Open Agriculture. 2019; 4: 322–345 DE GRUYTER

Research Article

W. Goldstein*, A.A. Jaradat, C. Hurburgh, L.M. Pollak, and M. Goodman

Breeding maize under biodynamic-organic conditions for nutritional value and N efficiency/N_2 fixation

https://doi.org/10.1515/opag-2019-0030
received September 1, 2018; accepted February 12, 2019

Abstract: An overview is given for an ongoing maize breeding program that improves populations, inbreds, and hybrids in the Midwestern USA. Breeding and selection occurred under biodynamic conditions in Wisconsin, on an organic winter nursery in Puerto Rico, a biodynamic winter nursery in Hawaii, and a conventional winter nursery in Chile. Emphasis is on improving protein quality, carotenoid content, competitiveness with weeds, nitrogen (N) efficiency/N_2 fixation, and cross incompatibility to pollen from genetically engineered (GE) maize. Philosophy is that the plant species is a responding partner in the breeding process. Adaptation and selection emphasizes vigor and yield under N limited conditions. The *Ga1* and *Tcb1* alleles were utilized to induce cross incompatibility. The program resulted in inbreds and hybrids with increased N efficiency and protein quality coupled with softer grain texture, more chlorophyll in foliage, and densely branched root growth in the topsoil relative to conventionally bred cultivars under N limited conditions. Grain protein quality was improved by utilizing opaque kernels that emerged in populations during the course of the program in surprisingly high frequencies. N efficiency was accentuated by breeding with landraces that may fix N_2 with microbes coupled with selection for response traits under N-limited conditions. When grown next to conventional hybrids, the best hybrids from this program have exhibited 30% more methionine and 16% more protein in grain and more protein/ha.

Keywords: methionine, gametophytic incompatibility, epigenetics, isolation by environment

1 Introduction

Biodynamic and organic farming practices intend to produce products that are healthier for the public and have greater benefits for the environment. Our question has been to explore how breeding and selection under low-mineral input, biodynamic/organic conditions might increase adaptiveness as well as nutritional value, protein quality, and nutrient efficiency. This paper will outline the philosophy, methods, and results of a maize breeding program for biodynamic/organic farmers and describe results from a few pertinent experiments relating to adaptation and nitrogen efficiency that have helped to guide its efforts.

N and nutritional value: Cereals have been bred and selected for their ability to respond to mineral nitrogen (N) fertilizer (Tsai et al. 1992). However, N fertilizer causes the accumulation of cereal storage proteins that have low contents of essential amino acids thereby reducing nutritional value. Use of N fertilizer increases the nutritionally least valuable α and γ zein content of maize grain, makes kernels harder (more vitreous), and reduces digestibility (Tsai et al. 1992; Masoero et al. 2011) and overall protein nutritional quality (Tsai 1979).

Methionine and lysine are two essential amino acids that positively affect protein utilization and the growth and health of animals and people. If an essential amino acid is missing, even in part, dietary protein is transformed less efficiently into new body tissues or into animal products, such as eggs. There is a need for high methionine and lysine maize for international and domestic markets, for feeding organic poultry (Goldstein et al.. 2008; Goldstein et al. 2012), and for dairy production (Lee et al. 2012). Increasing the quality of protein in maize in developing countries, where maize consumption can at times constitute the bulk of caloric consumption, could

***Corresponding author: Walter Goldstein,** Mandaamin Institute, Elkhorn, WI, USA, E-mail: wgoldstein@mandaamin.org
A.A. Jaradat, USDA-ARS North Central Soil Conservation Research Lab, Morris, Minnesota;
C. Hurburgh, Agricultural and Biosystems Engineering, Iowa State University, Ames, Iowa;
L.M. Pollak, Windy Acres Genetics, Guthrie Center, Iowa;
M. Goodman, Crop and Soil Sciences, North Carolina State University, Raleigh, North Carolina.

have major effects on human nutrition and health.

N efficiency/fixation and microbes: Recently, the senior author reviewed the literature associated with partnerships between maize and bacteria for nitrogen efficiency and nitrogen fixation (Goldstein 2016). Nitrogen use efficiency is affected by corn/microbial ecology. Bacterial and fungal species can secrete phyto-hormones, regulate ethylene levels, defend against pathogens, help the host to obtain nutrients from the rhizosphere, improve stress resistance, and enhance root development (Schulz 2006; Baca and Elmerich 2007; Rodriguez et al. 2009). Certain landraces of maize foster N_2 fixing bacterial colonies in their tissues and in the mucilage on their brace roots (Goldstein 2016; Van Deynze et al. 2018). These bacteria fix N_2 from atmospheric gas and the N that is fixed is then taken up by the maize plant and turned into plant protein.

In some cases substantial amounts of N_2 may be fixed with the help of diazotrophic bacteria, but results depend on varieties (Goldstein 2016; Van Deynze et al. 2018). Nitrogen fixation can occur in the mucigel secreted by brace roots of the Mexican landrace Mixeno. This was first hypothesized by naturalist T.B. Hallberg (1995) and later confirmed, first by USDA-ARS (Hallberg 2016) and Mexican scientists (Vega-Segovia and Ferrera-Cerrato 1993; Gonzalez-Ramirez 1994; Gonzalez-Ramirez and Ferrera-Cerrato 1995; Martinez-Romero et al. 1997; Reis et al. 2004). Later it was confirmed and fixation was quantified using a range of methods by a team including the University of California-Davis, the University of Wisconsin, and Mexican researchers (Van Deynze et al. 2018). Typical effects of diazotrophic bacteria on crops include earlier flowering, higher N content in tissues and higher yields on N-limited soil (Khan et al. 2012).

Fusarium species, which are often the dominating endophytic fungi in maize, may play the role of a gatekeeper (Goldstein 2016), inducing N deficiency and antagonizing beneficial bacteria that can fix N_2. In the Goldstein (2016) review, evidence is presented that conventional breeding and production practices have inadvertently reinforced *Fusarium* dominance in conventionally bred maize. *Fusarium* endophytes may benefit maize plants by enhancing stress resistance and protecting them from other pathogens.

Since 1989 the senior author has bred and selected maize varieties for 45 growing seasons under low-input organic and biodynamic conditions, alternating summer nurseries in Wisconsin and winter season nurseries in Puerto Rico, Hawaii, or Chile. The program began in 1989 in response to farmer interest in improved open pollinated populations to improve nutrition for feeding animals. For the first 14 years, breeding consisted mainly in part-time breeding of open pollinated varieties. However, in 2002, listening sessions in 3 states revealed that organic farmers wanted access to non-genetically engineered maize hybrids that were adapted to conditions where weed competition is problematic and organic matter is the main provider of N. They wanted higher nutritional value (more protein, better protein quality, and more minerals, and better tasting grain). They did not want more than 10% lower yield than for conventional hybrids. In 2002, an inbred and hybrid breeding program began under the senior author's direction at Michael Fields Agricultural Institute (East Troy, WI). This program was transferred to the Mandaamin Institute (Elkhorn, WI) in 2011 when that institution was founded by the senior author. Over time, selection came to focus on high methionine content in grain, high general combining ability for yield, and nutrient efficiency under conditions where manure was either not applied or applied in low doses.

Breeding approach: The program utilizes an inbreeding-pedigree-based selection program coupled with early-generation-testing of grain yields of hybrids made with the breeding lines crossed with a parent that has a complementary heterotic pattern. Inbreeding was carried out usually for 6 to 8 generations or more to fix genetic traits. Occasionally, selected breeding lines or inbreds are recombined to make improved synthetic populations, from which inbreeding and selection occurred. Advice on field plot and breeding technique and breeding strategy were provided by cooperating corn breeders L.M. Pollak and K. Montgomery and implemented by the senior author.

The Mandaamin program is based on five inter-related working hypotheses or principles that are intended to be in consonance both with the holistic approach taken by biodynamic and organic agriculture as well as with general breeding practice, recent discoveries about the role of epigenetics in plant biology, and the actual maize breeding program. These are: 1) The important interactions that occur between the breeder, the plant, and the environment, as selection and fixation of traits occur, can be enhanced by the breeder. 2) To achieve elite performance it is important to enhance and combine both genetic and epigenetic factors that regulate whole body performance and enable fitness. 3) Breeding and selecting under low input, stressful conditions is critical both for forming and identifying the best performers for organic farms. 4) The maize plants are active players in the breeding process as they exercise something like creative biological intelligence to shape viable, dynamic, whole bodies that are more or less adapted to their environment.

5) Selection should efficiently identify the plants with the most fit, best-adapted, and most productive bodies.

If new maize inbreds and hybrids are to become commercially acceptable, they must perform well for many different criteria and many environments. Small breeding programs with limited resources for phenotyping and quantitative measurements rely on the efficiency and ability of the breeder to make correct, relevant observations and judgements during selection. This necessitated constant efforts by the senior author. He attempted to gain insight by applying qualitative breeding methods (Burbank and Hall 1939; Kraft and Kraft 1967) and adapting the phenomenological approach of J.von Goethe and Rudolf Steiner (Bockemuehl 1981; Holdrege 1996 and 2013) to the breeding process. An active, re-iterative process of observation and assessment of form, balance and general whole plant performance was exercised through the growing season in the context of different environments, to select the best adapted bodies. Observation included measurement of relevant traits, where feasible and appropriate, to assure that subjective assessments and judgements were grounded in reality.

As many of the crosses utilized in the Mandaamin Institute program were wide crosses between adapted commercial-grade inbreds and exotic landraces, changes in epigenetic regulation, genetic shifts or re-arrangements of the genome and plant-microbial relationships were expected. Attention was also paid to identify new and unexpected syntheses, patterns, and traits that emerged as a consequence of segregation, responses to stresses in previous generations or unusual crossing combinations.

2 Methods

Breeding stocks: The program benefited from breeding stocks from USDA-ARS and Iowa State University breeding programs in Ames, Iowa. It also utilized 1) cultivars identified by USDA-ARS cooperative Landrace Accession of Maize Program (LAMP) and developed by the Germplasm Enhancement of Maize (GEM) programs; 2) native US maize landraces and exotic landraces from the USDA Plant Introduction Station in Ames, Iowa; and 3) commercial, ex-Plant Variety Protection inbreds available from USDA-Plant Introduction. Earlier publications documented some of the diverse sources (Jaradat and Goldstein 2013, 2014, 2018; Jaradat et al. 2010). As the Mandaamin Institute program matured it came to mainly select from within and between its own breeding families or synthetics to develop new inbreds.

Breeding stocks for cross incompatibility. The genes *Ga1*, and *Tcb1* are found in teosinte (wild maize) and in landraces of sweet maize, field maize, and popcorn from Central America (Padilla-Garcia et al. 2012). These genes convey a trait called gametophytic incompatibility or cross incompatibility. The presence of these alleles, especially in the homozygous state, prevents pollination by maize pollen that does not possess the same alleles. Thus if maize has these genes it can avoid contamination by GE maize. The Mandaamin cross incompatible maize was bred by crossing our maize breeding lines with maize stocks possessing these traits. The *Ga1* trait was originally obtained from popcorn then transferred to white maize varieties which we obtained from Lawrence Darrah, (USDA-ARS Columbia, MO). The senior author made a stock population by intermating two of those inbreds with the trait (Mo508W x Mo506W). These are the older white inbreds K6 and H30, respectively, that have the *Ga1* allele backcrossed into them for 5 to 7 generations. In conjunction with Iowa State University and USDA-ARS (Moises Gonzales, L.M Pollak, M.P. Scott), the *Ga1* trait was backcrossed from this stock into a set of commercial-grade inbreds. The senior author then used these inbreds with the trait to backcross the trait into the different backgrounds of Mandaamin Institute's high methionine, N efficient maize while systematically testing for the active presence of the gene.

