“Biological nitrogen fixation: Innovative approaches to address global challenges”

An NSF task force’s report identified two significant hurdles the NSF needed to overcome to support high risk/high return proposals: 1) the conservatism of the peer review process, and 2) the reluctance of NSF program officers to fund research with a high potential for failure in an environment in which proposals with a high probability of success were not being funded because of limited budgets.
OPEC induced oil shortages in 1973-74 and 1979-80 led to the US rationing gasoline and focusing on energy conservation.
Source: Agricultural Prices, National Agricultural Statistics Service, USDA.
Mosaic Science Magazine, Fall, 1973

“In view of the many research studies in plant sciences, biology, and chemistry, and in view of the proliferation of new..."
World Population Growth, Actual and Projected, 1950-2050

## FUTURE PROJECTIONS

<table>
<thead>
<tr>
<th></th>
<th>2000</th>
<th>2050</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPULATION</td>
<td>6 Billion</td>
<td>9 Billion (maybe 10)</td>
</tr>
<tr>
<td>HUNGRY PEOPLE</td>
<td>0.8 Billion (17%)</td>
<td>1.4 Billion (17%)</td>
</tr>
<tr>
<td>N FERTILIZER</td>
<td>$90 \times 10^6$ MT</td>
<td>$165 \times 10^6$ MT</td>
</tr>
<tr>
<td>P FERTILIZER</td>
<td>$42 \times 10^6$ MT</td>
<td>$75 \times 10^6$ MT</td>
</tr>
<tr>
<td>FOOD PRODUCTION</td>
<td>$3.5 \times 10^9$ MT</td>
<td>$6.5 \times 10^9$ MT</td>
</tr>
<tr>
<td>WATER-STRESSED COUNTRIES</td>
<td>23</td>
<td>52 (10x flow Nile)</td>
</tr>
</tbody>
</table>

PE Fixen. 2009. Perspective on Current and Future Agricultural and Environmental Need for Enhanced Efficiency Fertilizers  
Plant Management Network.
WORLD AGRICULTURE CRISIS

HUNGRY PEOPLE > 800 million - 1 billion
POVERTY > 1.8 billion less than $1 per day
POPULATION > 160 people every minute, 8 - 10 billion 2040
GLOBAL CLIMATE CHANGE Faster than anticipated

WOMEN > 70% work in agriculture in low income food deficit countries
FERTILIZER > 12-fold increase in N
   (Non Renewable) 6-fold increase in P
CEREALS > provide 60% of caloric intake
LEGUMES > provide 35-50% of protein intake
MEAT > 40% of all grain fed to animals
WATER > 75% of all water use is for agriculture by 2040 need 10X Nile
FOOD > By 2030 cereal demand = 3.1 billion tons
       cereal production = 3.0 billion tons
Many recent reports from the government and other organizations point out the importance of agricultural research to meet future global challenges and call for increased funding.....

 ✓ The 2009 “A New Biology for the 21st Century”, a National Research Council report recommended increased support for agriculture.
 ✓ 2013 The Plant Science Decadal Vision, American Society for Plant Physiology, again called for increased support for interdisciplinary, plant-driven science.
 ✓ The National Bioeconomy Blueprint, released by OSTP, pointed out the potential of plant based bio-products.

 ✓ The Dec., 2012 report by the President’s Council of Advisors on Science and Technology (PCAST), “Report to the President on Agricultural Preparedness and the Agricultural Research Enterprise”, concludes that the nation is not prepared for future agricultural challenges and recommends major R&D investments achieved through expanding the role of competition at USDA and increasing support through NSF.
WHY NITROGEN [N₂]? 

