Year-end Final Report: ***"Relationships between corn plants and nitrogen fixing bacteria on an organic farm."*** A report summarizing the work done with funding from the Ceres Trust in 2010, 2011, and 2012.

Principal Investigator: Carrie K Young, Ph.D., Research Director, Michael Fields Agricultural Institute, PO Box 990 East Troy, WI 53120. Resigned from Michael Fields Agricultural Institute December 2012

Report Author: Allison Pratt-Szeliga, Crop & Soil Researcher, Michael Fields Agricultural Institute, PO Box 990 East Troy, WI 53120. 262-642-3303 ext 107. apszeliga@michaelfields.org

Summary: Corn requires large quantities of nitrogen for its growth. Commercial corn production relies on substantial inputs of synthetic fertilizer. Overuse of synthetic fertilizer causes the contamination of surface and ground waters. The hypoxic zone in the Gulf of Mexico shows the extent of this pollution. Dependence on inorganic fertilizer can lead to soil degradation, as well. Farmers who use synthetic fertilizer are subject to fluctuating and ever-increasing fertilizer prices, primarily because it is derived from natural gas. Worldwide, many farmers cannot afford synthetic fertilizer. In the near future, agricultural production needs to decrease its reliance on inorganic fertilizer. Healthy soils and organic fertilizers are part of the solution, but biological nitrogen fixation also has the potential to displace synthetic fertilizer use.

The relationship between legumes and nitrogen-fixing bacteria is well understood. These bacteria fix atmospheric nitrogen that would otherwise be unavailable to the plant. Now there is increasing evidence that many non-leguminous species have the ability to cooperate with soil microorganisms to obtain atmospheric nitrogen. The objective of our research is to determine the potential of corn to fix nitrogen biologically. We are investigating several diazotrophic bacteria and how they interact with various breeding lines and seed treatments.

Background: In 2009, plant samples were submitted to the University of California-Davis Isotope facility for stable isotope analysis. Using the 15N natural abundance technique, this analysis reveals how much nitrogen a plant is deriving from the atmosphere. 15N is more prevalent in soils, while 14N is a higher in atmospheric nitrogen. Low15N/14N ratios suggest that biological nitrogen fixation is occurring. A total of 49 samples (one plant sample per breeding line) were submitted for stable isotope analysis in 2009. As stated in the previous report from Michael Fields, results showed some fixation occurring in all lines, with higher rates of fixation in exotic and primitive Plant Introduction (PI) lines.

2010 planting selections were based on 2009 leaf chlorophyll scores, protein and essential amino acid content determined by near infrared spectroscopy, and the stable isotope analysis. The objectives of the experiments conducted in 2010: 1.) measure the response of different cultivars to endophytes and seed treatments; 2.) identify the best bacterial inoculants; 3.) evaluate the effects of biodynamic sprays; 4.) quantify N fixation; 5.) evaluate the 'Inbred Culture-Shock Syndrome'. Throughout winter and spring of 2011, leaf and grain samples collected from these experiments were prepared for submission for stable isotope analysis. Sample preparation entailed shredding corn stalks, drying samples, grinding leaf/grain samples in a Wiley laboratory mill, grinding samples in a SPEX 8000 D mixer mill, drying samples a

second time, and weighing 3 mg of sample material for submission. In this manner, 1,151 samples were prepared and submitted to the University of Georgia Stable Isotope/Soil Biology Laboratory for analysis. The Michael Fields Agricultural Institute (MFAI) received all results from the University of Georgia in fall 2011. Nitrogen fixation data from the 2010 samples shows some promising results, as well as consistency with the 2009 results.