Combining *Ga1* with *Tcb1* might provide a stronger barrier than just using *Ga1*. Therefore, an accession of Maiz Dulce, a Mexican sweetcorn landrace, was used as a second source for our breeding because it combines the *Tcb1* and *Ga1* genes (Padilla-Garcia et al. 2012). This sweetcorn was crossed with Pioneer inbred PHZ51 by Major Goodman (maize breeder at North Carolina State University), and the traits were stabilized into a homozygous condition. Dr. Goodman used pollen from the stabilized version to cross with Mandaamin breeding lines or inbreds, and the senior author subsequently backcrossed or crossed those crosses into those same or related lines.

Testing grain for methionine: In conjunction with Iowa State University cooperator Charles Hurburgh, using Near Infrared Spectroscopic technology (NIRS), the inherent correlation between protein content and methionine, lysine, and cysteine content was broken for the first time (Hardy et al. 2009; Jaradat and Goldstein 2013). These three amino acids are critical for human and animal nutrition. The natural correlation was broken by constructing unique sets of maize grain calibration samples from our breeding program, containing different levels of these amino acids at variable levels of protein. Calibrations were developed for the Bruins Omega G and the Infratec 1241 spectrophotometers. Using the calibration based on spectra associated with 1319 wet chemistry analyses the senior author was able to breed productive inbreds and hybrids with more methionine in their grain and softer, more digestible kernels than conventional hybrids. Wet chemistry utilized High Performance Liquid Chromatography (HPLC, by the Chemical Testing Lab of the University of Missouri) to determine amino acid content. The correlations between NIRS and HPLC results have been highly significant on all years tested, giving a cheap and accurate method for testing for these amino acids. The NIR spectra were significantly better correlated with the spectra data for methionine and lysine than a prediction using protein alone. For methionine the R value for correlation with protein was 0.669, but with spectra was 0.788. For lysine the R value with protein was 0.39 but with spectra was 0.771. For cysteine the R value with protein was 0.702 but with spectra was 0.764. The USDA-ARS corn breeding labs of L.M. Pollak and M.P. Scott estimated the amino acids concentration of grain samples from hybrids for the breeding program utilizing the calibrations on a Foss Spectronic 1241 spectrophotometer (Agilent Co Santa Clara, California).

Sites and management. Breeding nurseries and yield trials were generally grown after grass or legume crops on nearby organic farms. The main sites, their rotations, modes of fertilization, and prevalent stressors are listed in Table 1. The program bred under low-input conditions. Weeds and low levels of available nutrients were prevalent stressors, probably associated with organic farming in practice. In early years of the program, fertilization, when it took place, consisted in application of composted cattle manure. Biodynamic preparations were applied for crops grown in Wisconsin and Hawaii. In Puerto Rico, maize followed green manure crops of macuna or crotalaria or fallow on heavy clay mollisols with an organic matter content of approximately 2%. An organic winter nursery at the University of Puerto Rico or on a biodynamic farm in Hawaii, or at a conventional nursery in Chile, also enabled Mandaamin to have two breeding seasons each year. Since 2009, the program systematically tested inbreds for

Table 1: Site information for nursery and yield trial sites used for breeding in Wisconsin or Puerto Rico

Year(s)	Farms	Location	Soils	Preceding Crop	Fertilization	Prevalent Stresses
1989-2011	Nokomis, Goldstein	East Troy, Elkhorn, WI	alfisols and mollisols, sometimes low in available N, P and K.	alfalfa, grass, rye, oats, clover.	none or CCM	Annual weeds, low available N.
2009-2017	Lajas Experimental Station	Lajas, PR	mollisols, high in clay; sufficient in P and K.	mucuna, crotalaria or fallow	none or CCM	Insects, annual weeds, poor soil structure, waterlogging, low available N.
2012	Pounder	Delavan, WI	alfisols well stocked in N, P, K.	alfalfa + grass.	CCM	annual weeds.
2012	Gravel Pit	Whitewater, WI	inceptisol	old gravel pit	none	drought, low nutrients
2013	Mitten	Eagle, WI	alfisol, low organic matter	grass	none	low available N
2012-2017	Rohrer	Spring Prairie, WI	alfisols, low available N, P, K.	rye or alfalfa + grass.	none or CCHM	annual and perennial weeds, waterlogging, drought, low available N.
2013	Little Prairie	Little Prairie, WI	shallow mollisols. Variable depth.	perennial grasses.	none	perennial weeds, drought, low available N.
2013, 2014, 2016	Goldstein	Elkhorn, WI	alfisols.	alfalfa + grass, wheat,	none	annual and perennial weeds. Drought and low available N.
2017	Creek Field	East Troy, WI	mollisols; shallow to deep.	annual cover crop mix.	none	annual weeds, waterlogging, drought and low available N.
2013-2017	Zinniker	Elkhorn, WI	alfisols	alfalfa + grass	none or CCM	annual weeds, drought, waterlogging.

CCM = composted cattle manure; CCHM = composted chicken manure.

performance on field sites that are low in available N. Soils in Wisconsin consisted mainly in alfisols (Fox, McHenry, Miami silt loams) or mollisols (Sebewa and Warsaw silt loams) with organic matter contents generally ranging between 2 to 3%. Special, N-limited breeding nurseries were either grown on loamy sandy or gravelly, sandy soils with very low organic matter content of 1% or less (Eagle, WI 2013; Whitewater, WI 2012), and on medium textured soils with a history of low fertilization input (Little Prairie 2014, 2015, Elkhorn 2014 to 2018). In some cases maize was grown under N immobilizing conditions caused by grass thatch buildup or by following maize after maize or wheat (Little Prairie, 2014, 2015; Elkhorn 2014, 2016). Between 2002 and 2015, replicated, small plot trials were carried out in Iowa, first by L.M. Pollak and later by J. Edwards to test yields of hybrids from the program on multiple sites on various mollisols on both organic and conventional farms. Information from yield trials on all sites was averaged each year to determine which breeding lines to advance.

Seeding: Plot size for nursery grown plants on all sites was one row. Each row was 5.25 meters long and 0.75 meters apart from adjacent rows. Thirty seeds were planted in each row using an Almaco cone seeder mounted on a John Deere, Max-Emerge planter. Spacing between seeds was approximately 17.5 cm. Yield trials had the same spacing but each plot had two rows.

Selection of inbreds: Seed of breeding lines was pre-selected before planting for opacity and for carotenoids by selecting for as bright orange or yellow kernels as possible. Where feasible, breeding lines were grown and evaluated on two sites in a year. Observations included emergence, early plant vigor, competitiveness with weeds, canopy cover, leaf orientation and color, height to ear, root development, pollen production, synchronicity of anthesis and silk emergence, plant integrity at harvest, grain yield, kernel moisture content at harvest, and problems with diseases, insects, and lodging. Detailed notes were taken over multiple observations. Overall ratings were assigned at harvest to synthesize whole plant performance based on the breeder's experiences with the plants and as a first indication of which lines should be bred further.

When breeding lines were grown under N-limited conditions they were evaluated in early June for emergence, color, and height. In early phases of the program, values for each of these parameters were converted into a fraction of the mean overall value, which was averaged for a given breeding line. Only lines that were above the overall mean were self-pollinated and lines with unusual disease or insect infestations were avoided. Additional selection criteria affecting choice of progeny included brace root formation and mucigel production on brace roots, root tip autolysis (Goldstein 2016), N_2-fixation (isotopic natural abundance method), chlorophyll scores of foliage using a Minolta SPAD spectrophotometer, and grain protein and methionine in grain and per ha.

In 2014, inbreds, breeding lines, and hybrids grown in the Goldstein garden on a McHenry silt loam after wheat or maize, were tested in early September for leaf chlorophyll content and ear leaf N content by A.A. Jaradat.

Testing for cross incompatibility: In the breeding process, populations were developed that segregated for the presence or absence of the relevant incompatibility genes. To determine whether a plant had the alleles in an active state, we used two methods. In the first method, we self-pollinated the plants but also used pollen from plants to cross with a 'tester' plant that we knew had the *Ga1* alleles in a homozygous state. If the plant in question had at least one allele for *Ga1* it would make a cross with the tester plant. Otherwise the tester plant would be barren.

The second method was to take pollen from a plant that had blue aleurone kernels (blue sweet maize, bred by Dan Specht, McGregor, Iowa). That pollen was applied to the silks of the plant in question first. Then, afterwards, the plant was pollinated with approximately the same amount of pollen from itself. If the resulting ear had no or less than 7 blue kernels, it was kept for further breeding. If there were no active *Ga1* or *Tcb1* alleles in the plant the plant would have many blue kernels.

Finding new mutants with high methionine content: The senior author initially utilized the floury-2 (*fl2*) allele which was obtained from the USDA Maize Genetic Stocks Center and introgressed into relevant breeding lines. This allele is a known conveyer of high methionine and lysine in maize grain. Feeding trials with hybrid seed developed with this gene by the senior author's program were shown to replace the use of synthetic methionine for feeding broilers (Levendoski and Goldstein 2006) and layers (Jacob et al. 2008). However results showed that *fl2* routinely conditioned a 10% reduction in seed size and the resulting decrease in grain yield proved untenable for farmers, so its use was discontinued. The opaque-2 (*op2*) allele was not used because it reduces methionine content in grain (Scott et al. 2004).

In order to discover mutants that had enhanced grain methionine contents without reducing grain size, grain was selected from self-pollinated ears from within our breeding families for opaque kernels utilizing a light table. To determine whether opaque kernels were associated with seed weight reductions average weight was determined by weighing all seed in the opaque and translucent categories and dividing by the number of seed. Weight difference was estimated according to the formula ((translucent weight-opaque weight)/opaque weight) x 100. Only samples which averaged greater than 2% lower grain weight were kept for grow outs.

Methionine, lysine, and cysteine content of whole grain was determined by the near infra-red spectroscopic (NIRS) calibration mentioned above (Jaradat and Goldstein 2013) by C. Hurburgh (Iowa State University Grain Quality Lab, Ames, IA) or by M.P. Scott (USDA-ARS, Ames, IA). Occasionally samples were sent to the Chemical Testing Lab of the University of Missouri for determining amino acid composition with HPLC.

Yield testing: Crosses for hybrid yield trials were generally made starting at the S2 or S3 stage of inbreeding by crossing breeding lines with inbred testers from an opposite heterotic group. Between 2011 and 2013, replicated yield trials of 100 to 1000 hybrid entries took place on two to four organic sites in Iowa (in conjunction with Dr. Jode Edwards, USDA-ARS) and one site in Wisconsin. The best 10 to 25 hybrid selections from a testing cycle were submitted to yield trials in comparison with commercial hybrids and hybrids from other breeding programs managed by the U.S. Testing Network (USTN, Ames, Iowa). USTN trials took place in three growing zones (100, 105 and 112 day relative maturity) on organic sites in New York, Pennsylvania, Ohio, Indiana, Illinois, Iowa, Wisconsin, and Nebraska.

Between 2014 and 2017, replicated yield trials of up to 1000 hybrid entries took place on the Zinniker, Rohrer, Goldstein farms, located near Elkhorn, Wisconsin. The Zinniker Farm was considered to be a high fertility site as maize followed alfalfa on a medium textured soil with a high level of fertilization (estimated at 80-120 tons of cattle manure/hectare). On the Goldstein Farm maize followed alfalfa + grass or maize. The Rohrer Farm yield trials were regarded as nutrient limited, based on results with soil testing and observation of plant growth. Maize generally followed rye with a low (4.4-6.6 tons/hectare) level of fertilization with composted chicken manure.