- Plant N is the underlying source of all human nutritional N.
- Highly abundant but unavailable due to triple bond N ≡ N.
- Production of N fertilizer requires 2-4% of Earth's natural gas yearly output (nonrenewable) and is not efficiently used by plants.
- Overuse in developed world poses environmental problems.
- Lack of availability in developing world limits crop production.
- Legume symbiotic N₂ fixation is renewable and sustainable.
CONSEQUENCES OF TOO MUCH N IN ENVIRONMENT

Photo courtesy of NASA/Goddard Space Flight Center Scientific Visualization Studio: The above map shows concentrations of phytoplankton, the algal blooms that contribute to dead zones, in Gulf Coast waters.
MAJOR LEGUMES: PRODUCTION AND N2 FIXATION

- HA GROWN
- Mt PRODUCED: 843 MILLION
- Mt N2 FIXED: 24 MILLION
- N FERTILIZER $ VALUE: $20-30 BILLION
“It is shocking—not to mention short-sighted and potentially dangerous—how little money is spent on agricultural research.” – Bill Gates

The problems that limit the ability of federal agencies to fund long-range, risky innovative research (i.e., 1 the conservatism of the peer review process, and 2) the reluctance of program officers to fund research with a high potential for failure in an environment in which proposals with a high probability of success were not being funded because of limited budgets.) are not found in philanthropic organizations, which have stepped up to fill this gap and to drive innovation in biological, physical and social sciences.
Gates Meeting: “Enhancing biological nitrogen fixation in crop plants” April 19-21, 2012

Three topic areas discussed:
1. Developing a rhizobial symbiosis in cereals
2. The introduction and encouragement of diazotrophic bacteria and cereal crop interactions
3. Synthetic biology, the design of a new organelle to fix N in crop plants

The research needed to achieve a practical, field level application of any of these technologies is likely to require a long term commitment.
Two of three topics to be discussed:

1. Developing a rhizobial symbioses in cereals
2. The introduction and encouragement of diazotrophic bacteria and cereal crop interactions
3. Synthetic biology, the design of a new organelle to fix N in crop plants --- Dr. Luis Rubio (Madrid), Ray Dixon (Norwich)

Goals more tightly defined:

“Engineering the Sym pathway of cereals for recognition of nitrogen fixing bacteria” — John Innes Institute, Dr. Giles Oldroyd, Lead Investigator
Engineering the Sym pathway of cereals for recognition of nitrogen fixing bacteria

Questions:

- What is the sym pathway?
- What would one engineer into plants?
- The title appears to presuppose that cereals and other non-legume plants lack the ability to recognize nitrogen fixing bacteria, is this true?
- If successful, how far would this get you toward achieving the original goal of “Developing a rhizobial symbiosis in cereals”? 
What is the sym pathway?

Endomycorrhiza fungal infection

Rhizobium-induced nodule
We now know that several of the signaling steps are shared between the endomycorrhizal and rhizobial symbiosis.

Since the endomycorrhizal symbiosis is very widespread and arose some 400 mya, we assume that the mechanism of rhizobial infection evolved from the endomycorrhizal symbiosis, which does occur in cereals.

Ercolin and Reinhardt (2011) Trends in Plant Science
Endomycorrhizae and Rhizobia Produce Chemically Related LCO signals.

C  Myc-LCOs

Glomus intraradices

D  Nod factors

Sinorhizobium meliloti  Mesorhizobium loti

What would one engineer into plants?

The symbiosis signaling pathway of legumes. The common Sym pathway is required for nodulation and mycorrhization. Nodulation specific receptor kinases and transcription factors lie upstream and downstream of the common Sym pathway. Nod factor induced calcium oscillations are indicated. Where appropriate the names of orthologous genes from *Lotus* and *Medicago* are shown.
In two recent reviews, Venkateshwaran et al. (2013) and Delaux et al. (2013) argue that it is the conservation of a core set of symbiotic genes that determines whether plants are capable of entering into a symbiosis with either rhizobia or mycorrhizae. For example, they argue that Brassicaceae (e.g., Arabidopsis) lack many of these genes.

