Effect of Native American Bean-corn Biculture Planting on Free-living Bacterial Abundance and Plant Growth

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ABSTRACT
Maize, bean, and squash have been intercropped for thousands of years, sustaining Maya Indians and Native American tribes with bountiful harvests. Today it is widely recognized that this associated intercropping system derives much of its success from symbiotic bacteria (e.g. *Rhizobium*). These bacteria colonize the roots of leguminous plants, allowing them to fix atmospheric nitrogen into ammonia. However, the effect of this intercropping practice on the microbial community, independent of the effect of the symbiotic nitrogen-fixing bacteria, is not well understood. Therefore, a study was designed to model the effects of simultaneously intercropping bean and corn on the abundance of aerobic heterotrophic, free-living nitrogen-fixing, and symbiotic nitrogen-fixing bacteria, as well as plant growth and fecundity markers. In parallel, the benefits mediated by rhizobia were evaluated by inoculating a duplicate set of treatments with N-Dure, a rhizobia-containing inoculum. Native American varieties of pole-bean (*Phaseolus vulgaris* L.) and corn (*Zea mays mays* L.) were planted in monoculture and biculture treatments. All cultivations were maintained under greenhouse conditions for 52 days with daily watering and no additional fertilizer or microbial amendments. Although a significant increase in weight per plant was noted for the inoculated biculture when compared to either the inoculated bean or corn monocultures ($p \leq 0.05$), the abundance of heterotrophic and free-living nitrogen-fixing bacteria did not show a significant change from the related controls, with or without inoculation. However, symbiotic nitrogen-fixing bacteria, as measured by root nodulation, increased significantly ($p \leq 0.05$) for the inoculated biculture and single planting. Thus, these data confirm that corn benefited from this associated intercropping system as shown by an increase in plant biomass that can be attributed to *Rhizobium*. However, neither the legume-bacteria symbiotic relationship nor the increase in plant biodiversity resulting from this intercropping practice appears to have had significant effects on the abundance of the two common soil-associated bacterial groups evaluated, though further research would be necessary to fully assess the changes to heterotrophic bacterial diversity at the species level.

KEYWORDS
Three Sisters; Nitrogen-fixing Bacteria; Inoculation with Rhizobia; Plant Growth Promoting Bacteria; Soil Microbial Biota; Corn and Bean Simultaneous Planting.

1. INTRODUCTION

The practice of an associated intercropping, maize and bean specifically, can be traced to the Yucatan peninsula over 6000 years ago, where the Maya Indians employed a milpa system of agriculture: maize, common or lima bean, and native squash seeds haphazardly planted together in the same hole[1,2]. As staple agricultural crops were being domesticated between 12,000 and 6,000 years ago throughout North America[3], planting schemes involving maize, bean, and squash were being optimized. This successful planting scheme was shared with neighboring tribes, making its way to the Northeastern US between 1,000–1,200 A.D. [1,2,4], where it was known as the Three Sisters.

Scientists have since been able to shed light on why the intercropping practice was so productive.

A key reason for the success of the triumvirate of squash, maize, and bean is the fact that their above- and below-ground physical growth characteristics are complementary and in some cases facilitate each other.[5-7] Considering the above-ground characteristics, squash forms the lower canopy of this system and produces dense foliage that shades the ground, lowering the ground temperature while promoting moisture retention and weed suppression.[7, 8] Maize then forms the vertical canopy of this system,[5] serving as a structure on which the beans can climb; thus, the growth characteristics of corn complements those of beans.[9] Lastly, bean utilizes the unoccupied mid-story between squash and maize, in turn maximizing use of available sunlight for photosynthesis.[5, 10]

Considering the below-ground characteristics, the large lateral root architecture of corn complements the vertical taproot growth of beans and squash, resulting in efficient utilization [5, 11, 12] and sharing of resources.[13, 14] Second, arbuscular mycorrhizal fungi commonly associated with legumes have been credited with facilitating the transfer of nitrogen and other available resources to other non-leguminous plants,[13, 15] especially during root decomposition.[16] Third, *Rhizobium* species that are commonly associated with legumes have been shown to inhibit the growth of soil-borne pathogens (e.g. *Fusarium* spp., *Phytophthora* root rot).[17-19] Lastly, and arguably most importantly, bacteria symbiotic with leguminous plants fix atmospheric nitrogen into ammonia.[20, 21] This self-sustaining source of biologically available nitrogen is accessible to the bean, in turn promoting plant growth.[22] The symbiotic relationship between bean and *Rhizobium* may be enhancing the growth of the intercropped plants in three ways: (i) by reducing the effect of beans as competitors for nitrogen in the soil, and/or, (ii) by 'leaking' excess nitrogen into the soil via root exudates that the other plants can access[23] and/or, (iii) by making a useful form of atmospheric nitrogen available to surrounding non-legumes through the decomposition of bean biomass. The optimization of the conversion of atmospheric nitrogen to ammonia by *Rhizobium* has been the focus of novel sustainable intercropping practices, including the development and application of bacterial soil inoculants that promote plant productivity.[24-26] One study revealed a correlation between the abundance of indigenous rhizobia to the efficacy of the inoculant to promote root nodulation (i.e. low initial rhizobia population resulted in an increase in the quantity and mass of nodules).[27]