Where there was sufficient hybrid seed, replicated plots were grown on both high and low fertility sites (2016). The maximum number of replicates on each site was 3. Plot yields and grain moisture content were determined at harvest with a Gleaner K2 combine modified to weigh plot yields and with a portable moisture meter. At harvest a grain sample was taken from each plot and protein, oil, methionine, lysine, and cysteine content of whole grain were determined by NIRS.

Grain and protein yield, methionine and lysine content, and moisture data obtained from these trials were combined with information from per se testing of the lines, to determine which breeding lines to advance. The selection pressure generally ranged from 2 to 15% depending on the results. Only inbreds that produced hybrids with competitive yields to conventional hybrids were advanced.

Roots: In 2017 root monoliths were dug on various Mandaamin inbreds and conventional inbreds to examine to what extent differences in growth and N efficiency might relate to different patterns of root growth. Two sites were sampled, the Creek Field in East Troy, WI and the Rohrer farm in Elkhorn, WI. On both sites maize followed non-leguminous cover crops. Water logged conditions led to symptoms of N deficiency on both nurseries during early phases of growth. Monoliths were dug with a shovel at a radius of 20 cm from the crown of 3 plants of a chosen plot and to a depth of 20 cm. Roots were shaken free of large quantities of roots, washed with a pressure hose to expose root morphology, then photographed.

Conventional inbreds tested were from three companies and were highly inbred. Four N efficient Mandaamin inbreds were focused on. Their families were C4-6, C2B2, NG10-2-3-2, and LAT-7. The C4-6 and C2B2 were both derived from the 'C' family. The former had been self pollinated 8 generations; the latter 6 generations, then sib mated and bulked. The NG10-2-3-2 was an inbred derivative from a synthetic composed of different S6 lines of LMPNG28 (Jaradat and Goldstein, 2013). Plants from the synthetic were subsequently inbred for seven generations and the outcome was then sib mated and bulked. The LAT-7 pedigree is 25% Mixeno landrace (derived from USDA, Plant Introduction accession Ames 19897) and 75% Corn Belt dent inbreds. It was self pollinated for 4 generations.

N_2 fixation. Initially an inoculate was utilized that consisted of *Azospirillum* species provided by the TerraMax company (Minneapolis, MN). Then, starting in 2010, in conjunction with TerraMax, the senior author and TerraMax developed and tested an inoculate consisting of a unique consortium of 6 to 8 N_2 fixing bacteria, isolated mainly from maize or its rhizosphere, that inhabit different parts of the plant (around the roots, on the roots, inside the plant). These strains were selected based on their efficacy per se and in combinations on positively affecting protein uptake by putative N efficient maize cultivars in the field. Seed for our nurseries was inoculated with this mixture of diazotrophic bacteria on many but not all sites.

Microbial relationships: In Wisconsin in 2008 an initial experiment was directed by the senior author under organic conditions to test whether inoculating or not inoculating seed with commercial strains of *Azospirillum* *species* (rhizosphere/rhizoplane inhabiting diazotrophic bacteria) might result in higher grain protein content in grain for several maize cultivars grown without soil fertilization. It did. This stimulated research on how microbial relationships might be affecting N efficiency. N efficiency/fixation might be improved by 1) breeding with landraces that foster bacterial relationships, and 2) by shifting the maize/microbial balance both in the rhizosphere and inside the plant to favor beneficial bacteria rather than *Fusarium*. The senior author directed different experiments to test these two hypotheses, focusing first on the 'culture shock' phenomenon and then on putative N_2 fixing maize landraces.

Testing culture shock: Some conventional inbred cultivars perform poorly (e.g., they display yellow-green leaves and are relatively stunted) the first year they are grown under organic conditions, but when they are multiplied a second or more years under organic conditions their color and vigor improves. This 'inbred culture-shock syndrome' (Goldstein et al. 2012) might be due to accidental, undetected outcrosses, epigenetic changes, and shifts in the endophytic balance away from *Fusarium* domination towards beneficial or even diazotrophic bacteria.

To test this, in 2010, eight inbred or partly inbred cultivars that had been grown and selected under organic or conventional conditions were compared by the senior author on a Sebewa, silt loam soil near East Troy, Wisconsin. The experiment was grown after four years of an alfalfa + grass mixture and was not regarded as being N limited. The cultivars and their USDA Plant Introduction numbers were M632 (PI 648424), GEMS 2 (PI632413), GEMS 29 (PI 639054), GEMN 111 (PI 651538), LH123Ht (PI 601079), PHB47 (PI 601009), PHK42 (PI601495), and PHK76 (PI 601496). The latter four were conventional commercial inbreds which either had been produced multiple generations on an organic farm or were the original conventionally produced inbreds. The GEM lines were derived from crosses between exotic maize varieties and Corn Belt Dent inbreds. When we obtained them from USDA-ARS, they were conventionally grown and bred S3 lines. They were either further bred either under organic conditions by the senior author or under conventional conditions by USDA-ARS. M632 was the result of backcrossing the *dzr1* allele into A632 (BC5S4 generation) at the University of Minnesota. The resulting lines were grown side by side in 2010 with 3 replications on an organic field without fertilizer. The experiment was a split-split design with varieties being the main plot factor, source of varieties being the subplot factor, and plus or minus bacterial inoculation and plus or minus seed disinfection being sub-subplot treatments. Seed was disinfected with and without heat + clorox to disinfect *Fusarium* (Yates et al. 1997) and then either inoculated or not inoculated with the mixture of diazotrophic (N fixing) microorganisms. Due to short supplies of seed, sub-subplots were single rows 5.25 meters long and 0.75 meters wide, and 30 kernels were seeded in each row at the end of May, 2010. Chlorophyll content of leaves was analyzed with a Minolta Spad meter in on September 6-8th to assess N status of plants. Analysis of variance was carried out with JMP software (SAS, Cary, NC) by A.A. Jaradat.

Testing N_2 fixation and landraces. In 2009 the senior author grew cultivars with *Azospirillum* inoculum but under N limiting conditions on an organically managed field (Sebewa silt loam) that had been in cereal crops or non-leguminous cover crops and had not been fertilized for at least 4 years (Goldstein et al. 2012). Cultivars tested included 1) 15 accessions that were mainly either older, ex-commercial inbreds that had expired Plant Variety Protection registration or S1 to S3 generations of crosses between them, 2) 23 new commercial advanced breeding lines/inbreds from a cooperating seed company mostly at the S5 to S7 level of inbreeding, 3) 26 breeding lines from the MFAI organic breeding program generally derived from populations at variable levels of inbreeding (S1 to S3) and 4) 13 exotic landraces obtained from USDA Plant Introduction and selected because of their unusually high methionine and lysine content. Groups 1 and 2 had been previously selected under conventional management. Group 3 was in development mainly from populations grown for multiple years under organic management. Group 4 was a set of landraces chosen for their high grain methionine contents; none of which were the landrace Mixeno. Plots were single rows 5.25 meters long and 0.75 meters wide with 30 kernels. Plants in Group 4 were from southern latitudes and assumed to be day-length sensitive affecting days to flower. In order to synchronize their flowering dates with the other cultivars they were covered with a light-blocking tarp in the evening and morning to give a 12 hour day length period for 2 weeks after emergence.

Testing initial topcross hybrids with N efficient/fixing landraces: In 2009 a set of mostly Mexican exotic landraces was identified from the previous study that appeared to be fixing N_2 and they were crossed with inbreds. To test relationships between *Fusarium sp.*, maize varieties, and diazotrophic bacteria for these hybrids and for other cultivars, the senior author carried out randomized, complete block, replicated field trials in Wisconsin in 2010. These trials took place on a Sebewa silt loam soil that was not fertilized and had not been fertilized for several years.

Maize followed a crop of soybeans. In preceding years the field had been used mainly for cereal production with rye cover crops and no manuring.

The four factorialized seed treatments were with and without heat + clorox to disinfect *Fusarium* (Yates et al. 1997) and then that same seed treated with and without inoculation with the mixture of diazotrophic (N fixing) microorganisms. Main plot factor was hybrids, subplot treatments were with and without inoculation and sub-subplot treatments were with or without heat treatment. Sub-subplots were single rows 5.25 meters long and 0.75 meters wide with 30 kernels.

Seed of four commercial organic hybrids were utilized as checks for the experiment. They were obtained from Blue River Hybrids (Ames, Iowa) and Albert Lea Seed House (Albert Lea, MN) for experimental purposes. These were TR7245 x TR6331; FR3916 x FR6943; 0.574; and 0.59-06. A set of four putative N efficient hybrids made by crossing Mexican landraces with Corn Belt cultivars (PDF x LR15, B73 x LR15; NG x LR15; and PHK76 x LR8) that produced plants with similar flowering times to the commercial hybrids were identified in the field in the context of the randomized experiment. Both sets of hybrids were harvested, and analyzed for yield, protein production, and grain quality.

Analysis of variance was carried out by A.A. Jaradat with JMP software (SAS, Cary, NC) and differences in LS means were determined with Tukey HSD procedure with $\alpha = 0.05$ and Q = 2.62 and 3.11 for treatment and type of hybrid x treatment differences respectively. Orthogonal contrasts with single degrees of freedom were also used to test for differences due to disinfection or inoculation treatments for and between the conventional or putative N efficient hybrids.

Fixing the trait for N efficiency/N fixation: Inbreeding was carried out on the sites and using the methods listed above under the section 'Selection for N efficiency/N fixation'. After fixation of trait was apparently achieved the plants were backcrossed into relevant high methionine breeding lines and inbreds, some of which had the two allele cross incompatibility trait.

Testing cross incompatibility of inbred lines: In 2014 the senior author tested the ability of putative *Ga1* homozygous plants to avoid pollination by exercising cross incompatibility. Thirty nine inbreds, 5 hybrids, and 1 commercial homozygous *Ga*1 hybrid were tested in un-replicated plots in an isolation alternating 4 rows of the putative cross incompatible varieties with 2 rows of a normal *ga1* homozygous tester with a broad range in flowering dates. The tassels of the *Ga1* lines were removed before pollination and at harvest ear and kernel set on those lines was examined.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results

The emergence of opaque, high methionine kernels: By 2005 NIRS methods for detecting methionine in seed in an affordable way had been developed. The senior author had come to the realization that *fl2* hybrids produced insufficient yields due to their low seed weight (see testing grain for methionine and finding new mutants with high methionine content sections above). In the summer of 2006 opaque kernels were found in breeding families FS8AS.SS and AR21B (Jaradat and Goldstein 2013) after seed of these families had been advanced on a Sebewa silt loam which had previously been in an alfalfa + grass mixture. This unexpected result caused the senior author to retrospectively examine the texture of the initial seed of the sets of breeding lines from 2005. For the FS8AS.SS family, there were the following results: In 2005 an average of 10 ears for each of 33 sub-families were grown and sib pollinated. The ears from each family were bulked and there was an average of 0.3 opaque kernels per subfamily. A random sample of seed from each of those subfamilies was planted in 2006 without selection for opaqueness. An average of 6 ears from each family were self-pollinated in 2006 based on agronomic performance of the plants. The average number of opaque kernels found in each ear of those plants was 16. The average maximum amount of opaque kernels found amongst ears within a subfamily was 49. The average total number of opaque kernels found from each subfamily was 90.

Similar results were obtained with the large AR21B breeding family. In 2005 we grew and sib pollinated an average of 15 ears for each of 24 subfamilies. The ears from each family were bulked and there was an average of 5 opaque kernels per subfamily. This was due to one outlier subfamily with 89 opaque kernels; the other families averaged 0.7 opaque kernels. A random sample of seed from each of those subfamilies was planted in 2006 without selection for opaqueness. We self-pollinated an average of 9 ears from each family in 2006 based on agronomic performance of the plants. The average number of opaque kernels found in each ear of those plants was 27. The average maximum amount of opaque kernels found amongst ears within a subfamily was 89. The average total number of opaque kernels found from each subfamily was 231. Without the outlier subfamily the total number was 175.