Venkateshwaran, et al, Symbiosis and the social network of higher plants, Current Opinion in Plant Biology, Volume 16, Issue 1, February 2013, Pages 118-127

Two possible hypotheses to explain the lack of rhizobial symbiosis in some plants (e.g., monocots) are that

- They lack the ability to recognize the Sym signals (e.g., Nod factor)

and/or

Yan Liang, Yangrong Cao, Sandra Thibivilliers, Jinrong Wan, Kiwamu Tanaka, Jeongmin Choi, Changho Kang, Gary Stacey (2013) Non-legumes respond to Rhizobial Nod Factors by suppressing MAMP-triggered innate immunity. Science 341: 1384-1387
P(M)AMPs (Pathogen/Microbe Associated Molecular Patterns) are small molecular motifs consistently found on pathogens. They are recognized by pattern recognition receptors (PRRs) in plants and animals, leading to the induction of innate immunity.

Mechanisms of Plant Defense

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ergosterol</td>
<td>Fungi</td>
<td>Emumera et al., 2004</td>
</tr>
<tr>
<td>Xylanase (TKLGE)</td>
<td>Fungi</td>
<td>Ron et al., 2004</td>
</tr>
<tr>
<td>Pep-13</td>
<td>Oomycetes</td>
<td>Brunner et al., 2002</td>
</tr>
<tr>
<td>EF-Tu (Elf 18)</td>
<td>Bacteria</td>
<td>Zipfel et al., 2006</td>
</tr>
<tr>
<td>LPS</td>
<td>Bacteria</td>
<td>Erbs et al., 2003</td>
</tr>
<tr>
<td>Flagellin (Flg22)</td>
<td>Bacteria</td>
<td>Gomez-Gomez et al., 2002; Meziane et al., 2005</td>
</tr>
<tr>
<td>Chitin</td>
<td>Fungi</td>
<td>Felix et al., 1993 Ramonell et al., 2002</td>
</tr>
</tbody>
</table>
ROS assays on soybean leaf discs from 2 weeks old plants

Control Chitin (640µg/ml)

Xylanase (1µM) (Pep 1, Elf18)

Chitin (640µg/ml)

flg22 (1µM)

Control chitin flg22 chitin+flg22

Use a combination of MAMPs to elicit a stronger response and to activate MTI with both bacterial (flg22) and fungal (chitin) MAMPs (synergistic effect; Aslam et al., 2009)
Identification of chitooligomers involved in synergistic effect with flg22

Histogram representing ROS production after 20 minutes of flg22 and diverse chitin oligomers (1µM) treatments.
Different letters represent the statistical difference between the treatments with a p-value=0.05
NOD FACTOR (NF) REDUCED FLG22-TRIGGERED ROS PRODUCTION IN SOYBEAN LEAVES

PRETREATMENT ENHANCED THE SUPPRESSIVE EFFECT

![Graph showing suppression of ROS production](image_url)
Nod Factor and chitotetraose (C4) reduced ROS production in soybean leaves. C8-triggered ROS production in soybean leaves:

- **flg22**
- **C8**

Graph showing ROS production (% of control) with significant differences indicated by asterisks. **H2O**, NF, C4.
One nanomolar of NF reduced FLG22-triggered ROS production in soybean.
NF AND C4 REDUCED FLG22-TRIGGERED ROS AND MAPK ACTIVATION IN ARABIDOPSIS LEAVES

Graph showing RLUX1000 (Relative Light Unit) for different treatments:
- H₂O
- NF
- C4
- flg22
- flg22 + NF
- flg22 + C4

Bar graphs with asterisks indicating significant differences.

Western blot analysis showing the following:
- H₂O
- flg22
- NF
- flg22 + NF
- C4
- flg22 + C4

Antibodies:
- Anti-phospho-p44/42-MAPK
- Non-specific band

Quantitative data:
- 1.0
- 0.67±0.17
- 0.88±0.02
NF and C4 reduced FLG22-triggered innate immunity as measured by in planta bacterial growth.
NF AND C4 REDUCED OTHER MAMP-TRIGGERED ROS PRODUCTION IN ARABIDOPSIS
NOD FACTOR INDUCED FLS2 AND EFR PROTEIN DEGRADATION BUT DID NOT REPRESS THEIR TRANSCRIPTION.

