Seed endophytes, rhizophagy, nutrient density, nitrogen efficiency and fixation in corn.

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Cereal crops, including corn, have been bred to produce high yields in conjunction with inputs of soluble nitrogen (N) fertilizers. However, N fertilizers cause pollution of surface and ground water with nitrate and produce a potent greenhouse gas, nitrous oxide. The problem is generally regarded as intractable within the present social/political context. The Mandaamin breeding program represents a long-term effort to breed corn adapted to the needs of organic farmers. This includes breeding for corn that is better able to obtain N from soil and air in order to reduce nitrification and to prevent economic and environmental problems with N fertilizers.

In this presentation we will a) describe the Mandaamin breeding program and its early results including studies in three states together with the University of Illinois and University of Wisconsin-Extension, b) research done by Rutgers University with bacterial endophytes and how that work helps explain results from the breeding program; and c) how practical questions are being answered regarding the usefulness of the cultivars developed by the breeding program.

The breeding program: Mandaamin Institute's breeding methods employ a field-based phenomenological approach to breed and select fit, whole bodies for improved inbreds and populations. The institute utilizes pedigree-based inbreeding. Breeding has taken place for 51 seasons under biodynamic conditions in Wisconsin USA, on an organic and biodynamic winter nursery in Puerto Rico, and Hawaii, and conventional winter nurseries in Chile. Crosses are made between landraces or teosinte with adapted inbreds to reintroduce valuable traits. Early-generation-testing is carried out to determine grain yields in hybrid combinations. Selection criteria include protein quality, carotenoid content, vigor, competitiveness with weeds, grain yields, and nitrogen (N) efficiency/N_2 fixation.

Mandaamin Institute's timeline, milestones, and cooperators are as follows: The program began in 1989 and for 14 years created open pollinated populations for farmers. In 2002 breeding began to produce inbreds and hybrids together with USDA-ARS. From 2004 to 2007 it was confirmed through experimentation that N efficiency and soft kernelled seeds with elevated methionine and trace minerals were emerging in multiple populations and inbreds (USDA-ARS, Iowa State University). In 2009 N_2 fixing landraces were discovered and the work began to breed the trait into adapted varieties. In 2018 trials began with the University of Illinois in three states. In 2020 seed-borne bacterial endophytes were found to be colonizing roots and being excreted out of root hairs in Mandaamin Institute's varieties (Rutgers University). Also, In 2020 Mandaamin Institute's first commercial hybrids were sold by a cooperating seed company (Foundation Organic Seed). In 2021 Mandaamin Institute's first commercial hybrids with a putative N_2 fixing parent will be sold.

Results: The program resulted in inbreds and hybrids with greater adaptation to nutrient-and-weed limited organic/biodynamic conditions, competitive yields, increased N efficiency and grain nutritional quality and softer grain texture. There is often more chlorophyll in foliage, rhizophagy, densely branched root growth in the topsoil, larger tassels, and greater phenotypic and genomic plasticity than for conventional inbreds. Part of Mandaamin Institute's nutrient efficiency is due to inbreds with broad, fibrous rooting systems adapted to extracting nutrients from soil.

Yield trials have been carried out at the Institute and in conjunction with organic farmers and the University of Wisconsin-Extension. There are generally similar grain yields for Mandaamin Institute's top yielding hybrids as with commercial maize. Somewhat lower yields under high input conditions, but often higher yields under low N input conditions.

Three state trials: Trials were carried out in conjunction with the University of Illinois in IL, IN, WI on organic farms under mostly manure fertilized conditions   Two regions, Central Corn Belt (IL, IN) and Northern Corn Belt (WI). There were 13 farms per year, two years (2018, 2019), unreplicated strip trials on each farm, two Mandaamin hybrids on each site. These hybrids were 17.C46 with a relative maturity (RM) of 105 days, and 17.C2B2 with a RM of 110 days. High yielding commercial hybrids served as checks. Grain was harvested for yield and analyzed at the University of Illinois in Urbana-Champaign, Iowa State University Grain testing lab, University of Wisconsin, and by a commercial lab. The data were analyzed using analysis of variance with main factors being regions and hybrids and with farm sites as replicates. Where differences between hybrids were statistically significant, we compared them with t-tests.