Although foliar and root complementation can at least partially explain the growth and performance benefits yielded by the Three Sisters planting scheme, it is also possible that the increase in plant biodiversity, specifically from a monoculture to a biculture or polyculture, facilitates a beneficial change to the soil microbial community[28-31] in ways that promotes microbial heterogeneity and reduction of pathogenic species.[17-19] In addition, Spehn *et al*. (2000) reported a significant positive correlation between plant species richness and microbial biomass. They also noted that when legumes were absent, microbial biomass declined significantly regardless of plant species diversity, suggesting that the presence of beans in a biculture or polyculture planting may impact the abundance of soil microbes. In addition to symbiotic bacteria (e.g. *Rhizobium* spp.), associated rhizosphere bacteria (e.g. *Pseudomonas* spp.) have been found to significantly promote the growth of non-legumes (e.g. maize, wheat, barley, mustard) either in combination with symbiotic microorganisms or independently.[32-34] This suggests that a change in the composition of the bacterial community, such as an increase or decrease in free-living nitrogen fixers or other plant growth-promoting rhizobacteria (PGPR), may potentially benefit corn directly by converting soil-based resources into biologically available forms or facilitating plant absorption of insoluble minerals.

Focusing specifically on legume-maize intercropping experiments, several reports provide evidence indicating significant benefits for maize, the legume, or both plants when they are intercropped,[35-38] though contradictory evidence exists as well.[39-41] It is widely accepted that rotating[42] between or intercropping a cereal and legume crop, coupled with an adequate population of symbiotic nitrogen-fixing bacteria and/or PGPR have generally positive influences on agriculture. However, it is not clear whether these are mutually exclusive: are rhizobia solely responsible for the improved growth and performance experienced by the Three Sisters? Or have bean and/or corn facilitated a positive, synergistic effect on the general heterotrophic bacterial community that is known to accompany a diverse planting? Noting that squash appears to serve this intercropping system only by promoting weed suppression and moisture retention, the scope of initial testing was reduced to focus on the effects of corn on beans and vice-versa, making the experimental design more manageable. As such, the purpose of this study was to investigate whether soil microbial biota can be identified as the major mediators of enhanced plant growth. The three features of the soil microbial biota investigated were the abundance of: (i) symbiotic leguminous nitrogen-fixing bacteria (e.g. *Rhizobium* spp. and *Bradyrhizobium* spp.), as measured through nodulation of the bean root system; (ii) free-living nitrogen-fixing bacteria, (used as an indicator of a change in the plant growth promoting bacterial population) and (iii) heterotrophic bacteria (used as a general indicator of large-scale effects on the bacterial community).

Our hypotheses are that intercropping corn and bean will promote conditions, independent of inoculation, that: (a) result in the increased nodulation of the bean root system indicating an increase in the symbiotic nitrogen-fixing bacterial population; (b) result in a decrease in the abundance of free-living nitrogen-fixing bacteria, which in turn represents a general change in the composition of the free-living heterotrophic bacterial community; and, (c) facilitate enhanced, concomitant growth of bean and corn in response to these effects.[29] To address these hypotheses, plant performance was assessed for corn and bean under three cultivation conditions: (1) corn and bean planted singly (i.e. one plant per pot); (2) two corn or bean plants cultivated in an individual pot (hereafter referred to as “monoculture”); and, (3) one corn and one bean plant cultivated in the same pot (hereafter referred to as “biculture”). To assess the role of symbiotic nitrogen-fixing bacteria, we inoculated a duplicate set of the aforementioned treatments with N-Dure, a commercial soil inoculum containing *Rhizobium* and *Bradyrhizobium* spp. Total heterotrophic and free-living nitrogen-fixing bacteria were quantitated for all treatments.

2. MATERIALS AND METHODS

Native American varieties of *Phaseolus vulgaris* L. (Fortex pole-bean, Hinterland Trading) and *Zea mays mays* cv. Mandan Bride (Organic Mandan Bride corn, Hirt’s Gardens) were grown under temperate greenhouse conditions during the months of July and August 2013 in Elmhurst, IL. Plants were grown in a previously unused fertilizer enriched potting soil (NPK 12:9:7; Schultz®, Infinity Lawn and Garden, Inc.; nutrient content was assumed as advertised by the manufacturer) for 52 days, with water supplied on average every 24 hours and without additional fertilizer.

3. SEED SELECTION AND PREPARATION

Based on a 30-day preliminary study demonstrating size-dependent germination success (data not shown), bean seeds weighing 0.43g – 0.55g were selected; this correlation was not demonstrated by the corn seeds, therefore these seeds were randomly selected. All seeds were rinsed in sterile water prior to being forcibly germinated. Germination was forced by arranging the seeds between damp paper towels inside new polyethylene zipper storage bags, and storing those bags in a dark growth chamber maintained at 30°C. Upon visual inspection, only seeds that produced a well-formed radicle were selected for planting.