**Graphs and Images:**

- **Top Left:**
  - NF: -MG132: 1.0, +MG132: 0.6±0.1
  - +MG132: 1.2±0.1
  - Ponceau S: 1.2±0.1

- **Top Right:**
  - NF: -MG132: 1.0, +MG132: 0.6±0.1
  - +MG132: 1.2±0.1
  - Ponceau S: 1.2±0.1

- **Middle Left:**
  - NF: -MG132: 1.0, +MG132: 0.66±0.07
  - +MG132: 0.84±0.08
  - Ponceau S: 0.83±0.08

- **Middle Right:**
  - NF: -MG132: 1.0, +MG132: 0.6±0.1
  - +MG132: 1.2±0.1
  - Ponceau S: 1.2±0.1

- **Bottom Left:**
  - NF: -MG132: 1.0, +MG132: 0.66±0.07
  - +MG132: 0.84±0.08
  - Ponceau S: 0.83±0.08

- **Bottom Right:**
  - Graph showing relative transcript levels of FLS2 for H₂O, NF, and C₄.
NOD FACTOR LED TO A LOSS OF FLS2::GFP ON THE PLASMID MEMBRANE

GFP  |  FM4-64  |  Merge
---|---|---
-NF  | ![GFP image] | ![FM4-64 image] | ![Merge image]  
+NF  | ![GFP image] | ![FM4-64 image] | ![Merge image]
NF REDUCED MAMP-TRIGGERED ROS PRODUCTION IN CORN AND TOMATO

**Com**

**Tomato**

[Graphs showing RLU x 1000 (Relative Light Unit) for different treatments in Corn and Tomato]
High Affinity (~1 nM) Chitin and Lipo-Chitin Signaling Pathways in Plants

**ALL PLANTS**

- **Chitoolctaose receptor**
- **Chitin**

**SYMBIOTIC PLANTS**

- **Non-symbiotic NF receptor**
- **Symbiotic NF receptor**

**Induction of plant defense pathways**

**Suppression of plant innate immunity**

**Induction of symbiotic development**
Three topic areas discussed:
1. Developing a rhizobial symbiosis in cereals
2. The introduction and encouragement of diazotrophic bacteria and cereal crop interactions
3. Synthetic biology, the design of a new organelle to fix N in crop plants

Note that of these three topics, topic 2 was the only one not chosen by the Gates foundation for funding. Primarily because there were few advocates of this approach at the meeting.
The introduction and encouragement of diazotrophic bacteria and cereal crop interactions

I originally ranked this topic as the more achievable goal among the three discussed?

Because agricultural relevant systems already exist in nature, have been harnessed for practical use and support a small but growing commercial inoculant business (much more prevalent in South America)

Special Issue of Plant and Soil Volume 356, July 2012. “The role of biological nitrogen fixation by non-legumes in the sustainable production of food and biofuels”
N$_2$-fixation associated with grasses

A brief ‘cyclical’ history

- “Azotobacterin” in Russia / *Azotobacter paspali* associated with *Paspalum* - Döbereiner
  - 1972 Brown - concluded inoculation responses due to hormonal effects of the bacteria
- The rhizosphere acetylene-reduction/ inoculation era
  - 1972 Döbereiner, Day and Dart - ARA associated with roots/*Spirillum lipoferum*
  - 1976 Smith et al. Science - inoculation responses to *Azospirillum* in USA
  - 1979 Tien et al./Okon et al. - inoculation responses due to hormonal effects
- The endophyte/sugar cane era
  - 1986/88 Baldani/ Döbereiner *Herbaspirillum/Gluconacetobacter*
  - 1980’s/1990’s Boddey/Urquiaga et al - large amounts of N$_2$ fixed

Recurrent, although sporadic, reports of biological nitrogen fixation supported by $^{15}$N incorporation---consensus in Brazil is that sugarcane receives $\sim$20% of its nitrogen from associative nitrogen fixation—some reports in wild grasses up to 60-70%. Rumors of corn genotype with high fixation rates???
Colonization of grass roots by diazotrophic endophytes

*Azoarcus* spp., *Herbaspirillum* spp., *Gluconacetobacter diazotrophicus*, some *Azospirillum* and rhizobia, *Klebsiella*, *Pseudomonas*