Results: The difference in yield between the hybrids were not statistically significant. However, the 17.C46, which was the earliest maturing hybrid, yielded less in the fuller season, Central Corn Belt sites and the 17.C2B2 averaged 10% less yield than the checks over all sites. The Mandaamin hybrids had lower test weights and seed density because they are softer textured Mandaamin Institute's corns.

The two Mandaamin hybrids had 10 and 18% more protein, 32% more oil, 44 and 63% more zeaxanthin, 48 to 150% more β-cryptoxanthin, 38 to 65% more β-carotene, 10 and 15% more lysine, 33 and 42% more methionine, and 10 and 16% more cysteine in their grain than did the commercial checks. These differences were all statistically significant at the 95% security level. They also had 23 to 25% higher methionine contents in their protein than did the checks. Furthermore, mineral analysis revealed that the Mandaamin hybrids had 17 to 24% more iron, 22 to 32% more manganese, 13 to 54% more copper, and 11 to 12 more zinc in their grain than did the checks.

In summary, the high methionine and carotenoid corn hybrids differed clearly from commercial hybrid checks by delivering significantly more methionine, carotenoids, and trace minerals at comparable, but somewhat lower yield levels. However, diet modeling and financial budgeting showed that the value of the Mandaamin crops per acre for poultry production were equivalent to the conventional hybrids based on the higher methionine content in the grain reducing the need for soybean meal.

Plant partnerships with endophytic bacteria and research at Rutgers University: Early isotope based research showed that certain landraces of corn had the ability to fix N_2. After extensive breeding efforts, N_2 fixing inbred families were developed such as C4-6 and LAT-7. These varieties appear to be fertilized with N when they are not fertilized. Both of these families produce mucoid secreting brace roots and these may harbor N_2 fixing bacteria that feed the plant with N. However, the C4-6 family appears to be N efficient from emergence on, long before brace roots are formed. Furthermore, hybrid trials on five sites suggest that application of manure to C4-6 derived hybrids reduced yields but increased yields of a conventional hybrid and other Mandaamin hybrids. These facts suggest that N efficiency in C4-6 is more complex than brace root colonization.

Research by James White's microbiology group at Rutgers University (see literature list below) help explain some of the breeding program results. The Rutgers research, carried out mainly with wild or semi domesticated plants, showed that 1) endophytic bacteria can induce rhizophagy cycles caused by seed-borne endophytes; 2) these bacteria increase mineral nutrient uptake and obtain N from the atmosphere; 3) the dialogue between bacteria and plant takes place in root hairs and in plant shoots; 4) This biochemical dialogue involves ethylene and NO/nitrate production and N2 fixation by bacteria, superoxide/hydrogen peroxide production by plants, N and increased N uptake.

Essentially a special mix of selected endophytic bacteria come from the mother plant, live in the seed, and multiply in root cells in root tips and in shoots, including in trichomes (hairs). The plant cells attack the bacteria with reactive oxygen and degrade bacterial cell walls and membranes, releasing proteins and minerals that are absorbed by the plant. The surviving bacteria stimulate branching of roots and production of root hairs. The bacteria are expelled through root hairs into the soil, where they grow cell walls again. There they presumably feed on soil and may be taken up by subsequently growing roots.

Grass roots show numerous roots tip meristems. These root tip meristems are the sites of internalization of microbes and extraction of nutrients from microbes in the rhizophagy cycle. Rhizophagy increases mineral uptake by plants. The presence of endophytes in maize is associated with N uptake from the air.

The plant/microbial partnership is based on a biochemical dialogue. The intracellular bacteria living in roots secrete ethylene . Ethylene triggers plants to grow root hairs and to supply the bacteria in the roots and hairs with carbohydrates. But the plant also produces superoxide which oxidizes the bacteria. In defense, the bacteria use the plant-supplied carbohydrates to fix atmospheric N_2. The bacteria then secrete antioxidant N (nitric oxide or ammonia) which neutralizes superoxide and protects their integrity. Nitrate is consequently produced and absorbed directly into plant cells

- NO (nitric oxide) + 2O (superoxide) → NO_3 (nitrate)
- 2NH3 (ammonia) + 9O (superoxide) → $2NO_3$ (nitrate) + 3H2O (water).

