Biofertilizer: Genetically Engineered *Azospirillum brasilense* for extended ammonia production

Tim Schnabel, PhD – Stanford Bioengineering

3rd February 2022

The New York Times

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- Farmers may have to use fewer nutrients or pass on the costs
- Gas crisis, export restrictions hit nitrogen fertilizer supply

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Business of Sport

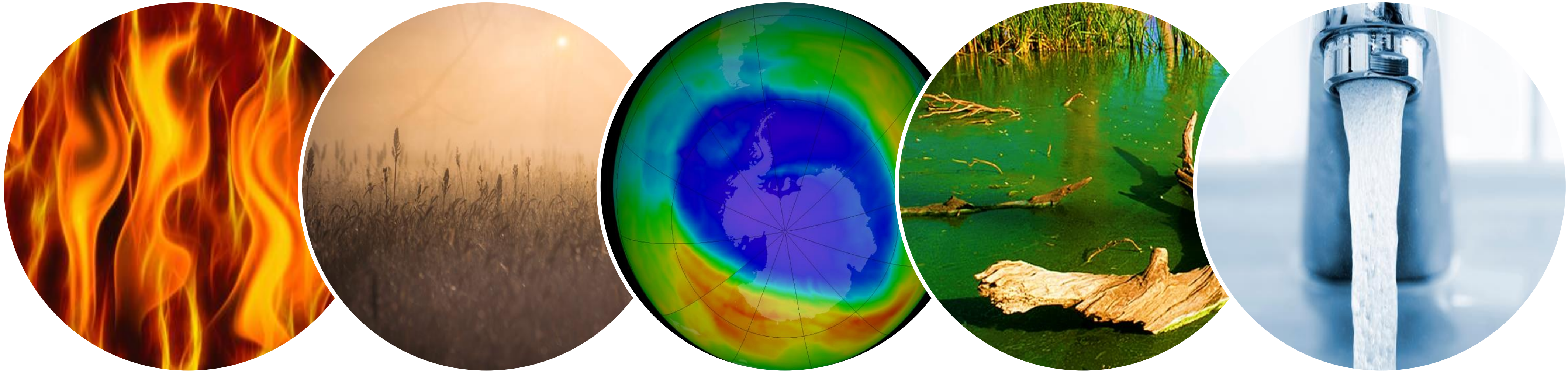
Poorest face food crisis amid fertiliser shortage

26 November 2021

THE WALL STREET JOURNAL.

Synthetic N fertilizer damages the planet

energy intensive — **greenhouse gases** — **ozone depletion** — **ecosystem leaching** — **groundwater poisoning**



- **energy intensive:** 300 atm, 500^{o}C, 1-2% global energy
- **greenhouse gases:** N_2O is 300X more potent than CO_2
- **ozone depletion:** one NO destroys 100,000 O_3
- **ecosystem leaching:** N-driven hypoxia, acidification, biodiversity loss
- **groundwater poisoning:** NO_3 implicated in cancers and birth defects

(Smith et al 2020, Solomon et al 2007, Lary 1997, Norton et al 2019, Ward et al 2018)

NAE GRAND CHALLENGES FOR ENGINEERING

NATIONAL ACADEMY OF ENGINEERING

The National Academy of Engineering (NAE) has named “Managing the nitrogen cycle” one of 14 Engineering Grand Challenges of the 21st century.

Engineering biology for a new solution

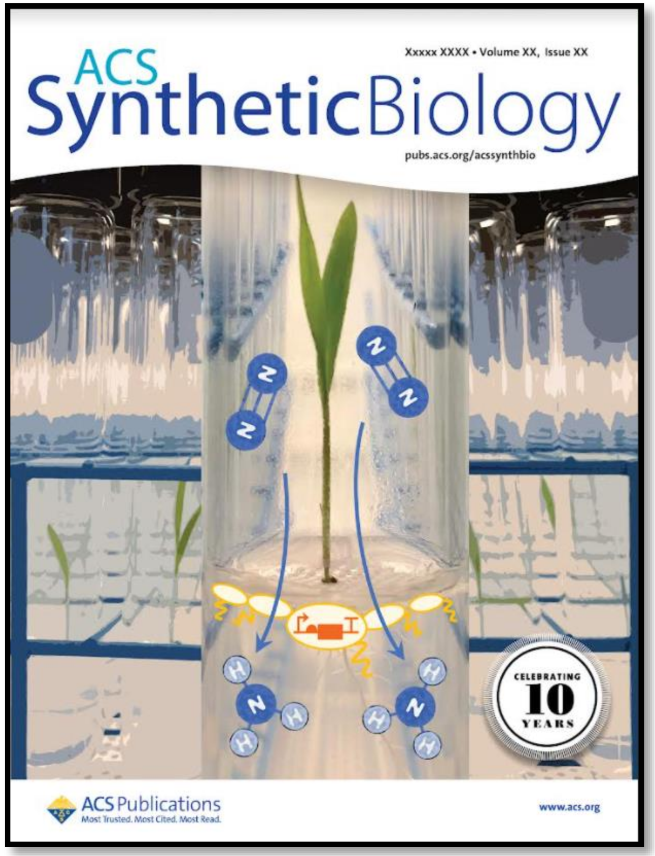
Schnabel T, Sattely E. 2021. Engineering post-translational regulation of glutamine synthetase for controllable ammonia production in the plant symbiont Azospirillum brasilense. Applied Environmental Microbiology.