In 2012 planting selections were based on the results of the stable isotope analysis and the yield trials executed in 2011. Soil samples were pulled from fields designated for 2012 corn breeding. Traditional weak acid soil analysis was conducted by A&L Labs concurrently with a biologically available, water extracted soil analysis, conducted by BioSystems. The field location had high P, high K, high Mg, but very low N. Balanced, adequate fertility is essential in making side by side comparisons by avoiding the confounding effects as the result of shortage of other minerals. Due to high P, K and Mg, manure was not an option for nitrogen source. Following soil test recommendation, and in compliance with our Organic Systems plan, Gypsum, Copper Sulfate and Boron were applied and incorporated to the field location. The field was measured and staked and a portion was allocated to the Low Nitrogen experimental area. The balance of the test field received feather meal applied at the rate of 300# per acre providing the normal rate of nitrogen.

Based on 2011 results we chose 9 varieties of cultivars to test and compared how different cultivars respond to different bacterial inoculants in the same growing season on a soil with low fertility compared to a soil with sufficient nitrogen. We were limited in test entries by the quantity of seed needed to plant two row plots, three replicates in four treatments (normal or low nitrogen level and with or without bacterial inoculum). Diverse cultivars and commercially released organic varieties were utilized. Additional rows of each entry were planted in the breeding nursery to be used for self-pollinated seed maintenance.

All 2010, 2011, and 2012 experiments were conducted in organic fields, and replicated three times as split-plot, randomized complete block designs. All experiments had two-row plots, with the exception of the 2010 experiment outlined in objective three (due to a shortage of seed). Here we apply the results of the stable isotope analysis to the objectives laid out in 2010, as well as the results of yield trials executed in 2011.Here we apply the results of the stable isotope analysis to the objectives laid out in 2010, as well as the results of yield trials executed in 2011, and the results of 2012.

Objective 1: Identifying and selecting the best cultivars and measuring their response to endophytes. This experiment measured responses in corn to different seed treatments: 1) no seed treatment; 2) chlorox and heat to kill any fungi living in the seed; 3) a mix of bacteria consisting in class 2, non-dangerous, N-fixing bacteria and fungi applied as a seed treatment (see list below); 4) seed disinfection with heat and chlorox, with later inoculation with the microorganisms. Inoculum was applied as a powder to seed (about 1gm per 30 seeds). Bacteria used were a combination of 1) *Azospirillum brasilense*; 2) *Azospirillum lipoferum*; 3) *Azotobacter*; 4) *Burkholderia unamae*; 5) *Gluconacetobacter diazotrophicus*; 6) *Herbaspirillum seropedicae*; 7) *Paenibacillus brasiensis*; 8) *Paenibacillus durus*;9) *Glomus intraradices* (mycorrhizae).

The goal of this experiment was to determine if disinfection of seeds to try to kill fungi (*Fusarium* species) would then allow for colonization of beneficial bacteria. *Fusarium* can survive in the seeds of an infected plant to colonize the plant's offspring, inhibiting colonization of other bacteria. There is competition among bacterial endophytes. If *Fusarium* can be eliminated, diazotrophic bacteria might become established and aid in nitrogen fixation.

The results of the stable isotope analysis for this experiment are inconclusive. However, compared to non-PI lines, the PI lines had a stronger response to the disinfection+inoculation treatment. One cultivar in particular (PI line 1) had a significant increase in it's percentage of nitrogen derived from air (%Ndfa). Three crosses with PI line 1 as a parent had an average of 35% more Ndfa in the disinfection+inoculation treatment over the control (see Table 1).

Table 1.

%Ndfa - PI line 1 Crosses 2011				
	D	**D+I**	**I**	**none**
Commercial 1 x PI line 1	**15.0**	**25.0**	**10.7**	**18.2**
MFAI x PI line 1	**29.9**	**35.0**	**21.9**	**23.7**
Commercial 2 x PI line 1	**24.6**	**26.3**	**26.0**	**22.2**
avg.	**23.2**	**28.8**	**19.5**	**21.4**

treatment D = seed disinfection treatment I = inoculant

For many cultivars in this experiment the %Ndfa was higher for the control than for the inoculation-only treatment, but not for the disinfection-only treatment. This suggests that, without seed disinfection, *Fusarium* species often outcompete other bacteria.