This relationship is not confined to roots. In aerial parts, bacteria were most common in non-photosynthetic cells of leaves and bracts, parenchyma, and especially trichomes (plant hairs). In fact, bacteria could be seen within nuclei of non-photosynthetic cells of many plants. Staining showed evidence for the following chemicals in association with intracellular bacteria: 1) ethylene, 2) reducing sugars, 3) superoxide, 4) hydrogen peroxide, 5) nitric oxide, and 5) nitrate. Furthermore, the cultured bacteria from tissues could fix N_2 or live in N-free media.

Experiments at Rutgers with ^{15}N enriched atmospheres showed strong N assimilation from the air in multiple species, including fescue and reed grass. Most assimilation was in aerial parts. Seed inoculated with labelled bacteria accumulated the bacteria in foliar tissues, especially around plant cell nuclei. Sterilizing the surface of seeds before planting resulted in plants with poorly formed trichomes, no bacterial infection, and no staining for nitric oxide or nitrate.

Studies of endophytes in aerial parts extended to several grasses including reed grass (*Phragmites australis*), crab grass (*Digitaria sanguinalis*, tall fescue (*Festuca arundinacea*) and red fescue (*Festuca rubra*). Studies showed that in the grasses bacteria are cultivated within nuclei where sugars fuel nitrogenase and bacterial replication. The bacteria are released into the cytoplasm of the cell in vesicles. Bacteria, once released from nuclei, begin to secrete ethylene. Bacteria in the cytoplasm are exposed to host-produced superoxide in the cytoplasm. Bacteria respond to superoxide with secretion of antioxidant forms of nitrogen. Such nuclear symbioses were seen in the epidermis and in simple trichomes .Furthermore, grasses have serrated epidermal cells and bacteria accumulate in the wall serrations

Studies with Mandaamin inbreds at Rutgers showed that their seedling roots were strongly colonized with bacteria. These bacteria were associated with production of root hairs and were excreted from root hairs. Such seed-borne rhizophagy was not found in conventional inbreds. Furthermore, visual microscopic inspection showed differences in the kind of microbial colonization between Mandaamin Institute's inbreds. Mandaamin's leading N_2 fixing inbred, C4-6, appeared to be forming dense accumulations or plugs of some kind of material in the roots, associated with bacteria. This inbred appears in the field to have dense trichomes on its leaf sheaths, and it also has densely branched roots that seem resistant to fusarium root rot.

Paradigm shift: Reflection on these findings indicates that maize is a capable farmer and selectionist, too. The implications lead away from a top-down approach to breeding to partnership breeding. The low N input, moderate stress, biodynamic /organic environments under which maize was selected may have also enhanced opportunities for emergent, maize-plant-driven, evolution/variation/selection. It seemed that maize is doing creative parts of the breeding work itself through emergent evolution. Maize has the ability to adapt to environments and to shape creative partnerships with a team of microbes. The role of the breeder can be redefined as being to select good, adapted, body forming partnerships but breeder capacities need to be continuously refined by fully engaging in learning from the plant/microbial team.

When plants employ rhizophagy and foliar endophytes, the mode by which plants obtain N expands beyond obtaining water soluble nitrate and ammonia to include microbial biomass-N, extractable organic N, and microbially responsive N_2 fixation in growing organs.

Practical questions: Pertinent questions arise from these results. How N efficient are the Mandaamin hybrids? How much can farmers reduce fertilization? How well do soil tests predict N relations for N efficient corn? To begin to address these questions Mandaamin Institute carried out strip test and randomized experiments on working organic and conventional farms. Research support was given by the University of Illinois, University of Wisconsin Extension, USDA-ARS and financial support came from USDA-NIFA-OREI and SARE programs and from the Ceres Trust. This research compared Mandaamin hybrids with a commercial hybrid check. It addressed the question: "how do different farming systems affect N uptake from soil and air?" Therefore, N uptake and $\delta^{15}N$ measurement were critical measurements. The $\delta\ ^{15}N$ value assesses the ratio between the two natural isotopes ^{14}N and ^{15}N. The higher the value the greater the concentration of ^{15}N. Soil organic matter, and especially soil microbial biomass and easily available N (composed mainly of dead microbes) have higher ^{15}N content than air and hence higher $\delta^{15}N$ values. This is because bacteria consume soil organic matter and accumulate ^{15}N as they selectively release more^{14}N than during nitrification and denitrification (isotopic fractionation). Our previous studies in 2009 and 2010 (Goldstein et al., 2019) showed that the grain resulting from many of our inbreds and populations had higher $\delta^{15}N$ values than conventional inbreds and hybrids. But the grain of very high chlorophyll, putative N fixing populations had very low $\delta^{15}N$ values.