- Press: Using nature’s miracle bugs to help feed the world

Schnabel T, Sattely E. 2021. Improved stability of engineered ammonia production in the plant-symbiont Azospirillum brasilense. ACS Synthetic Biology.

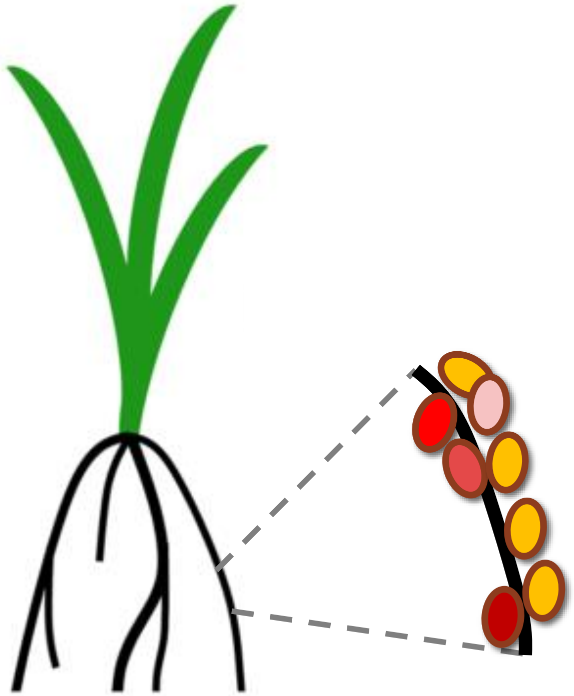
- Cover of ACS synthetic biology November 2021 issue (right).

Schnabel T, Sattely E. 2021. Optimal deactivation of glutamine synthetase in Azospirillum brasilense balances strain health with ammonia production rate leading to plant growth promotion in soil. In preparation.