4. EXPERIMENTAL DESIGN

To investigate the effect of associated intercropping on corn and bean growth, five treatments were imposed (N=3): Corn and bean were either planted alone (referred to as C or B), in monocultures comprised of two corn (CC) or two bean (BB) plants in the same pot, or in a biculture comprised of one corn and one bean in the same pot (CB). To identify the effect of rhizobia on plant performance and on the abundance of key microbial communities in each condition, an identical set of treatments was established that were inoculated with N-Dure, an alfalfa/true clover inoculant (INTX Microbials, LLC) containing *Bradyrhizobium japonicum* and *Rhizobium* species. Both corn and bean radicle-producing seeds were gently rolled in the inoculant prior to planting using sterile tweezers, and the surface of the soil was amended with approximately 1g of inoculant post planting. Unplanted soil controls with and without N-Dure were maintained under the previously mentioned conditions. All forcibly germinated seeds were gently placed in the soil to a depth of 2–4 cm, radicle down, and approximately 5 cm apart in the pot if planted in monoculture or biculture treatments.

5. PLANT MEASUREMENTS

The plants were grown for 52 days, at which time the experiment was terminated and various measures of plant and bacterial growth were recorded. For corn, the following data points were collected: stalk height as determined from just above root system to tallest point of the top leaf, number of leaves with visible leaf collars excluding visibly deteriorated lower leaves, presence of exposed tassel, and aboveground biomass. For bean, the following data points were collected: number of trifoliate leaves excluding visibly deteriorated lower leaves, number of flowers and bean pods, and aboveground biomass. The root systems of all bean plants were destructively sampled for root nodules. To ensure comparable results across replicates, a minimum nodule size (diameter ≥ 1 mm) and time limit threshold (90 minutes) was established, and nodules subsequently enumerated.

6. BACTERIAL MEASUREMENTS

Prior to using the newly procured potting soil, a three-point most probable number (MPN) dilution technique carried out to a 10^{11} dilution[43] was executed at T_0 for both general heterotrophs and free-living nitrogen-fixing bacteria. After collecting the aboveground measurements, the numbers of culturable aerobic heterotrophic and free-living nitrogen-fixing bacteria were enumerated using the same MPN technique. The 10^0 soil inoculation sample (0.5g) was collected from the center of each pot approximately 1–2 cm below the surface, or between mono- or biculture treatments where applicable. Aerobic heterotrophs were enumerated using a half-strength nutrient broth (Difco). Free-living nitrogen-fixing bacteria were enumerated using a modified Ashby's nitrogen-free broth containing the following per liter: mannitol 20g; K_2PO_4 0.2g; $MgSO_4 \cdot 7H_2O$ 0.25g; NaCl 0.2g (Ashby, 1907); Tanner's trace metals 5ml; and $CaCO_3$ 3g.[44] Individual broth tubes were scored positive if visible growth was observed after an incubation period of 48 hours at 23°C for aerobic heterotrophs, or after an incubation period of 7 days at 23°C for free-living nitrogen fixers.

7. STATISTICAL ANALYSIS

A series of Student's t-tests were performed to evaluate the effects of the associated intercropping system and the inoculation treatments on the plant and microbial measurements. In addition, an evaluation of the correlation between two quantitative variables (e.g., nodules per bean plant versus weight per plant) and the inoculation treatment or associated intercropping system was performed using analysis of variance (ANOVA), noting data were normally distributed (Shapiro-Wilk; $p > 0.05$). Where the ANOVA indicated significant effects or interactions, a Tukey's HSD post-hoc multiple means comparison test was used to identify the specific differences in among treatment conditions. Significance of difference was evaluated at $p \leq 0.05$ for all statistical analyses. Data analyses were performed in Microsoft Excel® (version 14.3.9, 2011) and in R©, freeware made available by The R Foundation for Statistical Computing (version 3.0.1, 2013).

8. RESULTS

Although the productivity of various intercropping designs has been studied at length,[22, 35-41] the focus of this research was placed on the microflora. We sought to evaluate the microbial response and subsequent impact on plant performance of a corn-bean biculture planting scheme. In this study we attempted to identify the effects of intercropping Native American varieties of bean and corn on the growth and fecundity of each individual of the biculture or monoculture. These results were then compared to the abundance of bacteria enumerated for each replicate, inoculated and not inoculated with rhizobia, with the goal of revealing correlations between plant performance and a change in the composition of the microbial community.

9. PLANT BIOMASS AND CHARACTERISTICS

Bean-Corn Biculture

The corn plants present in the inoculated biculture demonstrated a statistically significant increase in weight per plant when compared to the inoculated corn monoculture (**Figure 1**; p=0.025); this represented the second largest mean biomass weight recorded for corn, following singly planted corn. These data also revealed a statistically significant 44% increase in corn biomass weight when comparing the inoculated to the un-inoculated biculture planting treatments (**Table 1**, p=0.034). The bean plant present in the inoculated biculture planting treatment however, was not statistically different from the inoculated bean monoculture (**Figure 2**; p=0.30). In addition, a 51% increase in bean biomass weight was noted when comparing the inoculated to the un-inoculated biculture planting treatments, but again this was not statistically significant (**Table 2**, p=0.117). Out of all un-inoculated treatments containing bean or corn, lowest biomass values were recorded for the biculture, though this was not significantly different from either the bean or corn monoculture treatments, respectively.