The opaque trait was associated with higher methionine content (Figure 1).The trait emerged and persisted in a variety of inbreds and breeding families in subsequent years and by 2008 we had identified opaque kernels in a wide set of 13 breeding families. These were mostly derived from GEM crosses of normal, translucent-kernelled landraces with normal commercial inbreds (Jaradat and Goldstein 2013 and 2014). These kernels had little seed weight reduction and had high nutritional quality with strong positive associations to several trace elements (Jaradat and Goldstein 2013, 2014, 2018). In 2008 the senior author measured weight of opaque and normal translucent kernels from 410 ears segregating for the trait from 12 different breeding families. The average weight difference was 1.32% in favor of the translucent seed.

The opaque trait varied in its expression, stability, heritability and effect on increasing methionine and lysine in the grain. In many cases it proved mostly to be heritable and stable, but in the AR21B family the stability was variable according to subfamily (see Figure 2). Crosses of the most promising lines with homozygous lines showed the trait was not allelic to *fl2* but appeared to be allelic to *fl1*. Subsequently, there has been continued spontaneous occurrence of opaque kernels in populations, breeding lines, and in inbreds. In 2008 the senior author plotted the frequency of ears with different percentages of opaque kernels on the ear for 14 different breeding families or subfamilies. There was no indication that the trait segregated in any kind of clear Mendelian pattern. The trait often has proven to be fixable and stable when plants were grown under organic conditions, and had full expression in many of the backgrounds it has appeared in, but there has been variation for appearance and methionine content. Opaqueness has also appeared to emerge as piebald or translucent sectors and fully opaque kernels in inbred breeding lines derived by selfing lines from commercial inbreds LH123Ht (Figure 2, right), PHK42 and Mandaamin inbred FN. In some cases this has been associated with apparent loss of orange endosperm (see Figure 2, right).

The senior author utilized these opaque kernels for breeding inbreds with grain that was high in methionine and lysine. In 2011 wet chemistry (HPLC) analysis was done by the University of Missouri Chemical Testing Lab on open pollinated grain from six of our new opaque kernelled hybrids. They averaged 10.9% protein, 0.26% methionine, 0.22% cysteine, 0.35% lysine. Three conventional hybrids grown in the same randomized experiment averaged 8.1% protein, 0.17% methionine, 0.17% cysteine, and 0.31% lysine.

Large differences in grain quality for our hybrids relative to conventional hybrids confirming these results are shown in Table 2 for different experiments in 2015. Furthermore, between 2011 and 2016, M.P. Scott analyzed 1,250 grain samples of the Mandaamin Institute hybrids with NIRS in comparison to 149 samples from hybrid controls grown in the context of 9 replicated yield trials

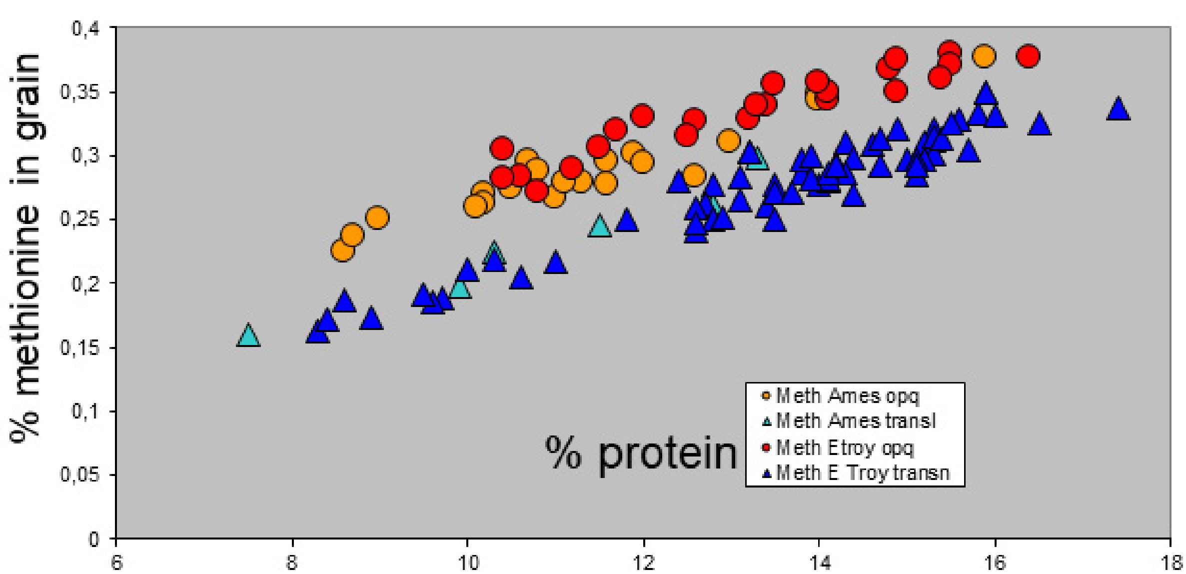

Figure 1: Relationship between protein and methionine content of normal translucent and opaque NG family lines grown in Ames, IA and E. Troy, WI in 2008

Figure 2: Picture on the left shows ears of breeding line AR21B-1-7-7-7-2-3-2 still unstable for the opaque trait after 6 generations of self-pollination. The picture on the right shows piebald opaque kernels from an inbred line derived from selfing the commercial inbred LH123Ht

Table 2: Quality results from experiments comparing hybrids made by crossing Mandaamin x Mandaamin inbred, Mandaamin x conventional inbreds, and conventional x conventional inbreds in 2015

Top hybrid trial Rohrer Farm, Elkhorn, Wisconsin, 2015.

		Protein		Oil		Lysine		Methionine	
	no entries	average	std dev	average	std dev	average	std dev	average	std dev
					% of total dry matter				
Mand x Mand	120	9,85	0,88	4,89	0,37	0,37	0,025	0,272	0,019
Mand x Conv	21	9,01	0,55	4,65	0,41	0,353	0,026	0,244	0,016
Conv x Conv	12	8,32	0,76	4,08	0,32	0,309	0,015	0,219	0,013
% diff. Mand vs. Conv		16,5		19,9		19,7		24,3	

N-efficent hybrid trial Zinniker Farm, Elkhorn, Wisconsin, 2015.

		Protein		Oil		Lysine		Methionine	
	no entries	average	std dev	average	std dev	average	std dev	average	std dev
					% of total dry matter				
Mand x Mand	90	10,93	0,79	5,02	0,24	0,373	0,023	0,287	0,020
Conv x Conv	7	9,51	1,12	4,47	0,17	0,313	0,012	0,229	0,021
% diff. Mand vs. Conv		15		12,5		19,2		25,4	

New hybrid trial Zinniker Farm, Elkhorn, Wisconsin, 2015.

		Protein		Oil		Lysine		Methionine	
	no entries	average	std dev	average	std dev	average	std dev	average	std dev
					% of total dry matter				
Mand x Mand	78	11,13	0,84	4,99	0,27	0,375	0,024	0,283	0,020
Mand x Conv	6	10,63	0,50	4,85	0,17	0,354	0,010	0,263	0,013
Conv x Conv	6	8,91	1,13	4,49	0,27	0,312	0,028	0,208	0,020
% diff. Mand vs. Conv		24,9		11,1		20,2		36,1	

on organic farms. On average the Mandaamin Institute hybrids had grain with 0.274% methionine while conventional had 0.210%. This is a 30% difference. On average our hybrids had 10% protein while conventional had 8.6% protein for a 16% difference. The percent methionine in the protein ranged from 2.14% to 3.14% for the different years and sites. Methionine in protein decreases as protein content increases. But regression showed that at the same protein level the Mandaamin grain had approximately 0.56% or 22% more methionine in its protein than did the conventional maize. This is critical for formulating protein efficient feed.

Cross Incompatibility: The putative cross incompatible lines generally did not allow themselves to be pollinated by maize possessing the *ga* allele. Of the cultivars tested, 35 showed no pollinations, 6 had a few kernels that were pollinated in the ear, and 4 had occasional whole ears that were pollinated.

Culture Shock: Plants from seed that had been grown or bred under organic conditions were earlier flowering and showed greater early vigor (Figure 3). Cultivars differed significantly for their chlorophyll scores ($P < 0.001$). Figure 4 shows that inbreds from the organic/biodynamic source had consistently higher chlorophyll content of leaves in early September during grain fill with an average positive increase of 16% relative to the conventionally grown/bred lines (source effect significant at $P<0.0001$). There no significant effects of disinfection or inoculation with bacteria. Box and whisker graphs in Figure 4 illustrate modes, and data ranges and dispersion associated with different cultivars from different sources. In 7 out of 8 cases the distribution boxes for the organic/biodynamic seed were smaller than for the corresponding conventionally bred and grown cultivar. In 6 out of 8 cultivars the standard error values were also lower for the organic/biodynamic variant. The respective standard error values for the conventional and organic/biodynamic were 0.59 and 0.79 for GEMN111; 1.48 and 1.74 for GEMS2; 1.54 and 1.28 for GEMS29; 2.13 and 1.72 for LH123Ht; 1.64 and 1.54 for M632; 1.59 and 0.89 for PHB47; 2.72 and 1.55 for PHK42; and 1.27 and 0.44 for PHK76.

N_2 fixation and landraces. In 2009 the senior author noted differences between cultivars that were inoculated with *Azospirillum* strains but grown under N limiting conditions on an organically managed field (Sebewa silt loam) that had not been fertilized but had been in cereals or non-leguminous cover crops for at least 4 years (Figure 5; Table 3). Leaf chlorophyll scores averaged 37 for the older commercial inbreds or recent breeding lines derived by crossing them, 40 for the new commercial breeding lines which were close to being finished inbreds, 45 for the early MFAI breeding lines derived from organically managed populations, and 54 for the exotic landraces. The percent of lines within each group with scores of 50 or over (showing N sufficiency) were 8% for the older commercially derived lines and the newer commercial lines/inbreds, 34% for the MFAI breeding lines, and 65% for the exotic landraces Multiple races from Mexico and South America produced dark green leaves with levels of chlorophyll scores in the high 50's and low 60's that suggested that they had been heavily fertilized. Inspection of several of these varieties relative to the check B73 inbred suggested that these plants actually had invested less in root dry matter than side by side conventional lines, so difference could not be explained by having larger, more extractive roots (Figure 5).