SattelyLab

Host microbe: *Azospirillum brasilense* Sp245



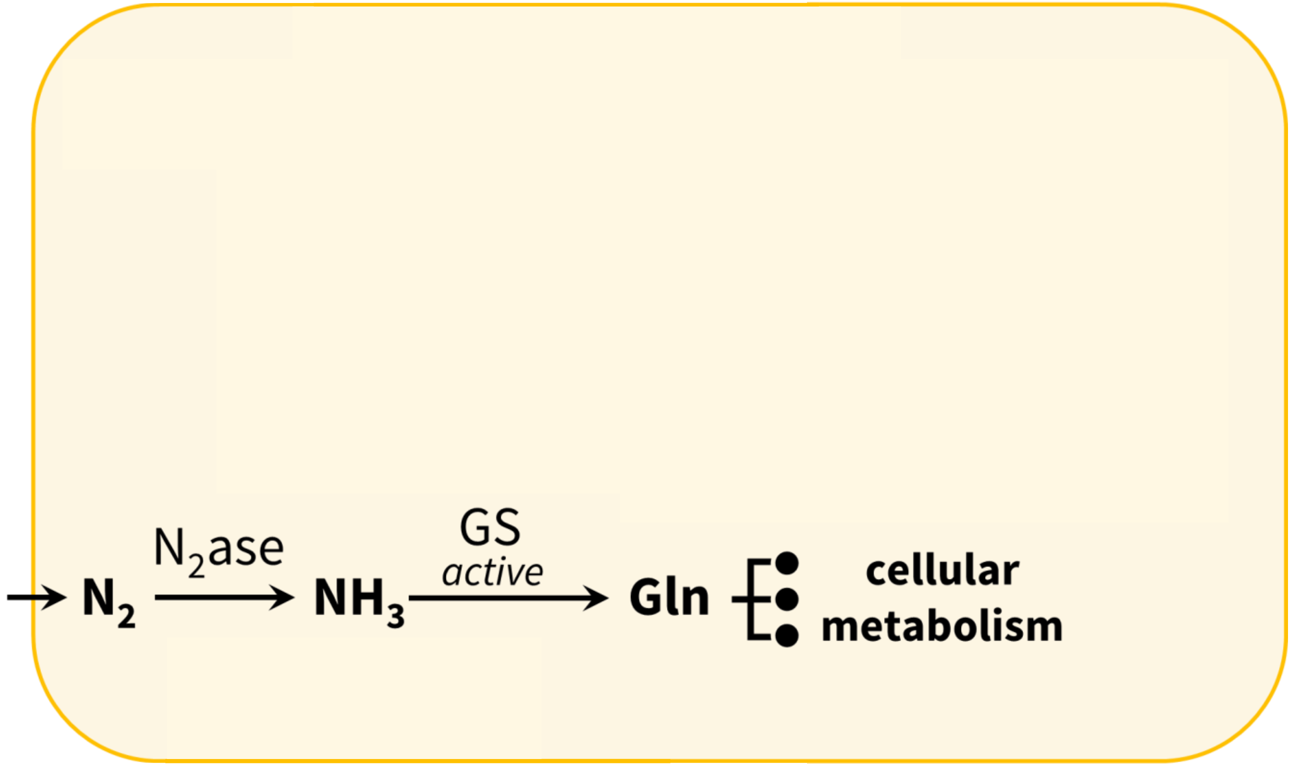
Genus: *Azospirillum*

- BSL-1, well-studied, gram negative, alpha proteobacteria, soil bacteria and symbiotic colonizers of grasses, broad-host range, nitrogen-fixing, phytohormone producing (Steenhoudt and Vanderleyden 2000)
- At least 22 species found around the globe, including some in the US (Cassan et al 2020, dos Santos Ferreira et al 2020).

Species: *brasilense*

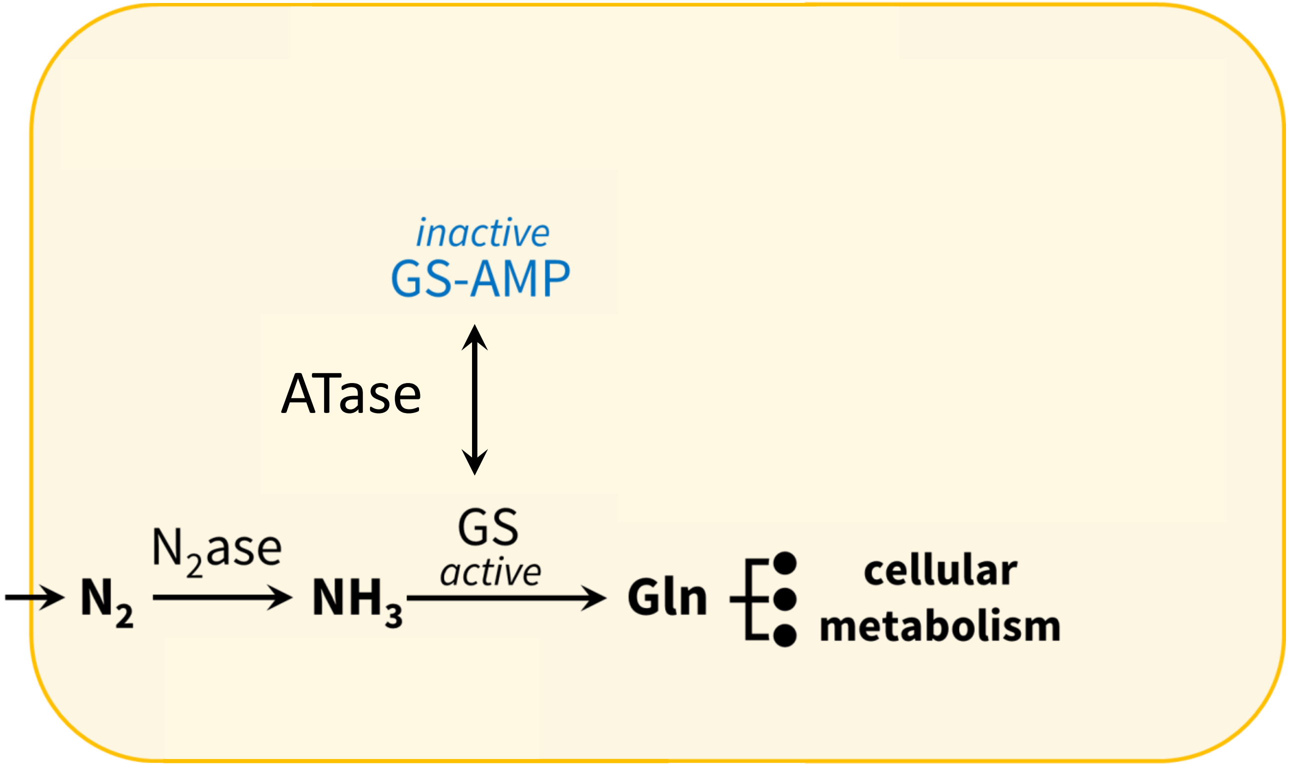
- Commercialized in many countries (~100 products, Cassan et al 2020), mostly in South America, but also products in the US.
- Strain Sp245 was originally isolated in 1983 from grass roots in Brazil (Baldani et al 1983).
- *Azospirillum brasilense* Sp245 was recently renamed to *Azospirillum baldaniorum* sp. nov (dos Santos Ferreira et al 2020).