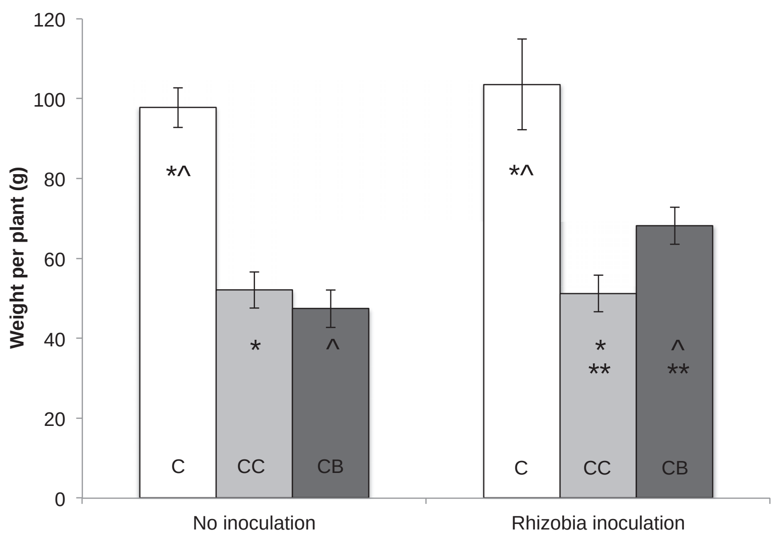

Figure 1. Comparison of mean aboveground biomass weight per corn plant for each inoculation treatment across each planting scheme. Empty bars represent single corn plantings (C), light-grey bars represent monoculture corn plantings (CC), and dark-grey bars represent corn-bean biculture plantings (CB). Bars are mean values ± SE, N=3. Significant differences are denoted with an *, ^, and ** ($p \leq 0.05$, Student's t-test)

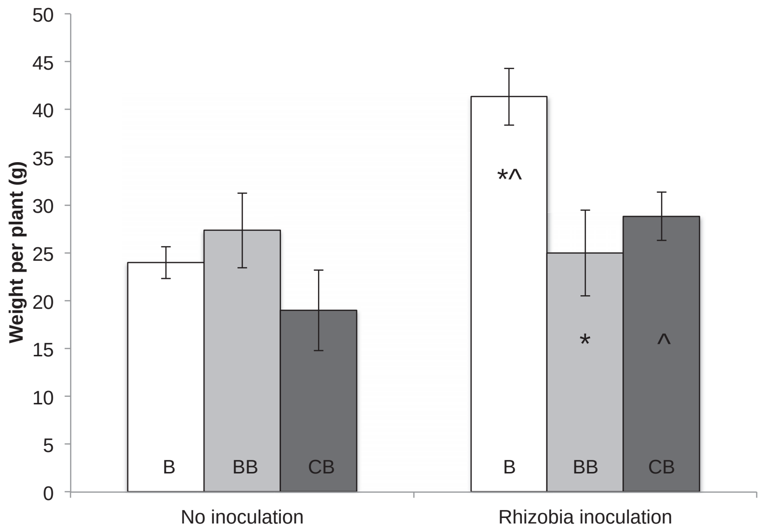

Figure 2. Comparison of mean aboveground biomass weight per bean plant for each inoculation treatment across each planting scheme. Empty bars represent single bean plantings (B), light-grey bars represent monoculture bean plantings (BB), and dark-grey bars represent corn-bean biculture plantings (CB). Bars are mean values ± SE, N=3. Significant differences are denoted with an * and ^ ($p \leq 0.05$, Student's t-test)

The inoculation had a significant positive effect on the number of root nodules found on the biculture bean, representing a 690% increase from the un-inoculated biculture bean (**Figure 3**; p=0.043). While, nodulation of the bean root system for the un-inoculated planting treatments was found to be similar between the biculture and the singly planted bean (p=0.754).

No significant differences were noted with the additional plant characteristics recorded for the biculture bean (number of trifoliate leaves, number of flowers, number of seed pods, nodule count; **Table 2**) or the biculture corn (number of collared leaves, presence of tassel; **Table 1**).

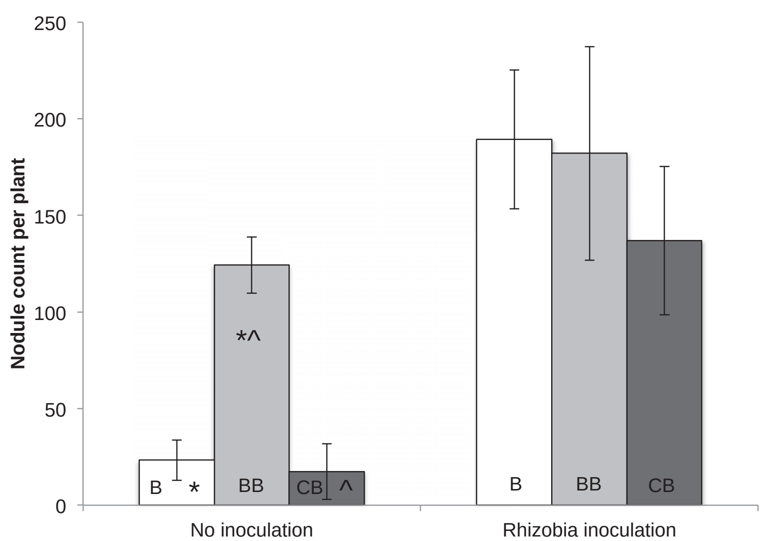

Figure 3. Comparison of mean nodule count per bean plant for each inoculation treatment across each planting scheme. Empty bars represent single bean plantings (B), light-grey bars represent monoculture bean plantings (BB), and dark-grey bars represent corn-bean biculture plantings (CB). Bars are mean values ± SE, N=3. Significant differences are denoted with an * and ^ ($p \leq 0.05$, Student's t-test)