In spring 2011, seed samples from several of the 44 cultivars in this experiment were submitted to Dr. Charles Bacon, USDA-ARS researcher specialist in *Fusarium* in corn at the University of Georgia to look at the effects of the four treatments on endogenous *Fusarium* in seeds. We have not received the results from those submissions. This experiment was not carried out in 2011.

In 2012, we chose 9 cultivars, based on their measured the results of 2010 and 2011. These cultivars were subjected to four treatments (normal or low nitrogen levels in soil and with or without bacterial inoculum).

Objective 2: Identify the best bacteria for inoculation. To identify the best bacterial inoculants, nine species were obtained from the companies Premier Tech and TerraMax. These bacteria, alone or in combination, composed the treatments for experiments in 2010 and 2011. In 2010, treatments were 1)*Azospirillum brasilense* + *Burkholderia;* 2) *Azospirillum spp* + *Azotobacter*; 3*) Azospirillum brasilense;* 4) *Azospirillum lipoferum;* 5*) Azotobacter;* 6)*Burkholderia unamae*; 7) *Gluconacetobacter diazotrophicus*; 8) *Herbaspirillum seropedicae*; 9*)Paenibacillus brasiensis;* 10) *Paenibacillus durus;*11) *Glomus interradices*. (mycorrhizae); 12) all of the microorganisms combined. Nine varieties were tested with these treatments: three Mexican landraces, four cultivars from the Michael Fields breeding program, one commercial inbred, and one organic commercial hybrid as a check. (One of the nine varieties was excluded

from the 15N results do to insufficient data). The results of the 15N data show a consistent increase in %Ndfa for three of the treatments: 3, 6, and 12 (see Table 2).

Table 2.

% increase in Ndfa over control for various treatments 2011			
	3	6	12
all cultivars	5%	13%	6%
PI lines	-8%	7%	9%
MFAI lines	10%	9%	8%

Treatment one (*Azospirillum brasilense* + *Burkholderia*) had a decrease in nitrogen fixation compared to the control (no bacterial inoculant). However, when these two bacteria were isolated (i.e., treatment three and six) they resulted in an increased amount of %Ndfa in six out of the eight cultivars tested. This raises the question of whether beneficial inoculants can compete with each other, leading to a decrease in nitrogen fixation. Indications of endophytic competition are consistent with the findings of the previous experiment.

The most significant increase (44%) in %Ndfa occurred with the commercial hybrid check. Treatment twelve had an average of 40.2 % Ndfa, compared to 27.9 %Ndfa for no bacterial treatment.

Biological nitrogen fixation does not necessarily translate to increased yield. If a plant is fixing nitrogen, it does not always signify that it's assimilating more nitrogen, it may only mean that it'coming from a different source (atmospheric nitrogen). However, yield trial data from 2011 does show a yield increase in some lines with bacterial treatments. The 2011 yield trial consisted of 18 varieties: the same three landraces (PI lines) from 2010, the same commercial hybrid check, and fourteen populations (MFAI breeding lines and MFAI lines crossed with various PI lines). The trial had five treatments: 1.) a combination of *Azospirillum brasilense, Burkholderia unamae, Azotobacter, Paenibacillus brasiensis,* and *Paenibacillus durus*; 2.) *Azospirillum brasilense* and *Burkholderia unamae*; 3.) *Azotobacter*; 4.) *Paenibacillus brasiensis,* and *Paenibacillus durus*; 5.) no bacterial inoculant. The 2011 yield trial results shows a slight decrease in yield for the MFAI cultivars with bacterial treatments compared to the untreated control. In contrast, yield increases occured for all but treatment four in lines that had genetic material from landraces (albeit minor increases for the PI crosses) (see Table 3).

Table 3.

% increase in yield over control for 3 treatments 2011			
	1	2	3
PI lines	5%	19%	12%
MFAI x PI crosses	5%	2%	3%

The 2011 growing season had a prolonged dry spell were plants began to exhibit signs of drought stress and the 2012 growing season experienced extreme drought conditions. Nitrogen

availability most likely decreased during this dry period when soil moisture was extremely low. One could hypothesize that diazotrophic bacteria (interacting with maize that contains genetic material from landrace cultivars) aided in nitrogen fixation during the dry period, thus minimizing yield losses in comparison to plants with no bacterial treatment.