Genetic modifications to yield NH_3 excretion



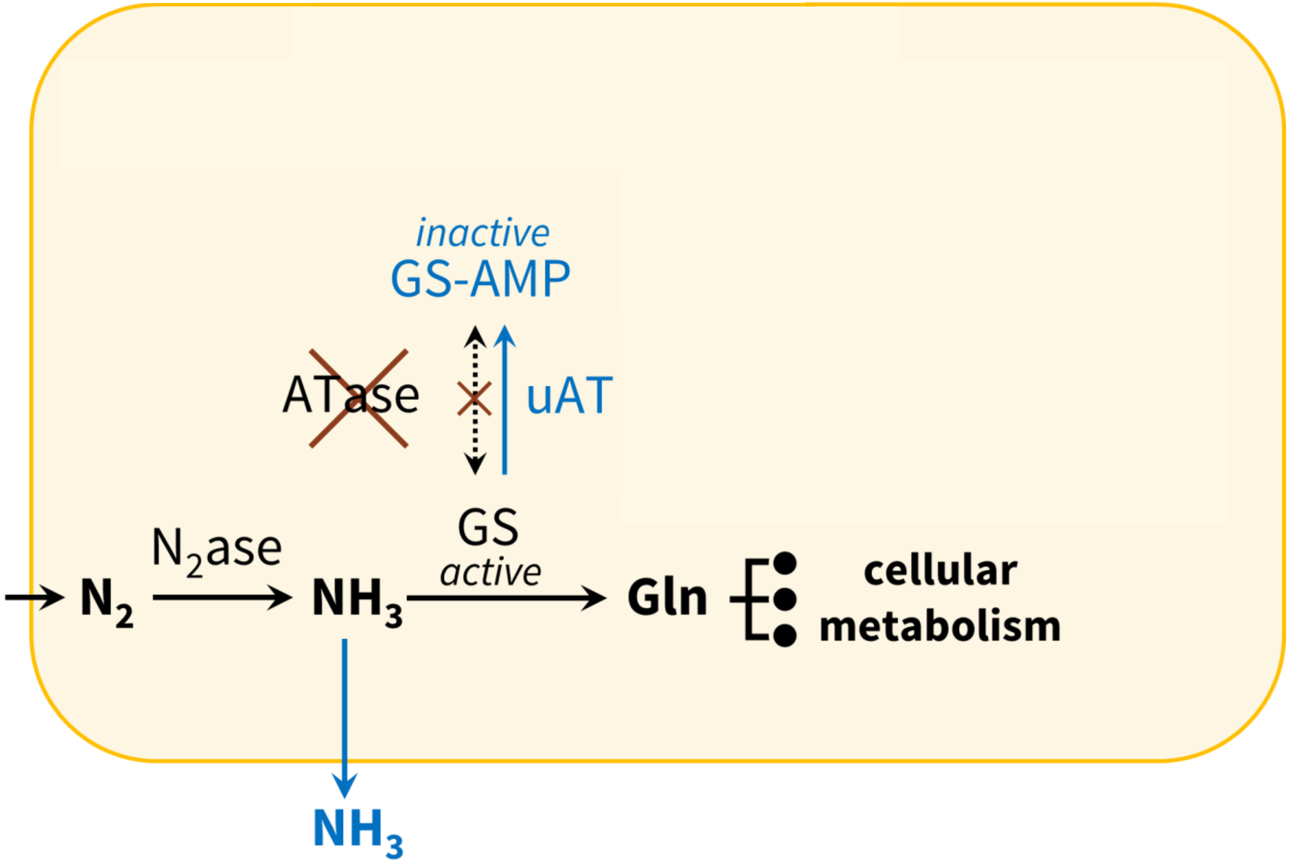
After N_2 is fixed into NH_3, it is assimilated by glutamine synthetase (GS)