Figure 3: Picture at the top shows M632 developed in our program (MF) relative to the original USDA accession. Picture at the bottom shows GEMS 29 developed in our program (MF) relative to the original USDA accession. Pictures were taken in July. Plus B indicates seed was inoculated with a mixture of diazotrophic bacteria

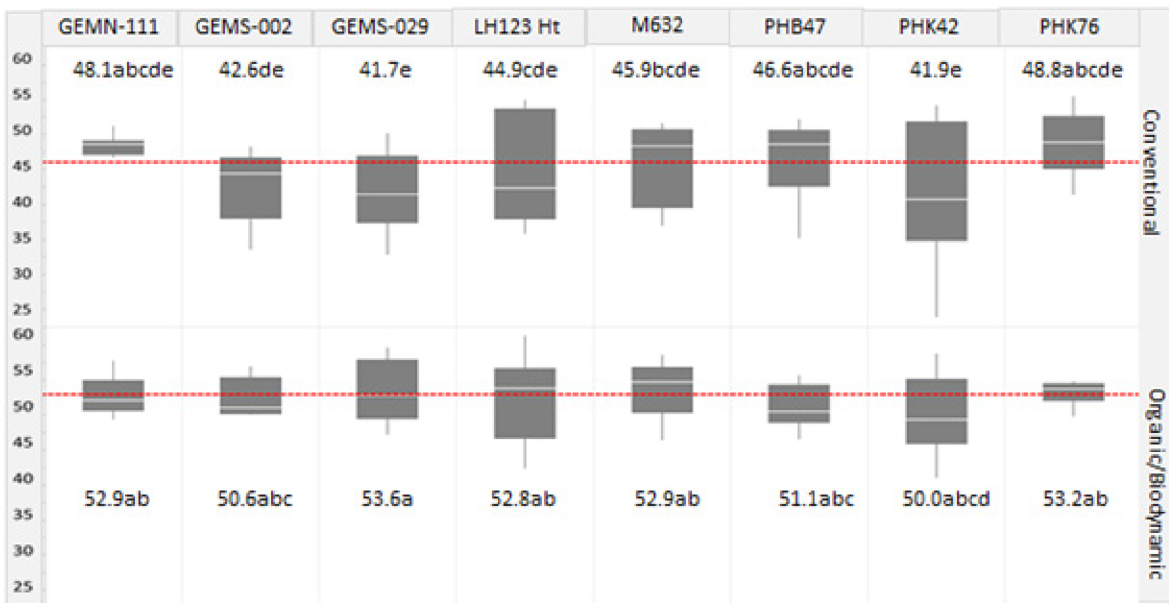

Figure 4: Spad meter chlorophyll scores presented in the form of box and whisker graphs. Data is for inbreds and breeding lines grown and selected under conventional and organic/biodynamic programs. The boxes comprise the first, second, and third quartiles of the data distribution and show the mode values. Whisker lines above and below the boxes indicate the range in the data. Red lines indicates the overall mean value for the conventional source cultivars (45.1) and for the organic/biodynamic source (52.1). LS means and letters indicating significance of differences at P = 5% are next to their respective boxes

Figure 5: Conventionally bred inbred B73 on the far left and accessions of two Mexican landraces (LR 36 and LR8) on the right with similar flowering times. Plants were grown on a N-limited site near Elkhorn Wisconsin in September, 2009. The chlorophyll score for B73 was 43 and the dry root weight per plant was 130 grams. The chlorophyll scores for the two Mexican accessions were 56 and 59 and the root weights per plant were 57 and 100 grams of dry matter, respectively

Utilizing the natural abundance method (Boddey et al. 2001) the decreased δ [15]N signatures on N from grain samples indicated that some of the dark leafed landrace cultivars might have fixed up to half of their nitrogen from the air. On the other hand, isotope results also suggest longer-term selection of breeding lines under biodynamic/organic conditions increased in δ [15]N isotope ratio in the grain and tops, which may indicate greater mineralization of N from soil organic matter.

Testing initial topcrosses with N efficient/fixing landraces:: The senior author tested four conventional hybrids and four hybrids made between N-efficient Mexican landraces identified in 2009 and Corn Belt Dent inbreds. The two landraces used in crosses for the comparison had the lowest δ [15]N ratios in the 2009 trials (LR8 and LR15 with ratios of 3.8 and 3.21, respectively).

Hybrid type and treatment effects are shown in Tables 4 and 5. On average, the conventional hybrids had 23% lower plant population density than the N efficient hybrids. They averaged 9% less grain and 28% lower protein and valuable amino acids (lysine + methionine + cysteine)/ha. The conventional grains also had 18% less lysine, 29% less methionine, and 17% less cysteine on a per se basis than the N efficient, and there was 10% lower methionine in the protein than for the N efficient grain.

The differences between N efficient and conventional hybrids might be partly explained by differences in plant density. So grain yields were re-calculated using plant population density as a covariate. However, that adjustment had little effect on LS means or treatment differences (Table 4).

Disinfection to remove *Fusarium* had major effects on the conventional hybrids (Tables 4 and 5). Where no disinfection occurred there were no differences in plant density or grain yield between the conventional and N efficient hybrids (Table 5, fifth contrast). However,

Table 3: Data for chlorophyll scores for leaves and N isotope distribution in grain for maize grown in 2009 on an N limited site near Elkhorn, Wisconsi

	Overall results			Results from Isotope study		
Cultivar	**no. of cultivars**	**ave. chlorophyll score**	**ave. % of plants with score > 50**	**no of cultivars**	**range in delta 15N**	**highest % NDA**
Old commercial inbreds	15	37	8	15	3.9 to 5.4	27
New commercial inbreds	23	40	8	9	3.9 to 5.8	32
Our breeding lines and inbreds	26	45	34	15	4.6 to 6.1	35
Landraces	13	54	65	10	3.2 to 6.1	48

Table 4. Effects of types of hybrids and seed treatments on agronomic and grain characteristics.

	Population	raw grain yield	pop adj grain yld	protein/ha	Met Lys Cys/ha	Protein	Met in Protein	lysine	methionine	cysteine
						Significance level P				
Type of hybrid	<0.0001	0,125	0,145	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Treatment	<0.0001	0,001	0,001	0,001	0,001	0,063	0,300	0,378	0,120	0,031
Type x Treatment	<0.0001	0.177	0.169	0.054	0.112	0.035	0.046	0.704	0.297	0.121
						LS Means				
	plants/ha	**kg/ha**	**kg/ha**	**kg**	**kg**	**%**	**%**	**%**	**%**	**%**
Conv	45,847b	7,202a	7,353a	463b	39b	7.49b	2.40b	0.283b	0.179b	0.170b
N effic.	59,655a	7,952a	8,077a	643a	54a	9.53a	2.66a	0.343a	0.253a	0.204a
D	47,023b	6,234c	6,341c	445b	38b	8.26a	2.55a	0.308a	0.224a	0.184a
D + I	49,190b	7,019bc	7,153bc	517ab	44ab	8.42a	2.54a	0.312a	0.215a	0.186a
nothing	58,000a	8.120ab	8,274ab	611a	50a	8.99a	2.49a	0.311a	0.214a	0.193a
I	56,796a	8,933a	9,092a	640a	54a	8.36a	2.54a	0.321a	0.212a	0.185a
1) Conv.	58,640a	8,607ab	8,795ab	607a	49ab	8.49ab	2.34b	0.296b	0.196b	0.184bc
2) Conv. + D	33,746b	5,468c	5,574c	334b	29c	7.12c	2.43b	0.277b	0.173b	0.165d
3) Conv. + I	54,030a	8,623ab	8,804ab	537ab	46ab	7.24bc	2.37b	0.280b	0.172b	0.166d
4) Conv. + D + I	36,973b	6,111bc	6,238bc	374b	33bc	7.09c	2.46b	0.278b	0.174b	0.165d
5) N effic.	57,350a	7,635abc	7,754abc	615a	52a	9.49a	2.65a	0.346a	0.251a	0.202ab
6) N effic. + D	60,300a	7,000abc	7,108abc	554ab	47abc	9.40a	2.67a	0.339a	0.251a	0.203ab
7) N effic. + I	59,562a	9,244a	9,380a	743a	63a	9.48a	2.71a	0.342a	0.257a	0.205a
8) N effic. + D + I	61,407a	7,928abc	8,067abc	659a	55a	9.74a	2.62a	0.346a	0.255a	0.206a

D = disinfection; I = inoculation. Conv = average of 4 conventional hybrids. N effic. = average of 4 N efficient hybrids.

without disinfection, the N efficient plants still had 17% more protein, 12% more methionine in protein, 16% more lysine, 28% more methionine, and 14% more cysteine in their grain than the conventional hybrids (differences all significant at P < 0.0001). That resulted in 16% higher protein yields and 17% higher yields of the valuable amino acids/ha for the N efficient hybrids.

Disinfecting the seed of conventional hybrids (see first contrast, Table 5) directly decreased plant stands by 37%, grain yields by 33%, protein yield by 38%, and yield of valuable amino acids by 35% (differences significant at P < 0.0001). Protein, lysine, methionine, and cysteine content in the grain of conventional hybrids was decreased 4-6% relative to no disinfection.

On the other hand, disinfection had no statistically significant effect on the N efficient hybrids (see second contrast, Table 5). Though there were 11-12% decreases in grain, protein, and amino acid yields/ha associated with disinfection, those differences were not significant (P ranged from 0.23 to 0.26).

Inoculation with diazotrophic bacteria had no effect on the yields of grain or grain components for conventional hybrids (see Table 5, third contrast). But inoculating conventional hybrids depressed the grain contents of protein, lysine, and cysteine by 3 to 8% (P ranged from 0.08 to 0.10).

All in all, the highest levels for all parameters were found in plots where N efficient hybrids were inoculated with diazotrophic bacteria (Table 4). Inoculating the seed of N efficient hybrids increased yields of protein and valuable amino acids by 19 to 20%/ha (P = 0.07) (see Table 5, fourth contrast). Grain yields were also 11% higher with inoculation (P = 11%). Grain components were not significantly affected.

Table 5. Orthogonal contrasts for disinfection or inoculation treatments for conventional and N efficient hybrids including magnitude of response and level of significance.

	Population	raw grain yield	pop adj grain yld	protein/ha	Met Lys Cys/ha	Protein	Met in Protein	lysine	methionine	cysteine
Effect of disinfection on Conv. (2&4) vs. (1&2)										
magnitude in %	(37)	(33)	(33)	(38)	(35)	(10)	4	(4)	(6)	(6)
P level for t test	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0,04	0,005	0,134	0,076	0,072
Effect of disinfection on N effic. (6&8) vs. (5&7)										
magnitude in %	4	(12)	(11)	(11)	(11)	1	(1)	(0)	(0)	0
P level for t test	0,327	0,229	0,229	0,265	0,221	0,729	0,359	0,875	0,879	0,811
Effect of Inoculation on Conv. (3&4) vs. 1&2).										
magnitude in %	(1)	5	5	(3)	0	(8)	1	(3)	(6)	(5)
P level for t test	0,875	0,640	0,639	0,775	0,967	0,083	0,359	0,083	0,215	0,099
Effect of inoculation on N effic. (7&8) vs (5&6).										
magnitude in %	3	17	17	20	19	2	0	0	2	1
P level for t test	0,499	0,111	0,110	0,068	0,072	0,488	0,856	0,473	0,872	0,320
Contrast of N effic. vs Conv. without disinfection (5&7) vs. (1&2).										
magnitude in %	4	(2)	(3)	16	17	17	12	16	28	14
P level for t test	0,376	0,809	0,753	0,062	0,035	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Contrast of Neff vs. Conv. without Inoculation (5&6) vs. (1&2).										
magnitude in %	21	4	3	20	21	17	10	16	26	14
P level for t test	0,002	0,726	0,762	0,065	0,03	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Contrast of N eff vs Conv with inoculation (7&8) vs. (3&4).										
magnitude in %	25	14	14	35	33	25	9	19	32	19
P level for t test	< 0.0001	0,081	0,090	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Numbers desribing contrasts that are clustered with & in parenthesis (1&2 etc.) refer to treatments listed in the type x treatment section of Table 4. Numbers describing magnitude that are in parenthesis are negative.

The pronounced effect of inoculation on N efficient hybrids became clear when comparing results between N efficient and conventional hybrids with and without seed inoculation (sixth and seventh contrasts in Table 5). Differences between the N efficient and conventional hybrids were clearly apparent where no inoculant was applied. N efficient hybrids had 21% denser stands (P =0.002), 20 % higher protein yields/ha (P = 0.06) and 21% higher valuable amino acid yields/ha (P = 0.03). Protein, methionine in protein, and lysine, methionine, and cysteine contents in grain were higher by 17, 10, 16, 26, and 14%, respectively (P < 0.0001).

