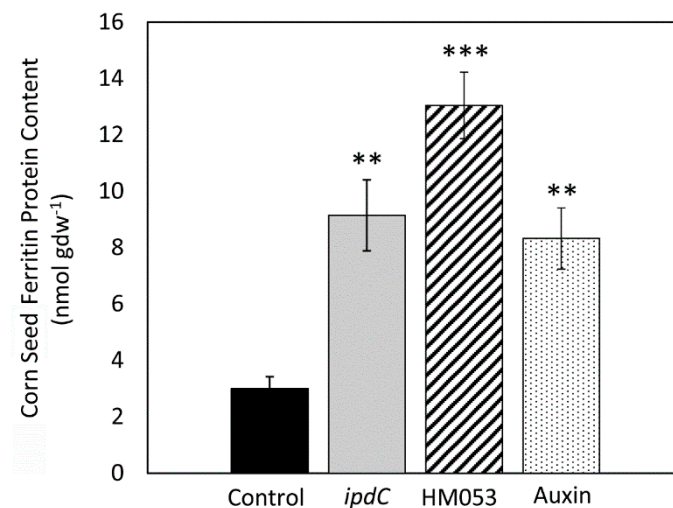


Figure 6. Seed iron-ferritin content as a function of treatment type. All treatment types showed significantly greater concentration of ferritin protein in maize seed relative to control with greatest elevation observed in the *A. brasilense* HM053-inoculated maize kernels. Data reflects $N = 4$ biological replicates for each treatment with error bars indicating \pm SE. Paired comparisons were made using the Student t-test between single treatments and non-inoculated control plants. Asterisks indicate significant differences (** $p < 0.01$, *** $p < 0.001$) in these comparisons.

Finally, while chemical treatments using auxin resulted in similar improvements in crop yield and crop nutritional value as *A. brasilense* HM053 inoculation relative to untreated control plants, we view chemical treatments as more intrusive to the environment, and likely a more expensive mitigation strategy than application using a liquid inoculant of bacteria.

4. Conclusions

The present study has demonstrated that certain PGPR can have beneficial effects in improving crop nutritional value. Specifically, *A. brasilense* HM053 a hyper N₂-fixing and high auxin producing strain of *A. brasilense* provided the greatest benefits in improving seed iron content with improved iron bioavailability when stored in ferritin. It is also noted that this bacteria strain improved crop yields significantly, possibly as a result of host iron assimilation during the growing season impacting carbon input via the photosynthetic machinery. Future studies will seek to translate our findings from pots to the field.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

Abbreviations

Fe	Iron
PGPR	Plant growth-promoting rhizobacteria
N ₂	Nitrogen
<i>A. brasilense</i>	<i>Azospirillum brasilense</i> rhizobacteria
IAA	Carbon dioxide
DAG	Days after germination
SE	Standard error
°C	Degrees Celsius
rpm	Rotations per minute
CFU	Colony forming unit
TLC	Thin-layer chromatography
UV-VIS	Ultraviolet-visible spectroscopy
HCl	Hydrochloric acid
M	Moles per liter

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