Genetic modifications to yield NH_3 excretion



GS can be post-translationally deactivated by adenylylation (reversible) via the bidirectional adenylyltransferase (ATase, *glnE*)

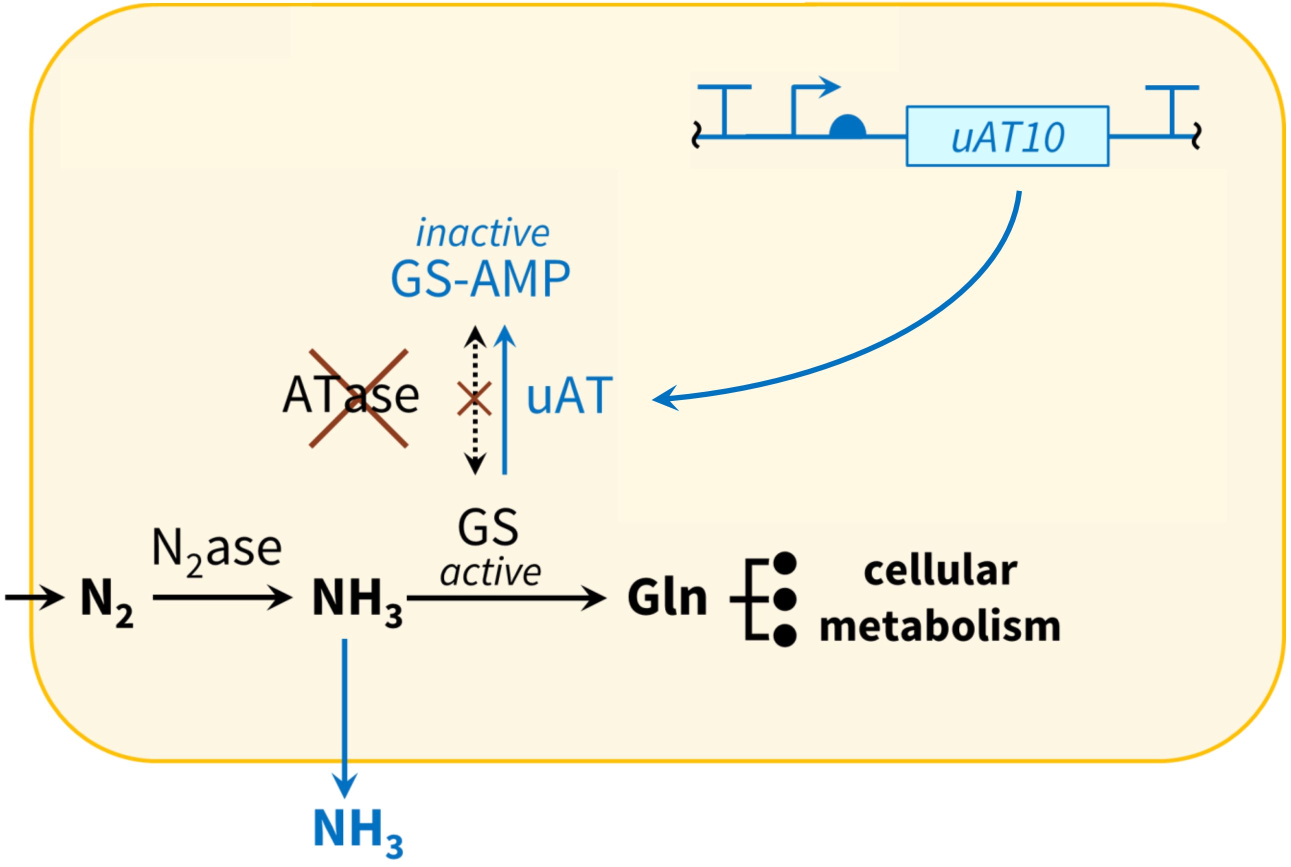
Genetic modifications to yield NH_3 excretion