However, the differences between these two types of hybrids were even larger for the contrast where seed had been inoculated. N efficient hybrids had 25% denser stands (P < 0.0001), 14% greater grain yield (P= 0.08), 35% higher protein yield (P < 0.0001); and 33% higher yield of valuable amino acids/ha (P < 0.0001). Protein, methionine in protein, and lysine, methionine, and cysteine in grain from the N efficient hybrids were higher by 25, 9, 19, 32, and 19%, respectively (P < 0.0001).

Breeding for N efficiency/N fixation. On the Metea loamy sand site nursery in 2013 there was variation in growth and plant color indicating variable levels of N stress (Figure 6). At harvest there was widespread bareness (fewer pollinated ears) due to N deficiency stress (Table 6) and large differences in ear size (Figure 7). The two conventionally bred check populations averaged 77% barren plants. In contrast the top 20% of N efficient breeding lines had 10 to 40% barren plants. These results do not prove N fixation but suggest some kind of N efficiency.

Both an early color score and the early breeders rating clearly and significantly (P > 0.1%) to 5%) distinguished plants that turned out to have more total ears and ears/plant. This was confirmed by partial least squares analysis of ears/plant by A.A. Jaradat.

In 2014, breeding lines that were grown on a McHenry silt loam without fertilization following either winter wheat or maize production showed symptoms of N deficiency on some of the breeding lines, but not all. Chlorophyll scores and N content of ear leaves taken in September showed that several inbred lines maintained high N content in ear leaves and relatively high chlorophyll scores under those conditions (see Figure 8).

In the breeding lines and hybrids derived from putative N_2 fixing landraces the senior author observed mucigel formation on brace roots and what appeared to be sequential self-digestion of the tip zone of the brace roots that produced the mucigel when roots appeared to reach a late stage of development. See Figure 9 and numerous photos in Goldstein 2016 for documentation of the phenomena in breeding lines derived from multiple Mexican landraces that appeared to be N efficient. This included but was not limited to the Mixeno landrace described by various authors (Goldstein 2016; Van Deyze et al. 2018).

By 2015 it appeared that we had fixed what we believe to be the N_2 fixation trait into an inbred called C4-6 that had very dark green foliage when grown under

Figure 6: Differences in seedling growth for different breeding lines grown on a low organic matter, loamy sand soil in 2013 near Eagle, Wisconsin

Figure 7: Differences in ear size for check population CG-SS at top and for population ‘C’at bottom when grown on a low organic matter, loamy sand soil in 2013 near Eagle, Wisconsin

Table 6: Comparison of ear production by top 30 breeding lines and 3 normal Corn Belt Dent checks when grown on a Metea sandy loam without fertilization near Eagle, Wisconsin in 2013

Cultivar	no breeding lines	plants/plot	total ears/plot	% of plants making an ear
normal Corn Belt dent populations	3	28	5	19
North American native x elite inbred populations	3	29	22	77
Mexican native x elite inbred populations	14	27	20	75
South American native x elite inbred populations	13	28	22	79

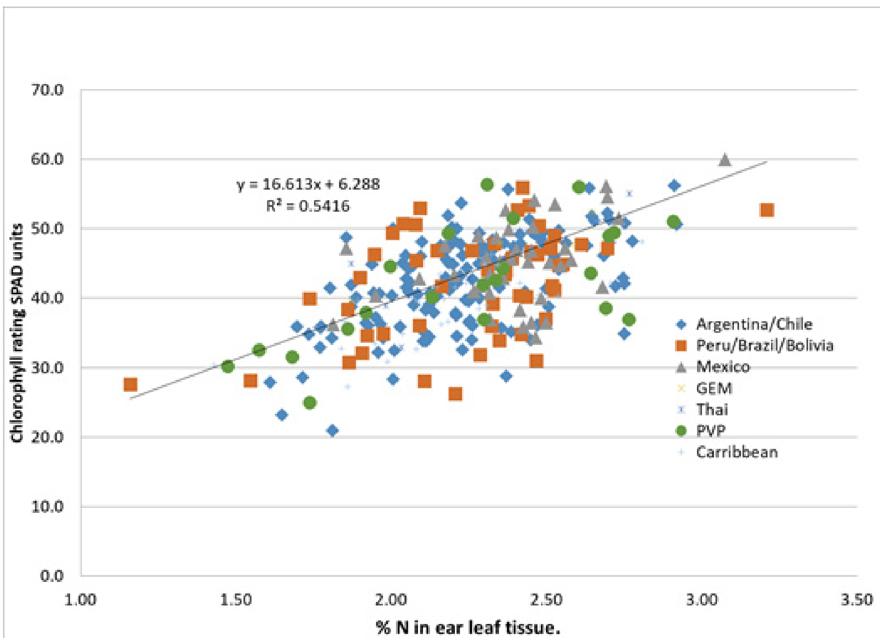

Figure 8: Relationship between N content of ear leaves and chlorophyll scores for 334 inbred lines grown under N-limited conditions in 2014 in the Goldstein Garden


Figure 9: Picture at the top shows inoculated plant of the of the C4-6 subfamily with autolyzing root tips in 2014 in the Goldstein Garden. Picture at the bottom shows non-inoculated plant without autolyzing root tips of a C4-6 derivative line grown on the Rohrer Farm in 2017

N-limiting conditions on several sites (Figure 10). C4-6 was a BC1-S4 line that descended by inbreeding from the highest yielding breeding line 'C' in the 2013 Eagle study (see ear photo in Figure 7). The 'C' line was a BC1-S1 line derived from sequentially crossing a Mexican landrace (not Mixeno) with a Corn Belt inbred, backcrossing to that inbred, and then self-pollinating the offspring one generation. By pedigree it was ¾ commercial maize belt inbred and ¼ Mexican landrace. The BC1-S3 predecessor line to C4-6 had a high leaf-N content (2.73%) and SPAD meter chlorophyll scores (52) in 2014 in the Elkhorn nursery (Figure 8).

Figure 10: The BC1 S3 breeding lines C4-5, C4-6, and C4-7 grown side by side after clover grass in 2015 on a McHenry silt loam near Elkhorn, WI. Note the 'N-type' with dark foliage is the central C4-6 line

Figure 11: Derivatives of the 'C' family grown on a N limited sites after rye near Elkhorn, WI in 2016. On the left side of this picture are various C6 subfamily inbred lines. On the right side are various C4-6 subfamily breeding lines

Comparisons of advanced sister breeding lines that possess or do not possess the trait in 2015 showed that the trait was associated with earlier flowering, and better ear set under drought and N stress conditions on two sites. Measurement showed that the 'N type' had darker foliage, faster growth rate before flowering, better synchronicity of flowering between anthesis and silking, fewer barren plants, but lower final total height than the sister lines that did not express the trait. Efficient extraction of N from soil under N limited conditions has been related in the scientific literature to the presence of large, extractive root systems (Wang et al. 2005; Miti et al. 2010; Li et al. 2017). However, field inspection of C4-6 and many of its descendent lines indicated that the rooting system was relatively small, enough so that subsequent selection needed to be practiced to remove susceptibility to root lodging.

Grow-outs and further selection of the C4-6 inbred subfamily in 2016, 2017, and 2018 in winter and summer nurseries showed the same growth type and dark foliage coloration relative to other breeding lines when grown under N limited conditions (see Figure 11). Exaggerated brace roots with weeping mucigel exudate occurred sporadically, and this has appeared to be independent of whether or not the seeds were inoculated with diazotrophic bacteria.

In 2015, the senior author crossed C4-6 inbred with many other inbreds and breeding families. In 2016 these hybrid crosses were grown under conditions with low and high fertility, first in Puerto Rico in the winter, and then F2 populations in Wisconsin in the summer. Some of the F2 generations made with this parent expressed dark foliage when grown under N limited conditions. Cursory observation was that some of the darkest F1 and F2 generations tended to appear when both parents were from the Mandaamin Institute breeding program rather than when C4-6 was crossed with conventionally bred inbreds, though there were exceptions.

Root systems. In 2017 the senior author observed root systems for a range of different inbreds from different breeding families developed at the Mandaamin Institute or conventional companies. Roots were observed under N-limited conditions on the Rohrer and Creek field sites. Conventional inbreds generally appear to have very tightly appressed, vertical roots (Figure 12) In contrast, the adventitious rooting systems of the most advanced Mandaamin inbreds were relatively spreading with broad

Figure 12: Root systems for conventionally bred cultivars grown on N limited sites in 2017 on the JR field (pictures at top and bottom left) and from the Creek Field (bottom right) Picture in top left) is LH206; picture in top right is LH123Ht; picture in bottom left is S7; picture in bottom right is S5. LH206 and LH123Ht were bred by the Holdens Seed Company (now owned by Bayer). S7 and S5 are commercial inbreds from a seed licensing company

crowns encompassing a larger volume of topsoil, and densely covered with secondary roots in the topsoil (see Figure 13).

4 Discussion

Selection under low-input, organic growing conditions appeared to induce the appearance of opaque kernels in multiple populations, breeding lines, and inbreds over time. These seed possessed elevated levels of methionine, cysteine, and lysine without reductions in seed weight. In contrast, we found that *fl2,* which has high methionine content in its grain, conditions maize to have seed weight reductions of 10%. This confirms finding by Lorenzoni et al. 1980 who found *fl2* caused reductions of 11%.

The occurrence of opaque kernels in the Mandaamin Institute breeding lines may be the result of genetic/epigenetically conditioned shifts in regulation. Opaque kernels appeared to emerge in unexpected quantities between generations, and frequencies lacked clear Mendelian patterns of segregation. Furthermore, in some cases, there were difficulties stabilizing the trait through

Figure 13: Root systems for Mandaamin Institute bred inbreds derived from different breeding families advanced on N limited sites in 2017 from the JR field (picture on bottom right) or from the Creek Field (other pictures). Picture in top left is from family C4-6; picture in top right is from family LAT7; picture in bottom left is from family NG10-2-3-2; picture in bottom right is from family C2B2

inbreeding. The emergence of this trait may have to do with shifts of epigenetic control of zein protein production in grain under N-limited conditions. At question is whether the Mandaamin program may be reversing the consequences of previous generations of breeding for cultivars that respond to N fertilization.

The trait is non-allelic to *fl2*. Floury2 has a defective signal protein that reduces α zein storage in protein bodies in grain (Coleman et al. 1995). Though in some cases the trait in our breeding lines seemed to be allelic to floury 1, floury 1 has not been found to affect α zein accumulation (Holding et al. 2007) nor to increase methionine content or other essential amino acids in grain (Nelson et al. 1966). Preliminary qualitative electrophoretic studies with inbreds from Mandaamin Institute by David Holding at the University of Nebraska relevant to normal genotypes suggest that our selections have reduced α zeins but higher levels of the nutritionally more valuable β zeins, δ zeins, and non-zein proteins in grain. Because the α zeins lack lysine, cysteine, and have only small amounts of methionine (Tsai 1979); these other protein fractions have much higher levels of essential amino acids and cysteine (Boundy et al. 1967).

In the case of LH123Ht and some other inbreds we see the emergence of seed with opaque and translucent sectors. This may reflect transponson activation. Some of these lines have been or are being coaxed towards becoming fully opaque with repeated selection.

Our inbred development program is combining protein quality with acceptable agronomic traits to produce competitive hybrids. Increasing protein yield makes sense because the Mandaamin Institute cultivars produce more high value protein without accumulating high proportions of α and γ zeins with low quality nutritional value (Frey 1951; Tsai et al. 1992).