Hurek et al. (1994) *J. Bacteriol.* 176: 1913
Predicted and demonstrated interactions

- Ingress and spreading
- Cellulases, pectinases
- ROS detoxification
- Type V secretion
- Type VI secretion
- ACC deaminase
- Phytohormone production
- ACC production and uptake
- Iron: Siderophore production and uptake
- Nitrogenase
- PHB / PHA synthesis
- Fumarate respiration, butane-diol, butanol fermentation
- Metabolic adaptations

Plant growth promotion, biocontrol, phytoremediation
Effect of inoculation of maize CD 304 with a commercial inoculant containing *Azospirillum brasílense* AB-V5 and Ab-V6 on grain productivity.
Maize plants inoculated with *Azospirillum brasilense* AB-V5 e Ab-V6 were more resistant to drought.
The introduction and encouragement of diazotrophic bacteria and cereal crop interactions

Was my original ranking justified....does this area indeed hold promise?

✓ There are clearly well supported reports in the literature, albeit sporadic, of significant levels of nitrogen fixation and incorporation in plants, although only a few in crop plants (e.g., sugarcane)

✓ However, these reports and, indeed, the entire area is met with some skepticism by the wider scientific community.

✓ I believe this is largely due to the fact that the field is dominated by phenomenological reports, with few mechanistic studies, and even fewer molecular/ genetic studies....
Setaria viridis – A model for the study of diazotrophic-plant interaction

Fernanda P. Do Amaral

Vania C. Pankievicz

Karina Freire de Eça Nogueira Santos

Fabio Pedrosa
Emanuela de Souza
Univ. of Curitiba, Brazil
Setaria

- *S. viridis* is a problematic weed
- Foxtail Millet (*S. italica*) was domesticated from *S. viridis*
- Foxtail Millet is a significant crop and dietary staple in Northern China
- *Setaria* has been used as a potential model species for understanding basic biological processes
- *Setaria* is a C4 plant
- *S. viridis* is a small plant, easily grown under greenhouse conditions, with a rapid growth cycle (60 days)
- *Setaria* has a recently sequenced genome

*S. viridis*
Green Foxtail
Phylogenetic position of *S. italica* and *S. viridis* relative to selected important grass species.

System development to grow *S. viridis* to study the bacterial colonization.

---

**Seed Sterilization** *(Brutnell, 2010)*

- Seeds germination
- Seedling Inoculation
- Seedling Planting

**Bacterial culture**

- **O.D**<sub>600</sub> = 1.0
- 13000 rpm (3X)
- 48h dark, 20''

**1ml of washed Bacterial culture per seedling for 30 min.**

**Turface**: 1:1 Vermiculite

**Greenhouse**: 16/8h 30°C

The plants were watered with Hoagland nutrient solution twice a week, nitrogen was applied depending on the experiment.

**Bacterial mixture**

- *H. seropedicae* Ram4::DsRed fusion
- A. *brasilese* FP2 nifH::gusA
Parameters analyzed

• Plant height
• Root weight
• Total root length (WinRhizo Scanner and Software)
• Number of tips of the root (WinRhizo Scanner and Software)
• Shoot weight
• Flag leaf area
• Number of seeds
• Number of tiller
• Bacterial Recovered
• Bacterial Colonization followed by microscopy
# Parameters analyzed during plant development