We replaced ATase with an engineered unidirectional adenylyltransferase (uAT), resulting in NH_3 excretion

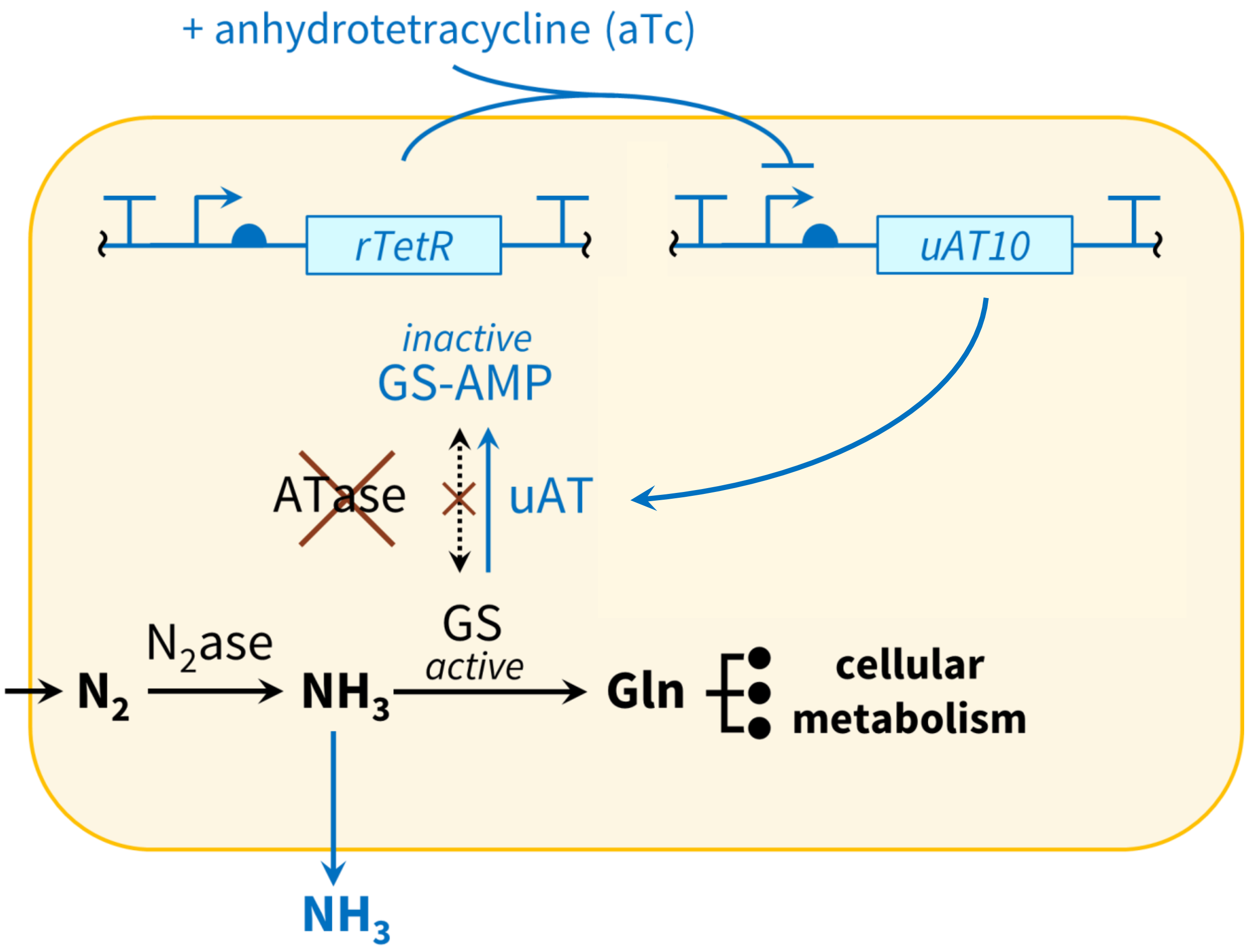
(Jaggi et al 1997, Jiang et al 2007, Schnabel and Sattely, AEM 2021)