Early assumptions were that 1) Mandaamin varieties have more active rhizophagy cycles.than conventional corn. Rhizophagy should affect a higher mineral and N uptake from soil. It should raise $\delta^{15}N$ values by increasing N uptake from microbial biomass and easily available soil organic matter. This should be especially apparent in root composition which is close to the high ^{15}N source. 2) In hybrids where N_2 fixation is also fostered by endophytes, $\delta^{15}N$ in grain would be lower than for conventional inbreds because the N taken up from the soil is progressively diluted with ^{14}N from the air. This should be especially apparent in tops and in grain. Hence, the expectation was that in the best N efficient hybrids the $\delta^{15}N$ levels are being simultaneously increased and decreased in the plant parts by these competing plant/microbial activities.

Three system study in 2019: Farms represented different farming systems. There were two farm sites each in three systems. The arable organic farms centered on production of crops with inputs of poultry manure. The cattle-based organic system was where cropping centered mainly on dairy or beef production and forages were a major part of production. Conventional monoculture was a dairy system where large quantities of slurry were applied to monoculture corn. Four hybrids were tested. These were the FOS8500 control which is a conventionally bred, high yielding commercial hybrid. Three Mandaamin hybrids were C2B2-1.C46 (=C2B2 x C4-6), 17.461 (=17 x C4-6), and 17.2B24 (=17 x C2B2). The first two of these hybrids were expected, on the basis of field and microbial observations, as being putative N_2 fixers with rhizophagy. Crops were grown and harvested. Soil samples were taken after planting for organic sites and tested for total C, total N, mineralizable N, and particulate organic matter-C. Soils were sampled under the corn in September on all sites and tested for protein, protein score, SLAN, nitrate, CO2 respiration, bulk density, and aggregate stability.

Results: Samples taken at planting time showed that the two sites in the organic arable organic systems had higher levels of available phosphorus and potassium. The two sites in the cattle-based system had higher levels of total C and N, greater amounts of potentially mineralizable N, and higher amounts of particulate organic matter. We modelled crop performance parameters with an analysis of variance that included farming system, hybrid, system x hybrid, and total organic N and C/N as covariates. The percentage of the total sum of squares accounted for by the factors soil organic N plus the C/N ratio were substantial. They were 36% for grain yield, 57% for stalks/acre, 51% for roots/acre, 45% for N uptake/acre, 66% for C uptake/acre, 45% for tissue %N, 87% for $\delta^{15}N$ in grain and 27% for $\delta^{15}N$ in roots.

Early soil tests were also correlated with soil test results in the fall. The vast majority of the variation for the nitrogen related parameters could be explained by total carbon and nitrogen and the relationship between them (99.6% for soil protein, 99.7% for protein score; 96% for SLAN; 75% for nitrate, and even 85% for CO2 respiration). Soil C, N, and C/N attributed less to the variation found in values for aggregate stability and bulk density but there, the young fractions of particulate organic matter C and potentially mineralizable N correlated better.

Fall soil test results were correlated with crop performance. Soil protein content and scores and soil structure (aggregate stability and bulk density) correlated strongly to grain, root yields and total N uptake. Soil under the Mandaamin hybrids had 18% lower nitrate and 13% higher aggregate stability than the control.

In line with these soil quality parameters the arable organic system produced the lowest yields and the organic cattle-based system produced the highest yields. The conventional monoculture system was least efficient at transferring N to grain. The FOS8500 produced 11% more grain but less stalk and root than the hybrids crossed with 17. On average, across the systems, the Mandaamin hybrids took up 5-19% more macronutrients (N, P, K, Ca, Mg, S) and 14-20% more micronutrients (Fe, Cu, Zn, Si, Al, Ti, Sr) per acre than the conventional hybrid.