<table>
<thead>
<tr>
<th>Germination</th>
<th>Germination Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radicle emerged from caryopsis</td>
<td></td>
</tr>
<tr>
<td>Coleoptile emerged from caryopsis</td>
<td></td>
</tr>
<tr>
<td>First leaf just at coleoptile tip</td>
<td></td>
</tr>
<tr>
<td>First leaf through coleoptile</td>
<td></td>
</tr>
<tr>
<td>First leaf unfolded</td>
<td></td>
</tr>
<tr>
<td>2nd leaf unfolded</td>
<td></td>
</tr>
<tr>
<td>3rd leaf unfolded</td>
<td></td>
</tr>
<tr>
<td>4th leaf unfolded</td>
<td></td>
</tr>
<tr>
<td>5th leaf unfolded</td>
<td></td>
</tr>
<tr>
<td>6th leaf unfolded</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leaf development</th>
<th>Leaf development Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>First tiller detectable</td>
<td></td>
</tr>
<tr>
<td>2nd tiller detectable</td>
<td></td>
</tr>
<tr>
<td>3rd tiller detectable</td>
<td></td>
</tr>
<tr>
<td>4th tiller detectable</td>
<td></td>
</tr>
<tr>
<td>5th tiller detectable</td>
<td></td>
</tr>
<tr>
<td>First node at least 1 cm above tillering node</td>
<td></td>
</tr>
<tr>
<td>Node 2</td>
<td></td>
</tr>
<tr>
<td>Node 3</td>
<td></td>
</tr>
<tr>
<td>Node 4</td>
<td></td>
</tr>
<tr>
<td>Node 5</td>
<td></td>
</tr>
<tr>
<td>Flag leaf just visible</td>
<td></td>
</tr>
<tr>
<td>Flag leaf fully enrolled (ligula just visible)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tillering</th>
<th>Tillering Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>First tiller detectable</td>
<td></td>
</tr>
<tr>
<td>2nd tiller detectable</td>
<td></td>
</tr>
<tr>
<td>3rd tiller detectable</td>
<td></td>
</tr>
<tr>
<td>4th tiller detectable</td>
<td></td>
</tr>
<tr>
<td>5th tiller detectable</td>
<td></td>
</tr>
<tr>
<td>First node at least 1 cm above tillering node</td>
<td></td>
</tr>
<tr>
<td>Node 2</td>
<td></td>
</tr>
<tr>
<td>Node 3</td>
<td></td>
</tr>
<tr>
<td>Node 4</td>
<td></td>
</tr>
<tr>
<td>Node 5</td>
<td></td>
</tr>
<tr>
<td>Flag leaf just visible</td>
<td></td>
</tr>
<tr>
<td>Flag leaf fully enrolled (ligula just visible)</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Main stem elongation</th>
<th>Main stem elongation Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early boot: flag leaf sheath extending</td>
<td></td>
</tr>
<tr>
<td>Flag leaf sheath opening</td>
<td></td>
</tr>
<tr>
<td>First awns visible</td>
<td></td>
</tr>
<tr>
<td>Beginning: tip of inflorescence emerged from sheath</td>
<td></td>
</tr>
<tr>
<td>One-fourth of head emerged and beginning of peduncle elongation</td>
<td></td>
</tr>
<tr>
<td>Middle of heading: half of inflorescence emerged</td>
<td></td>
</tr>
<tr>
<td>Three-fourths of head emerged</td>
<td></td>
</tr>
<tr>
<td>End of heading: inflorescence fully emerged</td>
<td></td>
</tr>
<tr>
<td>Beginning of flowering: first anthers visible</td>
<td></td>
</tr>
<tr>
<td>End of flowering: all spikelets have completed flowering but some dehydrated anthers may remain</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heading</th>
<th>Heading Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watery: first grains have reached half their final size</td>
<td></td>
</tr>
<tr>
<td>Early dough</td>
<td></td>
</tr>
<tr>
<td>Fully ripe: grain hard, difficult to divide with thumbnail</td>
<td></td>
</tr>
<tr>
<td>Overripe: grain very hard, cannot be dented by thumbnail</td>
<td></td>
</tr>
<tr>
<td>Grains loosening in daytime</td>
<td></td>
</tr>
<tr>
<td>Plant dead and collapsing</td>
<td></td>
</tr>
<tr>
<td>Harvested</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Harvested</th>
<th>Harvested Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant dead and collapsing</td>
<td></td>
</tr>
</tbody>
</table>
S. viridis Genotypes (~50 available)

Of the first 30 genotypes screened, only 3 showed a significant growth response to bacterial inoculation... hence, we conclude that plant genotype is a crucial factor.