Soil microorganisms, fungi, and bacteria, have the ability to access and digest nitrogen found in mineral form in the soil. Upon death and decomposition, the nitrogen captured in their body structure is released in the soil water solution and is available for plant uptake. As with nitrogen fixing rhizobium, the appropriate bacterial species are endemic in the soil but may be limited in population or proximity to the roots. Microbial species differ in their relationship with host root tissue, falling into three general categories: 1) Internal within root cell structure, protected and accessing root cellular metabolites, as in legume rhizobium relationships in soybeans, 2) closely adjacent and connected to the roots, on the root exterior, or 3) in the near proximity, close enough to respond to capillary action as the root drawn moisture from the soil. The proximity and degree of root contact and resulting accessibility and movement of soil minerals are microbial species specific.

In 2012, to ensure bacterial availability in the root zone, seed was inoculated before planting with a mix of bacteria consisting in class 2, non-dangerous, N fixing bacteria and fungi including *Azospirillum brasilense, Azospirillum lipoferum, Azobacter, Burkholderia unamae, Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Paenibacillus brasiensis, Paenibacillus and Glomus intradices* (mycorrhizae). Bacteria are mobile in the soil water solution. Fungal hyphae are far reaching and extend beyond the perimeters of a single plot. Field buffer rows between inoculated and non-inoculated plots. The planter box hoppers were blown out to eliminate, or at least reduce, inoculum carry over from inoculated into non-inoculated plots.

Objective 3: Evaluate effects of sprays. A 2010 experiment was conducted to clarify the effects of an unsprayed control and biodynamic sprays on N fixation. All cultivars in this study had the same inoculum that was used in objective one. The stable isotope analysis results show that the plots receiving biodynamic sprays had an average of a 24% decrease in nitrogen fixation compared to the control. All but one of the eleven cultivars in the experiment had a decrease in %Ndfa versus the control (Table 4).

Table 4. **%Ndfa**

	BD spray	control
MFAI line 1	**25.1**	**25.9**
MFAI line 1 x PI line 2	**10.5**	**26.9**
MFAI line 2 x MFAI line 1	**25.8**	**27.3**
Public line 3 x PI line 3	**22.8**	**31.4**
MFAI line 4 x PI line 4	**15.4**	**25.8**
MFAI line 4 x PI line 5	**23.2**	**29.8**
Public line 1	**12.9**	**15.2**
Public line 1 x PI line 6	**28.6**	**32.1**
Public line 2 x PI line 7	**22.8**	**30.5**
Public line 2 x PI line 8	**12.4**	**17.7**

MFAI line 5 x PI line 9	**33.7**	**26.7**
avg.	**21.2**	**26.3**

One of the hypotheses going into this study was that biodynamic soil and foliar sprays "might stimulate and enhance N fixation possibly due to hormonal effects on the plant and root system (Goldstein, 2010)." The results suggest the opposite. It may be hypothesized that the biodynamic sprays (potentized organic fertilizers) provide the plant with more readily-available nitrogen and/or bolster the plant's root system for increased uptake of mineralized N. If this is true, then the higher rates of biological nitrogen fixation in the control would indicate that the bacterial inoculants *do* fix nitrogen when it is less available.

We carried out a similar experiment in 2011, also replicated three times, as split-plot, randomized complete block design. There were eighteen cultivars: two commercial hybrid checks, four MFAI lines, and twelve crosses of MFAI or commercial lines with PI lines. Expanding on the 2010 trial, this trial included four treatments: 1.) biodynamic sprays with inoculants (the same ones used for treatment one of the 2011 yield trial in objective two); 2.) inoculants with no sprays; 3.) biodynamic sprays with no inoculants; 4.) no inoculants and no sprays. Isolating the yield results of the lines without genetic material from the landrace cultivars, one sees no statistically significant outcomes. However, the crosses made with PI lines showed an increase in yield with the inoculants and biodynamic sprays. Compared to the control, treatment one had an 8% increase in yield, treatment two had a 6% increase in yield, and treatment three had a 7% increase in yield. Without stable isotope analysis for this experiment, we cannot determine the rates of nitrogen fixation for the different treatments, their effect on yield, or the interaction between bacterial inoculants and biodynamic sprays.