Bean Single and Monoculture

In the un-inoculated treatment, bean root nodules were significantly higher in monocultures when compared to the single and biculture planting treatments (p=0.005, p=0.007, respectively), suggesting a facilitative effect of conspecifics on nodule growth.[45] The inoculation had a positive effect on the number of root nodules found on the single and monoculture bean, (711% and 47% increase, respectively), though this was only statistically significant for the singly planted bean (p=0.011). Furthermore, nodule count per plant was significantly, positively correlated with bean biomass weight (p=0.001), which agrees with the known symbiotic relationship between legumes and rhizobia.[46, 47]

The inoculated single bean planting treatments demonstrated higher biomass values when compared to the inoculated bean monocultures (p=0.010; **Figure 2**). Furthermore, highest bean biomass values were achieved under the single planting treatment that was inoculated, and were nearly double the biomass weight as those found in monocultures and bicultures. However, there were no significant differences noted in bean growth between the inoculated and un-inoculated monocultures, which generally agrees with the evidence showing that root nodule growth in monocultures were similar between inoculation treatments, and root nodules were positively correlated with growth.

No significant differences were noted with the additional bean plant characteristics recorded for either the single or monoculture bean (number of trifoliate leaves, number of flowers, number of seed pods, nodule count; **Table 2**).

Corn Single and Monoculture

Corn growth in the absence of bean was unaffected by the inoculation. The singly planted corn, regardless of the inoculation condition, demonstrated significantly higher biomass values when compared to the respective bicultures (inoculated, p=0.045; un-inoculated, p=0.002) and the corn monocultures (inoculated, p=0.010; un-inoculated, p=0.005; **Figure 1**). Highest corn biomass values were achieved under the single planting treatments, and were nearly twice the biomass weight as those in monocultures and bicultures. This difference can be attributed to the height of the stalk, which was statistically significant between the single and monoculture corn conditions (p=0.030). No other significant differences were noted with the additional corn plant characteristics recorded (number of collared leaves, presence of tassel; **Table 1**).

CORN	Weight per plant (g)		Height of stalk per plant (cm)		No. of leaves per plant		Tassel present	
Un-inoculated								
Single	97.8[a]	*±5.0*	105.3[a]	*±6.1*	6.7	*±0.6*	0.3	*±0.3*
Mono	52.1[a]	*±6.4*	110.3[a]	*±5.0*	5.0	*±1.2*	0.3	*±0.3*
Biculture	47.4[a,b]	*±4.7*	86.7[a]	*±0.9*	5.7	*±0.7*	0.3	*±0.3*
Inoculated								
Single	103.6[a]	*±11.3*	130.3[a]	*±11.1*	7.7	*±1.2*	0.3	*±0.3*
Mono	51.2[a]	*±6.5*	91.0[a]	*±4.1*	5.3	*±0.9*	0.0	*±0.2*
Biculture	68.2[a,b]	*±4.7*	106.0[a]	*±8.7*	5.3	*±0.3*	0.0	*±0.0*

[a] The sample means for the biomass characteristic were significantly different within the inoculation treatment. Details regarding which planting scheme pairs were significantly different are discussed in the RESULTS section and supporting figures.

[b] The sample means for the biomass characteristic of the planting scheme were significantly different across inoculation treatments (i.e., un-inoculated treatment compared to the inoculated treatment).

Table 1. Effect of inoculation treatment on **corn** biomass measurements across planting schemes. Mean values of the three replicates are presented supplemented by the standard error (*N* = 3 per planting treatment within each inoculation treatment)

BEAN	Weight per plant (g)		No. of trifoliate leaves per plant		No. of flowers per plant		No. of seed pods per plant		No. of nodules per plant	
Un-inoculated										
Single	24.0[a,b]	*±1.7*	6.7	*±0.7*	1.0	*±0.6*	0.7	*±0.7*	*23.3*[a,b]	*±10.5*
Mono	27.4[a]	*±5.5*	8.5	*±1.8*	2.0	*±0.8*	0.0	*±0.0*	*124.3*[a]	*±14.7*
Biculture	19.0	*±4.2*	6.3	*±2.3*	1.0	*±0.6*	0.3	*±0.3*	*17.3*[a,b]	*±14.4*
Inoculated										
Single	41.3[a,b]	*±5.1*	12.7	*±2.3*	2.3	*±0.9*	1.7	*±1.7*	*189.3*[b]	*±35.9*
Mono	25.0[a]	*±11.0*	9.0	*±2.0*	2.5	*±1.5*	1.7	*±0.7*	*182.2*	*±55.4*
Biculture	28.8[a]	*±4.4*	10.3	*±0.3*	2.3	*±1.3*	1.7	*±0.9*	*137.0*[b]	*±38.4*

[a] The sample means for the biomass characteristic were significantly different within the inoculation treatment. Details regarding which planting scheme pairs were significantly different are discussed in the RESULTS section and supporting figures.