The Mandaamin hybrids had lower $\delta^{15}N$ values for their grain but high values for their roots relative to the conventional control. On that basis N derived from air in grain was preliminarily estimated to range on average from 26 to 34% for the Mandaamin hybrids and 12% for the FOS8500. The highest fixation occurred for the C2B2-1.C46 under the arable organic system (48%) but for the 17.461 and 17.2B24 the highest fixation was under conventional conditions (47 and 40%, respectively). The $\delta^{15}N$ values for grain correlated negatively with grain and stalk parameters and with mineral accumulation for multiple macro and micro elements.

Preliminary estimates of total N obtained from soil biomass and easily available soil organic matter relative to FOS8500 was based on natural isotope abundance in roots/stalks. Uptake was 16 to 26% higher for the Mandaamin hybrids.

Discussion and summary: The Mandaamin hybrids yielded 10-11% less grain but produced more stalks and roots than the conventional hybrid. Selection for inbred vigor and robust performance, and endophytic partnerships may have shifted towards investment in greater vegetative production. The stalks and roots of the Mandaamin hybrids were richer in N which means more protein and possibly better silage value.

In general the mineral content of the Mandaamin hybrids is higher than conventional hybrids and in many cases goes beyond any kind of concentration affect associated with a lower yield. In 2019, the Mandaamin hybrids took up 5-19% more macronutrients and 14-20% more micronutrients than the conventional hybrids. Experience from other sites shows that the relative uptake of individual minerals varies from site to site and year to year.

Clearly, soil organic C and N, C/N ratio, and soil protein content appear to be major determining factors for crop performance in this experiment, irrespective of how the different hybrids got their N. However, the Mandaamin hybrids do obtain their N differently than the conventional hybrid. N fixation and N and mineral accumulation from soil were greater for the Mandaamin hybrids than for the conventional hybrid. There was less nitrate under the Mandaamin hybrids which might be due to less nitrification . Aggregate stability was greater for the Mandaamin hybrids as well.

Results from Rutgers and Mandaamin research suggest that endophytic partnerships can result in N_2 fixation from aerial portions plus enhanced mineral uptake. Earlier studies (Goldstein et al. 2019) and other research done in 2020 on farm sites that is not reported here confirmed that the Mandaamin hybrids have considerably higher ^{15}N uptake from soil than conventional hybrids. This likely has to do with uptake of ^{15}N rich, easily available organic N and microbial biomass-N through the rhizophagy cycle. Lower $\delta^{15}N$ values for the conventional control hybrid probably have to do with such crops relying on uptake of NO_3 and NH_4 which have low $\delta^{15}N$ values.

According to the Rutgers studies wild plants and maize fix N from the air, making wild plant standards unreliable. Nitrogen derived from air calculations could be derived either by a) comparing Mandaamin hybrids with the FOS8500 check or b) by estimating the amount of N derived from the air in the grain by calculating the difference between the highest $\delta^{15}N$ achieved in the root or the stalk and the $\delta^{15}N$ found in the grain. The contrast in δ^{15} values should indicate the dilution effect within the plant due to N_2 fixation.

Our calculations appear to confirm that accumulation of ^{15}N from soil microbial biomass/organic matter and enhanced ^{14}N uptake through N_2 fixation are occurring simultaneously in roots and tops of the Mandaamin hybrids. Furthermore, the $\delta^{15}N$ values were negatively correlated for multiple macro and microelements suggesting that fixation and rhizophagy may be coupled.

The interpretation presented here is based on natural isotope literature on soil and atmospheric N pools (Craine et al., 2015). This perspective may become more refined when more knowledge is gained on bacterial endophytes and isotope partitioning in these hybrids. It is important to continue to gain pertinent information from these crops and their N and mineral uptake on multiple farm soils and sites to aid the learning process. Furthermore, Mandaamin corn should be grown in ^{15}N enriched atmosphere studies to confirm N_2 fixation.

The Mandaamin hybrids differ in their performance in different systems. Plants grown in the conventional system averaged the highest level of fixation but the Mandaamin hybrids had the lowest level of N accumulation from soil organic matter relative to FOS8500 when they were grown in that system. C2B2-1.C46 appeared to be the best hybrid at fixing N and obtaining N from soil organic matter but it did relatively poorly in the conventional monoculture system.

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