Aside from *inbreds*, Mandaamin Institute's mostly soft-kernelled maize *hybrids* also tested high in the essential amino acids lysine and methionine, and in cysteine. HPLC analysis of our soft-endosperm cultivars, grown side by side with conventional checks in 2011, suggested that grain for the former had 31% more protein, 53% more methionine, 39% more cysteine, and 13% more lysine than conventional hybrids.

To the senior author's knowledge, this grain quality is not otherwise available in the form of competitive cultivars. Dairy farmers recognize that high levels of methionine and lysine and improved digestibility of maize in the rumen are equally important drivers of dairy production (Schwab 2012; Seymour 2016) and the Mandaamin Institute maize possesses both traits. The maize from this breeding program should reduce or eliminate the need for synthetic methionine supplementation widely used by dairy producers and the organic poultry industry. It should thereby enable industry growth in the face of continued restriction of synthetic methionine (McEvoy 2015; Fanatico and Ellis 2016).

In 2016, poultry nutritionist Pierre Meyer (Alltech, Inc.) estimated the value of the Mandaamin high methionine maize for organic poultry producers. He utilized commercial linear programming systems, and current prices for organic maize and soybean meal and synthetic methionine. According to his studies our maize should save organic poultry farmers \$32/ton of feed (\$493 vs \$525) by reducing the need for organic soybean meal and synthetic methionine. As margins for poultry production are tight this is a significant saving. According to its nutrient composition the maize should be worth \$1.38/bushel more for organic poultry producers than normal organic maize when the latter is worth \$9.82/bushel.

This increase in potential value does not take into account other factors such as potential yield losses associated with growing the opaque maize or potential benefits due to feeding the other valuable bionutrients (carotenoids, trace elements) found in the opaque maize. Selection for bright yellow and orange kernels has resulted in bright endosperm coloration of many of the inbreds coming from the program. This is generally associated with elevated levels of lutein and zeaxanthin. These pigments, when fed to poultry, can be expected to increase yolk coloration.

Tests of putative cross incompatible breeding lines that had *Ga1* showed that most of them were highly effective at preventing pollination from *ga*. This positive finding supported the effort to develop more breeding lines and inbreds with the *Ga1* and *Tcb1* trait.

Our society's current maize production system is dependent on synthetic N fertilizer and is degrading our ground waters, rivers, oceans and atmosphere with nitrogenous compounds. The appeal and value of the Mandaamin Institute cultivars is that they may reliably produce larger quantities of high quality protein under low N input conditions.

A pertinent question is the extent to which N efficiency is due to plant inheritance for root efficiency and to what extent it is due to plant-microbial relationships. The culture shock syndrome describes a phenomenon where conventionally bred and grown inbreds do poorly under organic conditions, but gradually appear to adapt after seasons of production under organic conditions. Growing the breeding lines under our organic conditions, coupled with mild selection, led maize to be more efficient at producing chlorophyll and to flower earlier. There is a strong linear correlation between chlorophyll content and the N content of plants (Schepers et al. 1992). In our trial, chlorophyll increased across all cultivars with organic selection/growing, in consonance with a reduction in variation in chlorophyll content. This reduction suggests that results were not due to hidden outcrosses in previous generations which would have been expected to increase variation of the variants. We suggest these phenomena are due to adaptive shifts. Possibly they are caused by progressive changes in endophytic communities under organic conditions coupled with shifts in root morphology and efficiency at obtaining N from soil organic matter.

Data presented in Table 3 showed that breeding lines developed under organic conditions had higher $\delta^{15}N$ isotope ratio in the grain, which may indicate greater mineralization of N from soil organic matter. The senior author observed qualitative differences in the roots of Mandaamin inbreds under N limited conditions. Those inbreds produced broader crowns with greater branching/fibrous development in the topsoil region, capable of encompassing larger quantities of soil.

In seeking varietal stability for N efficiency and protein production we found landraces with high protein quality in their grain that appear to interact with diazotrophic bacteria and thereby obtain more N and make more grain protein. Use of natural abundance $\delta^{15}N$ ratios indicated that significant N_2 fixation occurred in N limited soils for some of these landraces. Top-cross hybrids with these landraces produced more protein and grain yield when inoculated with diazotrophs. This efficiency may be due to greater uptake of N from soil or air or both.

Conventional hybrids responded negatively to seed disinfection of *Fusarium* but did not respond positively or responded negatively to inoculation with diazotrophic bacteria. *Fusarium* may play a protective role for conventional hybrids by increasing emergence. In contrast, the experiment with disinfection and inoculation gave proof of concept that hybrids with certain Mexican cultivars could have positive relations with diazotrophic bacteria and thereby produce more protein.

More research should be done to clarify why the inbreds and breeding lines in the culture shock experiment did not respond to disinfection while negative effects of disinfection were observed in the conventional hybrids in the hybrid type x seed treatment experiment. This may be due to differences in soil health on the different sites affecting emergence, root health, and microbial/plant relationships. The culture shock experiment was grown on soil which had previously been in an alfalfa/grass mixture for four years; a situation regarded as conducive for organic maize production. The hybrid type x seed treatment experiment took place in a N limited field which had been in cereal/soybean and non-leguminous cover crops with no fertilization with manure for at least four years.

The results of these trials stimulated our efforts to introgress the N efficiency/fixation trait into already existing breeding lines with commercial value. Also, early phenotyping from the trial on Metea loamy sand produced results that supported the contention that pre-anthesis human observation can help select under N-limited conditions. This finding confirmed results from others (Lafitte and Baenzinger 1997; Miti et al. 2010).

Under the growing conditions in Wisconsin, multiple, different landraces of Mexican maize or their derivatives from crosses with Corn Belt inbreds, showed the phenotype of exaggerated brace root development with dripping mucilage (Goldstein 2016). Furthermore, N efficient breeding lines derived from crosses with putative N_2-fixing Mexican landraces often showed apparent autolytic harvesting of the colonies and roots, i.e., the brace roots self-digest. For photographs of this phenomenon in different breeding lines derived from different Mexican landraces see Goldstein 2016. Autolytic harvesting of microbial N is found in other grasses (White et al. 2012). If what has been observed in maize is an autolytic reaction to harvest microbial N, we could not find that it has been previously described in the scientific literature.

In 2014 a major breakthrough appeared to occur in the Mandaamin program with identification of the N efficiency/putative N_2 fixation trait in the C4-6 line. This C4-6 inbred was derived from what had been the most N efficient breeding family grown on the N limited Metea loamy sand in 2013. It had high leaf N and chlorophyll scores when grown in an N-limited nursery in 2014. The breeding family had also shown relatively very dark foliage in trials in 2015 on multiple sites and in subsequent years. High chlorophyll content in leaves is correlated with N content in the leaves. The high chlorophyll content which was apparent from 2013-2018 under soil conditions with limited N availability indicated that the maize might be N efficient or fixing N_2. Relative to other Mandaamin Institute inbreds it does not have an especially large rooting system (Figure 13) though this is being increased through selection. Nor does it always form exaggerated brace roots with exudation and autolytic tips (Figure 9). Subsequent measurement of chlorophyll in 2018 with a SPAD meter has shown that the best of the C4-6 derivatives may have 15% to approximately one third more chlorophyll than adjacent conventional inbreds. Furthermore, yield results for 2016 and 2017 (not reported here) indicate that the N efficient/putative N_2 fixing hybrids can yield higher amounts of grain and protein/ha than conventional hybrids on N-limited conditions, but not as well under conditions with high rates of manure application.

Conclusion: The results validate a breeding approach that simultaneously combines breeding for nutritional quality and for nutrient efficiency under biodynamic/organic conditions. The program utilizes the biological diversity of maize. Profound shifts occurred in the course of the program in the appearance and physiology of roots, foliage, and grain. The basis for N efficiency and protein quality in our cultivars appear to be due to shifts in root efficiency, microbial relationships, and protein metabolism. Results are greater nutrient value of grain and greater N efficiency for plants. They are probably based on the maize plant's responses to reducing fertilization, including fostering beneficial plant/microbial partnerships. They are also due to exercising the breeding and selection principles, methods, and environments described above. Results illustrate the potential and importance of breeding under biodynamic-organic conditions. The evolutionary plasticity and creative adaptability of the species to organic environments was amply demonstrated in the context of the program, and was the foundation for the progress made by our program. It is unclear whether these results are due to epigenetic and/or genetic mechanisms, or represent an applied example of genomic change under 'isolation by environment' conditions (Wang and

Bradburd 2014). This deserves further clarification. The fruitfulness of our approach should be tested by others to increase nutritional value and agricultural sustainability for maize and other crops.

Acknowledgements: The program was carried out with the diligent work of multiple technicians including William Barber, Terry LeMahieu, R.J. Octaviano, Chris Wills, Jasper Karnes, Joel Kabanuk, and Virginia Thomas. Many farmers provided advice and helped with field work to enable grow outs, isolations, nurseries, and yield trials. In particular we would like to acknowledge the assistance of farmers Don Adams, Dan Specht, John Pounder, James Rohrer, Mark Zinniker, Graham Adsit, and one anonymous Amish farmer. The senior author acknowledges USDA-ARS and Iowa State University staff for valuable advice, assistance, services and, in some cases, breeding stocks that have assisted and enabled the above described maize breeding program. This includes help from maize geneticists and breeders (Marvin Paul Scott, Jode Edwards, John Golden, Arnell Hallauer, Kendall Lamkey) at the USDA-ARS Corn, Insects, and Crop Genetics unit at Iowa State University; Mark Millard, the maize curator at the Plant Introduction Station in Ames, Iowa; and Michael Blanco and the staff of the USDA Germplasm Enhancement of Maize project in Ames, Iowa. Kevin Montgomery (Montgomery Consulting, Maroa, IL) gave valuable advice, assistance, services and, breeding stocks. David Holding from the University of Nebraska was helpful with electrophoretic studies of the proteins in our cultivars. Chris Wilbeck from Practical Farmers of Iowa and USTN cooperators helped us to trial our hybrids through the USTN network. Steve Mohr, Foundation Organic Seeds, has financed production of inbreds and hybrid production in multiple winter nurseries. The Biodynamic Association of North America funded travel costs for the Biodynamic Research Conference. Though assistance from all those mentioned above was helpful for the breeding project, none of the persons or organizations mentioned above bear any responsibility for any errors the senior author may have made in the conductance of the breeding program or in writing this paper. The senior author also acknowledges funding from USDA-ARS, USDA-NIFA-OREI, the Ceres Trust, Organic Valley, Clif-Bar Family Foundation, the Organic Farming Research Foundation, the Martina Mann Foundation, the Department of Agriculture, Trade, and Consumer Protection of Wisconsin, Allen Goldstein, Paul Conway, Gordon Edwards, Dana and Phil Burns, Christian Wessling, Dorothy Hinkle-Uhlig, the Summers and Kabanuk families and other, anonymous funders or contributors that have fostered the breeding program.

Conflict of interest: Authors declare no conflict of interest.