[b] The sample means for the biomass characteristic of the planting scheme were significantly different across inoculation treatments (i.e., un-inoculated treatment compared to the inoculated treatment).

Table 2. Effect of inoculation treatment on **bean** biomass measurements across planting schemes. Mean values of the three replicates are presented supplemented by the standard error (*N* = 3 per planting treatment within each inoculation treatment)

10. BACTERIAL ABUNDANCE

Neither the heterotrophic nor the free-living nitrogen-fixing bacterial abundance MPNs demonstrated a statistically significant difference from the unplanted control pots inoculated with or without rhizobia (**Table 3**), and the unplanted control pots at T_{52} were not significantly different from the T_0 controls. In addition, though not statistically significant, the abundance of free-living nitrogen fixers increased in the un-inoculated biculture when compared to the inoculum positive control pots (p=0.064, **Figure 4**).

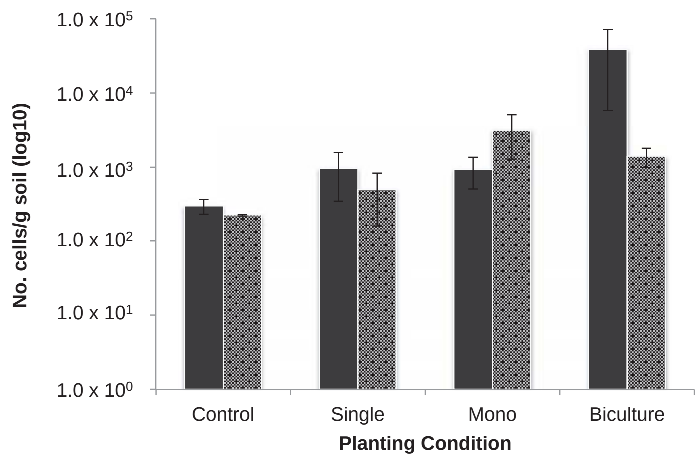

Figure 4. Free-living nitrogen fixing bacteria abundance using MPN enumeration technique. Results are presented for the combined bacterial abundances of corn and bean; i.e. single and monoculture MPN bars represent single bean + single corn and mono-bean + mono-corn, respectively. This was done for ease of reporting; no significant differences were noted for plant-based MPN. Mean abundance of free-living nitrogen-fixing bacteria for each planting scheme were compared across inoculation treatments. Dark-grey bars represent un-inoculated plantings, and light-grey-dotted bars represent inoculated plantings. Bars are mean values ± SE, N=3 ($p \leq 0.05$, Students' t-test and ANOVA)

	General Heterotrophs (MPN)		Free-living Nitrogen fixers (MPN)	
Un-inoculated				
Control	8.05×10^{10}	$\pm 2.95 \times 10^{10}$	2.96×10^{2}	$\pm 6.53 \times 10^{1}$
Single Bean	3.90×10^{10}	$\pm 3.55 \times 10^{10}$	1.62×10^{3}	$\pm 6.93 \times 10^{2}$
Single Corn	8.05×10^{10}	$\pm 2.95 \times 10^{10}$	2.90×10^{2}	$\pm 6.89 \times 10^{1}$
Double Bean	1.10×10^{11}	± 0	6.31×10^{2}	$\pm 2.17 \times 10^{2}$
Double Corn	1.10×10^{11}	$\pm 3.67 \times 10^{7}$	1.22×10^{3}	$\pm 5.63 \times 10^{2}$
Bean + Corn	1.10×10^{11}	± 0	3.86×10^{4}	$\pm 3.67 \times 10^{4}$
Inoculated				
Control	5.10×10^{10}	$\pm 2.95 \times 10^{10}$	2.24×10^{2}	$\pm 6.67 \times 10^{0}$
Single Bean	1.10×10^{11}	± 0	3.91×10^{2}	$\pm 2.78 \times 10^{2}$
Single Corn	1.10×10^{11}	± 0	5.98×10^{2}	$\pm 4.38 \times 10^{2}$
Double Bean	1.10×10^{11}	± 0	2.42×10^{3}	$\pm 9.81 \times 10^{2}$
Double Corn	8.05×10^{10}	$\pm 2.95 \times 10^{10}$	3.91×10^{3}	$\pm 2.71 \times 10^{3}$
Bean + Corn	3.79×10^{9}	$\pm 3.60 \times 10^{9}$	1.39×10^{3}	$\pm 4.59 \times 10^{2}$

Table 3. Effect of planting and inoculation treatment on microbial abundance. Mean values of the three point MPN are presented supplemented by the standard error

11. DISCUSSION

This study sought to investigate the impacts of a Native American inspired intercropping system on the microbial community, and to link the changes in the soil microbial biota abundance and the overall general heterotrophic community composition to the improved performance experienced by the individual plants. The Three Sisters planting scheme was purposefully deconstructed to focus on the interactions between the plants having the greatest reported effect on microbial communities, that being corn and bean. The microbe-facilitated interactions were assessed through the quantification of symbiotic leguminous nitrogen-fixing bacteria and free-living nitrogen-fixing bacteria, as evaluated through bean root nodulation and an MPN assay, respectively. The changes observed in the microbial community were then compared to growth and fecundity characteristics of both corn and bean, by planting treatment and inoculation treatment. The results of this study indicate that symbiotic nitrogen-fixing bacteria (i.e. rhizobia) are mediating most growth benefits realized by intercropped corn, while the bean neither gains nor loses in this biculture when compared to the monoculture system.