<table>
<thead>
<tr>
<th>Exp #</th>
<th>NAME</th>
<th>Genera</th>
<th>Species</th>
<th>#seeds</th>
<th>Germination Rate</th>
<th>GA added</th>
<th>Visual Plant Growth promotion response</th>
<th>Bacteria inoculated- RAM4</th>
<th>Bacteria inoculated FP2-7</th>
<th>Result</th>
<th>Day of analyses (40 DAI)</th>
<th>Next step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thompson</td>
<td>Setaria</td>
<td>viridis</td>
<td>100</td>
<td>34.8%</td>
<td>yes</td>
<td>na</td>
<td>1.0E+05</td>
<td>1.0E+05</td>
<td>only one plant left</td>
<td>na</td>
<td>harvest seeds</td>
</tr>
<tr>
<td>2</td>
<td>Estep ME035</td>
<td>Setaria</td>
<td>viridis</td>
<td>50</td>
<td>40.0%</td>
<td>yes</td>
<td>na</td>
<td>3.80E+06</td>
<td>8.80E+07</td>
<td>7 plants left</td>
<td>46 DAI</td>
<td>Experiment replica</td>
</tr>
<tr>
<td>3</td>
<td>S. viridis A10-1</td>
<td>Setaria</td>
<td>viridis</td>
<td>&gt;1000</td>
<td>90.0%</td>
<td>yes</td>
<td>++</td>
<td>3.80E+07</td>
<td>8.80E+07</td>
<td>only one plant left</td>
<td>40 DAI</td>
<td>follow colonization</td>
</tr>
<tr>
<td>4</td>
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<td>14</td>
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<td></td>
<td>4.20E+07</td>
<td>7.30E+06</td>
<td>only one plant left</td>
<td>na</td>
<td>harvest seeds</td>
</tr>
<tr>
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<td>4.20E+07</td>
<td>7.30E+06</td>
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<td>40 DAI</td>
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<td>50</td>
<td>29.2%</td>
<td>yes</td>
<td></td>
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<td>7.30E+06</td>
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<td>na</td>
<td>harvest seeds</td>
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<td>7.30E+06</td>
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<td>40 DAI</td>
<td>Plant to get seeds</td>
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<td>48.0%</td>
<td>yes</td>
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<td>4.20E+07</td>
<td>7.30E+06</td>
<td>4 plants left</td>
<td>40 DAI</td>
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<td>growing</td>
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<td>54</td>
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<td>measurements</td>
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<td>viridis</td>
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<td>1.04E+08</td>
<td>8.00E+05</td>
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<td>measurements</td>
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<td>viridis</td>
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<td>1.00E+05</td>
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<td>12/22/12</td>
<td>measurements</td>
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<td>Waselkov Vandali</td>
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<td>viridis</td>
<td>41</td>
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<td>no</td>
<td>growing</td>
<td>1.70E+08</td>
<td>1.00E+05</td>
<td></td>
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<td>viridis</td>
<td>72</td>
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<td>no</td>
<td>growing</td>
<td>1.70E+08</td>
<td>1.00E+05</td>
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<td>12/22/12</td>
<td>measurements</td>
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<td>1.00E+05</td>
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<td>12/22/12</td>
<td>measurements</td>
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</table>
Effects of inoculation of S. viridis A10-1 with H. seropedicae and A. brasilense

Soil: Promix 1:1 Sunshine
No nitrogen added

<table>
<thead>
<tr>
<th></th>
<th>No N</th>
<th>No N + BACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10.1 *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 + Bacteria</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.01

Graph showing:
- Inflorescence length (cm)
- Length of the third inter-node from the base
- Height (cm)

Photos showing plants labeled as No ENP and No EN + BACT.
Effects of inoculation of *S. viridis* A10-1 with *H. seropedicae* and *A. brasilense*

### Graphs

- **Left Graph**
  - X-axis: A10.1, A10.1+Bacteria
  - Y-axis: Height (cm)
  - Data points for Height (cm) for A10.1 and A10.1+Bacteria with error bars.

- **Middle Graph**
  - X-axis: A10.1, A10.1+Bacteria
  - Y-axis: Weight
  - Data points for Weight for A10.1 and A10.1+Bacteria with error bars.