Objective 4. Quantify N fixation. It should be noted that the 15N natural abundance technique is not an absolute measure of nitrogen fixation rates. Rates of fixation are based on the assumption that plants with higher delta 15N values are not fixing any nitrogen. In 2010, samples of various weed species were gathered to determine the delta 15N values of other non-leguminous plants where experiments were conducted. We did not have enough samples of these species to get an accurate assessment of fixation rates. Therefore, the estimates of nitrogen fixation are based on the delta 15N values of corn plants relative to one another.

Although this technique is not perfect, the results it gives are accurate enough to say with confidence that there are many plants in these experiments that were obtaining over 50% of their nitrogen through biological fixation. With the results we currently have, it is too difficult to ascertain the optimal conditions for these rates of fixation. The results of stable isotope analysis are consistent from 2009 to 2010 (Tables 5 and 6).

In the 2010 data, a wider range of delta 15N values is due to a larger sample population (49 samples in 2009 versus 1,151 samples in 2010). It is unclear whether higher rates of %Ndfa in 2010 are a result of the larger sample population, climate, planting site, or evolutionary breeding effects (covered under objectives one and five). However, among the four classifications of breeding lines, the average delta 15N values and rates of nitrogen fixation are similar from 2009 to 2010. In both years, commercial/private and PI lines had higher average rates of fixation

Table 5.

2009	Number of lines tested	Average delta 15N	Average standard deviation	Range	avg. %Nitrogen derived from air
PVP lines or conventional inbred derivations	15	4.74	0.42	3.94 to 5.41	12
Commercial inbreds from Cooperator & commercial hybrid checks	9	4.86	0.71	3.9 to 5.77	16
MFAI breeding lines > 5 years organic	15	5.36	0.46	4.62 to 6.14	13
HM Landraces (PI lines)	10	4.81	0.96	3.21 to 6.13	22

Table 6.

2010	Number of lines tested	Average delta 15N	Average standard deviation	Range	avg. %Nitrogen derived from air
PVP lines or conventional inbred derivations	10	4.77	0.7	2.31 to 7.11	22
Commercial inbreds from Cooperator & commercial hybrid checks	5	4.31	1	2.02 to 6.74	30
MFAI breeding lines > 5 years organic	18	4.81	0.9	2.53 to 7.23	22
HM Landraces (PI lines)	3	4.84	1.1	2.3 to 7.31	28

Table 7.

2012	Number of lines tested	Average delta 15N	Average standard deviation	Range	avg. %Nitrogen derived from air
PVP lines or conventional inbred derivations	1	4.30	.54	3.63 to 5.58	2.24
Commercial inbreds from Cooperator & commercial hybrid checks	2	4.20	.47	3.40 to 5.09	4.58
MFAI breeding lines > 5 years organic	3	4.35	.53	3.45 to 5.12	1.14
MFAI & public breeding lines crossed with HM Landraces (PI lines)	3	3.59	.68	2.23 to 4.88	18.41

compared to public and MFAI lines. In the commercial/private lines, higher rates of nitrogen fixation could possibly be the result of heterosis. The PI lines are derived from open-pollinated corn, which would indicate a different reason for their response to bacterial inoculants. It is likely that the landraces we are studying were developed to grow well in nitrogen-deficient tropical soils. Also, when public and MFAI lines were crossed with PI lines, their rates of nitrogen fixation increased. This evidence implies that there is some contingency on genotype with nitrogen fixation.