The first hypothesis sought to identify the effect of simultaneously intercropping corn and bean on the symbiotic leguminous bacterial population as realized through an increase in the number of nodules found on the biculture bean root system, focusing primarily on the un-inoculated planting treatment. If the benefits of intercropping are independent of inoculation, then perhaps bean is driven to fix greater amounts of atmospheric nitrogen to support its metabolic needs, as well as those of the associated crop. Unfortunately, plant performance was not significantly different between either plant in the un-inoculated biculture compared to the monoculture (same conditions). Therefore, the hypothesis could not be supported by the data gathered. This was unexpected noting that Dawo *et al*. (2007) witnessed highest biomass yields when *Phaseolus vulgaris* and *Zea mays* were simultaneously intercropped and not inoculated with symbiotic leguminous bacteria. The replication and sampling plan was originally designed to support microbiological practices, yet may have generated insufficient data on which to accept the hypothesis, noting that Dawo *et al*. planted a $432m^2$ field at densities ranging from 50,000 – 100,000 plants ha^{-1}. However, additional research has revealed differing results for similar un-inoculated, intercropped maize-legume systems. For example, Martin *et al*. (1990) reported highest corn yields from outdoor plots when it was monocropped vs. simultaneously intercropped with soybean. Risch and Hansen (1982) reported similar results from outdoor plots with a bean-corn-squash polyculture, noting that the per plant yields of both corn and squash decreased significantly, though bean yield benefited significantly. Although no benefits were realized by the un-inoculated bicultures, significant plant growth was observed for the biculture corn when coupled with significant nodulation of the bean root system, but only when this planting treatment was inoculated. This was not completely unexpected, recognizing previous research had reported benefits realized by corn when the maize-legume bicultures were collectively inoculated with symbiotic leguminous bacteria.[22, 25, 26] The results of this research suggest that the inoculated biculture corn indirectly benefited from either the increased nodulation of the bean as a result of an increased abundance of symbiotic nitrogen-fixing bacteria or through a change in the composition of plant-growth promoting rhizobia. Noting that the inoculated biculture bean performed equally as well when planted in monocultures, and the biculture bean appeared to recover from the reduced growth experienced under the un-inoculated condition (i.e. the inoculated biculture bean witnessed a 52% increase in biomass weight from the un-inoculated biculture bean, though this was not statistically significant), this further suggests that symbiotic nitrogen-fixing bacteria may also be ameliorating the competitive effects of corn on bean when these plants are intercropped. The results witnessed during this experiment differ from those reported by Searle *et al*. (1981), where no effect on corn grain yield was noted and legume biomass decreased when these plants were intercropped and inoculated.

The second hypothesis sought to link a change in the abundance of free-living nitrogen-fixing bacteria regardless of inoculation treatment to benefits realized in the biculture, and concomitantly, both corn and bean growth would be facilitated.[29] However, no link could be established between a change in free-living nitrogen-fixing bacterial abundance and the aboveground plant growth and performance. Instead, the abundance of free-living nitrogen-fixing bacteria increased in the un-inoculated biculture treatment when compared to the related unplanted control pots, however this was not statistically significant. These results were unexpected noting that Keswani (1976) reported an increase in rhizosphere microbial populations when maize and soybean were intercropped and un-inoculated, while also noting a significant increase in maize yield compared to a monoculture. Therefore, the bacterial abundance assessment performed may have been an ineffective measure of the full contribution mediated by free-living soil bacteria under a biculture planting scheme. For example, *Pseudomonas denitrificans*, *P. rathonis*, *Bacillus laevolacticus*, *B. amyloliquefaciens*, and *Arthrobacter simplex*, all aerobic or facultative anaerobic heterotrophic soil species, have been found to promote plant growth of maize,[49] along with *Azotobacter* spp., an aerobic free-living nitrogen-fixing soil bacterium.[50] Noting no evidence of a significant change in abundance of free-living nitrogen-fixing bacteria, an evaluation of the change in species richness of general heterotrophic bacteria may have offered the expected correlation.