- **Right Graph**
  - X-axis: A10.1, A10.1+Bacteria
  - Y-axis: Number of seeds
  - Data points for Number of seeds for A10.1 and A10.1+Bacteria with error bars.

### Notes

- *Nps* indicates no significant difference.
- *p < 0.05* indicates statistical significance.
- *p < 0.01* indicates highly significant difference.

---

Exp2: Soil
Turface 3:1 Vermiculite
No nitrogen
Effects of inoculation of \textit{S. viridis} A10-1 with \textit{H. seropedicae} and \textit{A. brasilense}

Exp 2: Soil
Turface 3:1 Vermiculite
No nitrogen

Roots colonized after 40 days of inoculation (D.A.I)
Effects of inoculation of *S. viridis* A10-1 with *H. seropedicae* and *A. brasilense* – Low N

![Graph 1: Height vs. Treatment](image1)

![Graph 2: Shoot fresh weight vs. Treatment](image2)

![Graph 3: Root dry weight vs. Treatment](image3)
NifH-Gus-Staining could be observed on *S. viridis* A10.1 growing under sterile conditions.

Tip Box 11 D.A.I

Test Tube 15 D.A.I
**Setaria viridis**: A Model Grass to Explore Bacterial-Plant Growth Promotion and Associative N$_2$-Fixation.

**Objective:** To provide mechanistic insight underpinning host plant growth promotion.

**Approach:** Metabolic partitioning of new carbon into key pools was quantified using $^{11}$CO$_2$ administered to plants grown under normal nitrogen and nitrogen limitation. Azospirillum brasilense and Herbaspirillum seropedicae N$_2$-fixing bacterial strains were introduced under N-limitation.

**Results/Impact:** N-limitation causes stress to the plant resulting in changes in carbon metabolism. The presence of bacteria re-establishes normal carbon metabolism under N-limitation.
rhizobacteria in non-leguminous grass systems. To date, research on \( N_2 \)-fixing rhizobacteria in non-leguminous grass systems has only inferred that host plants acquire biological nitrogen based on growth characteristics, but without direct evidence of this. Our objective was to provide this evidence leveraging the power for measuring minute amounts of fixed radioactive \(^{13}\)NN.

**Approach:** A remotely operated \(^{13}\)NN pulsing station was recently installed at the BNL Plant Radiotracer Facility that taps \(^{13}\)NN as a by-product from the \(^{13}\)CO\(_2\) cyclotron production target and re-directs the \(^{13}\)NN tracer through the soil column.

**Results/Impact:** Based on \(^{13}\)N data, we calculated a cumulative \( N_2 \)-fixing rate of 125 \( \pm \) 36 nmoles per day. Approximately 30\% of that nitrogen is acquired by the host and moved to aerial tissues. We estimate this...
Biological transport of fixed $^{13}$N to aboveground *Setaria* shoots demonstrated.

Only $^{14}C$ signature is seen in shoots immediately after pulse.

Biological transport of radiotracer from roots-to-shoots is evident under high light.

A $^{14}N$ signature can be seen in shoots after biological transport.
CONCLUSIONS

- We seem to be in an era of increasing interest and appreciation for biological nitrogen fixation.
- The ongoing strong record of research advances in the area of biological nitrogen fixation provide optimism for the notion that this research can be translated for practical benefit.
- Changes in the agricultural industry have created a more receptive environment for biological products.
- However, challenges remain and agricultural research continues to be undervalued.
  - Our research suggests that it is an inability to couple Nod factor recognition to symbiotic developmental pathways that is the missing link in non-legumes, not an inability to recognize the NF.
  - We believe that non-symbiotic, associative nitrogen fixation continues to hold significant promise and research in this area will be stimulated by the adoption of Setaria as a model system.
Many people to thank...

**My lab:**

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- Z. Yan
- C. Nguyen
- Y. Cui

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- Tom Clemente
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- Roger Boerma
- Dong Xu
- Jianlin Cheng
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- Grover Shannon
- Alan Jones
- Simon Gilroy
- Jeff Doyle
- Susan Singer
- Kristin Bilyeu
- J.C. Hong

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- Gyeongsang National University, Jinju,
Thanks for listening...