Samples submitted included grain and leaf samples. Due to labor constraints, we were unable to obtain analysis of root samples in 2011. In 2012, samples of the fourth leaf, immediately following pollination (R-4 stage) were harvested, dried, ground, processed and submitted to University of California-Davis for N15/N14 nitrogen fixation stable isotope analysis.

Objective 5. Evaluate the inbred shock syndrome. Previous research at Michael Fields Agricultural Institute has shown that, in an organic field, inbreds bred under conventional conditions perform poorly compared to the same lines bred under organic conditions. As plants are bred under organic conditions they may “evolve” to thrive in an organic system. As stated earlier, one hypothesis is that the balance of endophytes in seeds and plants contributes to plant performance. Evidence of this was demonstrated by lower chlorophyll scores in conventional inbreds (16% lower) in 2010. An experiment was conducted in 2010 to study the responses of conventionally-bred and organically-bred inbreds to the treatments used in the experiment outlined in objective one: 1) no seed treatment; 2) chlorox and heat to kill any fungi living in the seed; 3) a mix of bacteria consisting in class 2, non-dangerous, N fixing bacteria and fungi applied as a seed treatment (see list below); 4) seed disinfection with heat and

chlorox, with later inoculation with the microorganisms. seed was applied as a powder to seed (about 1gm per 30 seeds). Microbes used were a combination of 1)*Azospirillum brasilense*; 2)*Azospirillum lipoferum*; 3)*Azotobacter*; 4) *Burkholderia unamae*; 5)*Gluconacetobacter diazotrophicus*; 6) *Herbaspirillum seropedicae*; 7) *Paenibacillus brasiensis*; 8) *Paenibacillus durus*;9) *Glomus intraradices* (mycorrhizae).

The stable isotope analysis of samples submitted from this experiment shows higher rates of nitrogen fixation with the inbreds organically-bred at MFAI compared to the conventional inbreds. With treatment two, organic inbreds had 10% higher average rates of nitrogen fixation compared to convetional inbreds with the same treatment. With treatment three, organic inbreds had 14% higher average rates of nitrogen fixation compared to conventional inbreds with the same treatment. With the combination of bacterial inoculants and disinfection (treatment four) organic inbreds had 20% higher average rates of nitrogen fixation compared to conventional inbreds with the same treatment. This evidence reinforces the hypothesis that endogenous bacteria play a role in nitrogen fixation and plants bred under organic conditions have better nitrogen use efficeincy.

Conclusion. The results from 2009, 2010, 2011 and 2012 are promising yet inconclusive. These experiments can be expanded and improved upon. Other research has shown that one of the bacteria we have tested (*Paenibacillus durus*) is able to fix atmospheric nitrogen despite the presence of nitrate. It is important to determine the rates of fixation under varying soil conditions and crop rotations for this bacteria and others that have shown potential.

In 2009, there were treatments for two promising bacteria (*Azospirillum brasilense* and *Burkholderia unamae*) individually and in combination. According to the stable isotope analysis, these bacteria perform better individually than when they are combined. In 2010, we only had one treatment with the two bacteria combined. In the future it would be helpful to have individual treatments for them to see if the results are similar to the 2009 results. In the experiments involving different inoculants we made efforts to avoid contamination by use of the same planter. With more thorough measures to avoid contamination it is possible that trial results would be more significant.

In the future, it would be valuable to include non-fixing reference lines. It is important to have confirmed lineage that are not fixing nitrogen as references in the same experiment with potential fixing lines. Lastly, the most striking result from the stable isotope analysis is the consistently higher rates of nitrogen fixation in Plant Introduction lines. These landraces showed the most potential to fix substantial amounts of atmospheric nitrogen. These cultivars are poorly adapted to our climate and have lower yields than commercial varieties. Nevertheless, they continued to have higher rates of %Ndfa when they were crossed with regionally-adapted cultivars. Continued improvement of some of these lines could enhance their nitrogen use efficeincy. It appears that genotype plays an important role in a plant's ability to work with symbionts.

More research is necessary to understand the role biological nitrogen fixation can play in improving nitrogen use efficeincy and in reducing the use of synthetic fertilizer for corn production.