References

Baca B.E. and Elmerich C., Microbial production of plant hormones. Pages 113-137 in: Associative and Endophytic Nitrogen Fixing Bacteria and Cyanobacterial Associations, Editors Elmerich C.and Newton W.E., Springer Pub., 2007

Bockemuehl J., In partnership with nature. Biodynamic Farming and Gardening Association. 1981, pp. 84

Boddey R.M., Polidoro J.C., Resende A.S., Alves B.J.; Urquiaga S., Use of the 15N natural abundance technique for the quantification of the contribution of N2 fixation to sugar cane and other grasses. Australian Journal of Plant Physiology, 2001, 28, 889-895

Boundy J.A, Waychek J.H., Dunler J.S., Wall R.J., Protein composition of dent, waxy, and high amylose corn, Cereal Chem., 1967, 44, 160-169

Burbank L. and Hall W., Partner of Nature. D. Appelton Century Co., NY. 1939, pp. 315

Coleman C.E., Lopes M.A., Gillikin J.W., Boston R.S., Larkins B.A., A defective signal peptide in the maize high-lysine mutant floury 2, Proc Natl Acad Sci USA, 1995, 92, 6828–6831

Fanatico A. and Ellis K., Organic poultry production, providing adequate methionine,ATTRA publication, 2016, https://attra.ncat.org/attra-pub/viewhtml.php?id=336

Frey K.J., The inter-relationships of proteins and amino acids in corn, Cereal Chem, 1951, 28, 123-132

Goldstein W., Partnerships between maize and bacteria for nitrogen efficiency and nitrogen fixation. Mandaamin Institute, Elkhorn, Wisconsin; published on the Internet, January, 2016. Bulletin 1, www.mandaamin.org

Goldstein W.A., Pollak L., Hurburgh C., Levendoski N., Jacob J., Hardy C., Haar M., Montgomery K., Carlson S., Sheaffer C. Breeding maize with increased methionine content for organic farming in the USA. pp 262-275 IN: U. Koepke and S.M. Sohn Ed. ISOFAR International Symposium on Organic Agriculture Proceedings. 12-14 March 2008. Dankook University, Korea, 2008

Goldstein W.A., Schmidt W., Burger H., Messmer M., Pollak L.M., Smith M. E., et al.., Maize breeding and field testing for organic farmers, pages 175-189, In: Organic Crop Breeding. Publisher Wiley-Blackwell, NY. 2012.

González-Ramirez L.P., Caracterización de microorganismos de mucigel de raíces adventicias y suelo rizosférico de maíz Olotón de la región Mixe, Oaxaca. Doctoral thesis, Universidad Autónoma Benito Juárez de Oaxaca, 1994, p. 95

Gonzalez-Ramirez L.P. and Ferrera-Cerrato R., Microbiology of adventitious roots of Oloton maize (Zea mays L.), Proceedings of First International Meeting on Microbial Ecology. CINVESTAV. IPN. Mexico, D.F., 1995

Hallberg T.B., Nitrogen fixing bacteria associated with maizes native to Oaxaca. P. 760 In: Tikhanovich I.A, Provorov N.A., Romanov V.I., Newton W.E., eds. 1995. Nitrogen fixation Fundamentals and Applications. Proceedings of the tenth International Congress on Nitrogen fixation, St. Petersberg, Russia. Kluwer, Netherlands, 1995

Hallberg T.B., Letter to Walter Goldstein. Quote from letter: "I found that, in October 15,1990, I received from Dr. Gabor J. Bethlenfalvay of the Agricultural Research Service in Albany, California a letter saying that: 'Now I would like to report that my microbiologist did find N2-fixing bacteria in the gel collected from the aerial roots of Otolon corn in Totontepec'. That was from material collected by him on a trip with me to see those corn plants I had described to him before and my suspicions. I have not seen this in other corn races.", 2016

Hardy C.L., Rippke G.R., Hurburgh C.R., Goldstein W.A., Calibration of near-infrared whole grain analyzers for amino acid measurement in corn. Cereal Foods World (supplement) 2009, 54: A45 (abstract), Access http://meeting .aaccnet.org/2009/default.cfm

Holding D.R, Otegui M.S., Li B., Meeley R.B., Dam T., Hunter B.G., et al., The Maize Floury1 Gene Encodes a Novel Endoplasmic Reticulum Protein Involved in Zein Protein Body Formation, Plant Cell, 2007, Aug; 19, 2569–2582. doi: 10.1105/tpc.107.053538

Holdrege C., Genetics and the manipulation of life: the forgotten factor of context (Renewal in Science). Lindisfarne Books, 1996, pp. 192

Holdrege C., Thinking like a Plant. A Living Science for Life. Lindisfarne Pr., 2013, pp. 153

Jacob J.P., Levendoski N, and Goldstein W., Inclusion of high methionine corn in organic pullet diets, Journal of Applied Poultry Research. 2008, 17, 440-445

Jaradat A.A., Goldstein W., Dashiell K.E, Phenotypic structures and breeding value of open-pollinated corn varietal hybrids, International Journal of Plant Breeding, 2010, 4, 37-46

Jaradat A.A., and Goldstein W., Diversity of maize kernels from a breeding program for protein quality: physical, biochemical, nutrients and color traits, Crop Science, 2013, 956-976

Jaradat A.A., and Goldstein W., Diversity of maize kernels from a breeding program for protein quality: II. Correlatively expressed functional amino acids, Crop Science, 2014, 1-24

Jaradat A., and Goldstein W., Diversity of Maize Kernels from a Breeding Program for Protein Quality: III. Ionome Profiling. Agronomy. 2018, 8, 9 Agronomy 8020009, https://doi.org/10.3390/agronomy8020009

Khan Z., Guelich G., Phan H., Redman R., and Doty S. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. Agronomy, 2012, 11 pages, doi:10.5402/2012/890280

Kraft K. and Kraft P., Luther Burbank, the Wizard and the Man. Merideth Pr., 1967, pp. 270

Lafitte H.R. and Baenziger M., Maize population improvement for low soil N: Selection gains and identification of secondary traits. In G.O. Edmeades et al. (editors.), Developing drought- and low N-tolerant maize. Proceedings of a symposium, March 25-29, 1996. pages485-489. CIMMYT, El Batan, Mexico. CIMMYT, Mexico City, 1996

Lee C., Hristov A.N., Cassidy T.W., Heyler K.S., Lapierre H., Varga G.A., et al., Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed metabolizable protein-deficient diet. J. Dairy Sci. 2012, 95, 6042–6056

Levendoski N. and Goldstein W.A., Alternatives to synthetic methionine feed trial. Poster presented at the 1st IFOAM International Conference on Animals in Organic Production St. Paul, Minnesota, USA, 23-26 August, 2006

Li Q., Wu Y., Chen W., Jin R., Kong F., Ke K., Shi H., et al., Cultivar Differences in Root Nitrogen Uptake Ability of Maize Hybrids. Front Plant Sci., 2017, 8, 1060, Published online 2017 Jun 20. doi: [10.3389/fpls.2017.01060]

Lorenzoni C., Fogher C., Bertolini M., Di Fonzo N., Gentinetta E., Maggiore T., et al., Short communication on the relative merit of opaque-2, floury-2, and opaque-2 floury-2 in breeding maize for quality. Maydica, 1980, 25, 33-39

Martinez-Romero E., Oswald-Spering U., Miranda M., Garcia L., Fuentes-Ramirez L.E., Lopez-Reyes L., Estrada P., Caballero-Mellado J., Towards the application of nitrogen fixation research to forestry and agriculture. In. Legocki, A., Bothe H., Puehler A., eds. Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture. Springer Verlag, Berlin, Heidelberg, NATO ASI series Vol G, 1997, 39, 187-190

Masoero F., Gallo A., Zanfi, C. Giuberti G., Spanghero M., Effect of nitrogen fertilization on chemical composition and rumen fermentation of different parts of plants of three corn hybrids, Animal Feed Science and Technology, 2011, 164, 207-216

McEvoy M., Memorandum to the National Organic Standards Board, Sept. 3, 2015. https://www.google.com/search?q=methionine+National+Organic+ruling+USDA+requirement&ie=utf-8&oe=utf-8

Miti F, Tongoona P., and Derera J., S1 selection of local maize landraces for low soil
nitrogen tolerance in Zambia. African Journal of Plant Science, 2010, 4, 67-81

Nelson O.E., Mertz T.E, Bates L.S., Second mutant gene affecting the amino acid pattern of maize endosperm proteins, Science, 1966, 150, 1469-1470

Padilla García J.M., Sánchez González J.J., Larios L., Ruiz Corral J.A., Parra J.R.,
Morales Rivera M.M., Gametophytic incompatibility in Mexican maize breeds, Revista Mexicana de Ciencias Agrícolas, 2012, 3 525-537

Reis V.M.P., Estrada-de los Santos S., Tenorio-Salgado J., Vogel M., Stoffels S., Guyon P., Mavingui V.L.D., Baldani M., Schmid J., Baldani I., Balandreau J., Hartmann A., Caballero-Mellado J., Burkholderia tropica sp. nov., a novel nitrogen-fixing, plant-associated bacterium. Int. J. Syst. Evol. Microbiol., 2004, 54, 2155–2162

Rodriguez R.J., White J.F. Jr., Arnold, A.E., Redman R.S., Fungal endophytes: diversity and functional roles, New Phytologist, 2009, 182(2), 314-30, doi: 10.1111/j.1469-8137.2009.02773.x

Scavia D., Bertani I., Obenour D.R., Turner R.E., Forrest D.R., Katin A., Ensemble modeling informs hypoxia management in the northern Gulf of Mexico. Proceedings of the National Academy of Sciences, 2017, 114, 8823-8828

Schulz B.J.E., Mutualistic interactions with fungal root endophytes. In: Schulz B.J.E., Boyle C.J.C., Sieber T.N., editors. Microbial Root Endophytes, Berlin, Germany: Springer-Verlag, 2006, 261-280

Scott M.P., Bhatnagar S., Betrán J., Tryptophan and methionine levels in quality protein maize breeding germplasm. Maydica, 2004, 303-311

Schepers J.S., Francis D.D., Vigil M., Below F.E., Comparison of corn leaf nitrogen concentration and chlorophyll meter readings. Commun. Soil Sci.Plant Analysis 1992, 23, 2173-2187

Schwab C., The Principles of Balancing Diets for Amino Acids and Their Impact on N Utilization Efficiency, 2012, http://dairy.ifas.ufl.edu/rns/2012/1SchwabRNS2012.pdf

Seymour W.T., Role of methionine and methionine precursors in transition cow nutrition with emphasis on liver function, 2016, http://dairy.ifas.ufl.edu/rns/2016/2.%20Seymour.pdf

Tsai C.Y., Tissue specific zein synthesis in maize kernel, Biochemical Genetics, 1979, 17, 1109-1119

Tsai C.Y., Dweikat I., Huber D.M., Warren H.L., Interrelationship of nitrogen nutrition with maize (Zea mays) grain yield, nitrogen use efficiency and grain quality. Journal of the Science of Food and Agriculture, 1992, 58, 1,1-8

Van Deynze A., Zamora P., Delaux P.M., Heitmann C., Jayaraman D., Rajasekar S., et al., Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota. PLoS Biol, 2018, 16(8), e2006352, https://doi.org/10.1371/journal.pbio.2006352

Vega-Segovia M.L. and Ferrera-Cerrato R., Microorganismas del mucigel, rhizoplano y rizosfera del maiz oloton de la region Mixe, Oaxaca. In: J. Perez-Moreno and R. Ferrera-Cerrato (eds). Avances de Investigacion, Area de Microbiologia de Suelos. PRODAF-IRENAT, Colegio de Postgraduados, Montecillo, Estado de Mexico, 1993, pp. 9-17

Wang I.J. and Bradburd G.S., Isolation by environment. Molecular Ecology, 2014, 23, 5649-5662

Wang Y., Mi G., Chen F., Zhang J., Zhang F., Response of root morphology to nitrate supply and its contribution to nitrogen accumulation in maize. Journal of Plant Nutrition, 2005, 27, 2189-2202

White J.F., Crawford, H., Torres M.S., Mattera R., Irizarry I., Bergen M., A proposed mechanism for nitrogen acquisition by grass seedlings through oxidation of symbiotic bacteria. Symbiosis., 2012, 57, 161-171

Yates I.E., Bacon C.W., Hinton D.M., Effects of endophytic infection by Fusarium moniliforme on corn growth and cellular morphology. Plant Dis., 1997, 81,723-728