The third hypothesis considers the potential impact on both corn and bean growth when it is intercropped, as witnessed through the combined effects of a shift in free-living heterotrophic bacterial community composition and an increase in symbiotic leguminous bacteria as realized through increased nodulation of the bean root system. This can be best assessed through the results obtained for the inoculated biculture corn. Although not statistically significant, the decrease in abundance of free-living nitrogen-fixing bacteria for the inoculated biculture, coupled with the increased nodulation of the inoculated biculture bean suggests the inoculated biculture corn benefited from the facilitation provided by this intercropping system. This can be supported through the finding that the biculture corn realized significant benefits when inoculated. This appears to indicate that the inoculated biculture bean produced excess fixed nitrogen, making this resource available to corn, the transference of which is commonly mediated by mycorrhizal fungi.[13, 15, 16, 24] Isotopic analysis providing evidence of the transference of nitrogen from legumes to maize has revealed a complex system of resource sharing between these intercropped plants,[13] and it is clear that legumes inoculated with *Rhizobium* and intercropped with a cereal have been found to generate significantly higher amounts of ammonia both under field and greenhouse growing conditions.[24] Specifically, Patra *et al.* (1986) reported that inoculated maize-intercropped legumes fixed approximately 32% of the total nitrogen utilized by maize.

Weight per plant across both inoculation treatments consistently demonstrated highest means for the single planting scheme. This was a reasonable expectation recognizing neither the single bean nor the single corn had to compete with other plants for soil-based resources. In effect, these treatments received access to twice the resources made available to either the monoculture or the biculture. The spatial arrangement and density of the legume-maize intercropping practice has been reported to significantly impact the yields of both plants, recognizing the competition for available growth limiting nutrients directly influences the success of this planting scheme.[51-53] In evaluating the single bean results, it appears the inoculation facilitated a significant increase in bean growth, suggesting that nitrogen was a limiting resource that was mitigated by an increase in nitrogen-fixation activities performed by the bean root nodules. However, when this trend was not similarly demonstrated by the monoculture, either through an increase in biomass weight or root nodulation on a per plant basis, this suggests that physical space between plants[51, 52] may be just as important as the inoculation.

Although this research did not reveal a significant increase or decrease in the abundance of free-living nitrogen-fixing bacteria, which suggests little change in the overall composition of the general heterotrophic community, symbiotic nitrogen-fixing bacteria appeared to be responsible for mediating the benefits realized by bean and corn. The debate lies in whether rhizobia alone are directly or indirectly responsible. Research has found that bacterial diseases are inhibited[54] and the soil bacterial composition is significantly impacted when legumes are intercropped with maize or other cereal crops without inoculation.[55, 56] Furthermore, Rhizobium inoculation has been linked to a decrease in bacterial diversity.[27]

Regardless of the inoculation treatment, when these two 'sisters' are planted simultaneously and in close proximity, the bean-corn intercropping system reaps rewards greater than or equal to a monocrop system. The physical growth synergies realized both above and below ground by corn and bean, coupled with mutualistic microbial interactions represent a complex set of factors that govern this intercropping system. In an effort to fully vet the contributions made directly or facilitated indirectly by the bacterial community will require further research. First, squash should be incorporated into the planting treatments to allow for a more complete evaluation of bacterial abundance changes as a result of intercropping all three 'sisters'. Second, alternative methods may need to be employed. It is possible the microbial community associated with a Three Sisters planting scheme changes over time, which would suggest the use of soil obtained from a garden where these plants have been intercropped for successive growing seasons. Third, it is possible that the sample sizes appropriate for microbiological assays may have been insufficient to achieve the levels of significance necessary when conducting plant assays. As a result, plant replicates and sampling would need to be increased in order to fully vet the currently suggestive trends in the microbiologically mediated performance of corn and bean. Lastly, even though the MPN did not reveal significant changes in the abundance of the microbial communities we evaluated, it is possible that the abundance of specific general heterotrophic species are impacted by this intercropping system, in turn positively affecting the composition of the entire soil bacterial community. Valuable insights could be ascertained through the use of molecular techniques, allowing for detection and discernment of important shifts in species that may contribute to the success of this intercropping system.

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Heather Miller, a non-traditional student who obtained her bachelor degree in business over 15 years ago, recently returned to school to pursue a new career path in the biological sciences. As an undergraduate at Elmhurst College, she realized a passion for microbial ecology, which was elevated while conducting this research. As such, she applied and has recently been admitted to the graduate college at Michigan State University, where she plans to complete her Ph.D. in microbiology/molecular genetics and environmental toxicology. Her attention will be focused on the microbial ecology of aquatic environments and the effects of eutrophication (e.g. urban and agricultural run-off) and natural resource extraction activities (e.g. high-volume hydraulic fracturing, oil and gas drilling, and mining) on important groups of bacteria capable of bioremediating these events. With this education, she plans to continue research either in an academic, governmental, or private industry setting, helping to identify environmentally sustainable energy extraction processes that consider the vital role of bacteria in maintaining healthy aquatic ecosystems.

PRESS SUMMARY

Many studies have sought to unravel the mechanisms by which the practice of intercropping corn and bean have benefited both plants, in turn providing explanation of its success and bountiful harvest for thousands of years. Although it is widely accepted that *Rhizobium* spp., the symbiotic nitrogen-fixing bacteria colonizing legume root systems, facilitates many of these benefits, it was unclear whether other bacteria also played a role. It was hypothesized that when bean and corn were intercropped, the abundance of free-living nitrogen-fixing bacteria would decrease while the abundance of general heterotrophic bacteria remained the same indicating that the planting practice itself triggered a plant-beneficial microbial composition response. Therefore, we prepared and maintained two planting conditions, with and without rhizobium bacteria inoculation, and evaluated the results.