Genetic modifications to yield NH_3 excretion



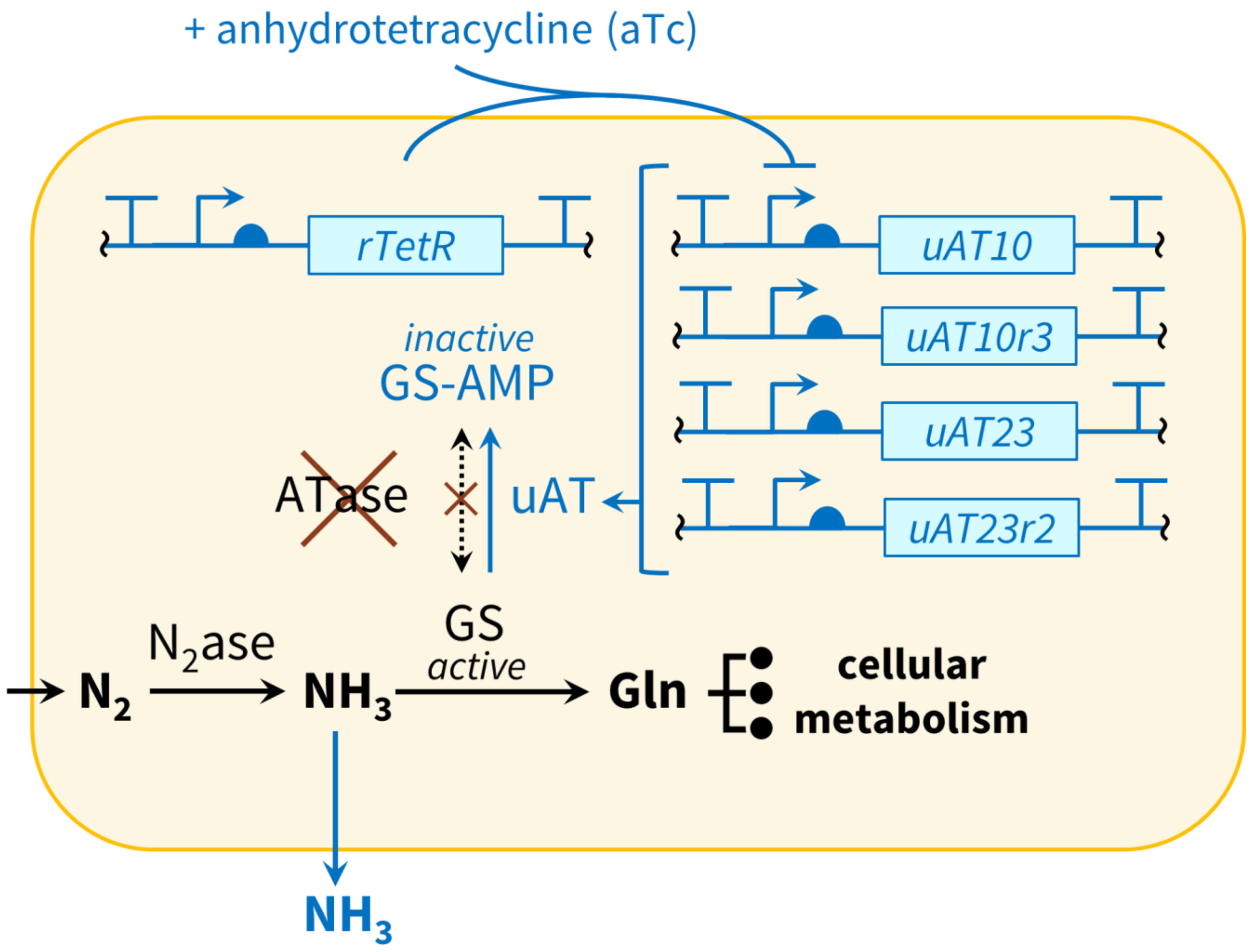
uAT is expressed from a synthetic circuit built with commonly used control parts.

Genetic modifications to yield NH_3 excretion



We can repress uATs in the presence of anhydrotetracycline using an rTetR control circuit.

Genetic modifications to yield NH_3 excretion



Multiple copies of uATs improve NH_3 production lifetime because they buffer against loss of function mutations.

(Tyo et al 2009, Schnabel and Sattely, ACS 2021)

Case study detail: uAT strain parts list

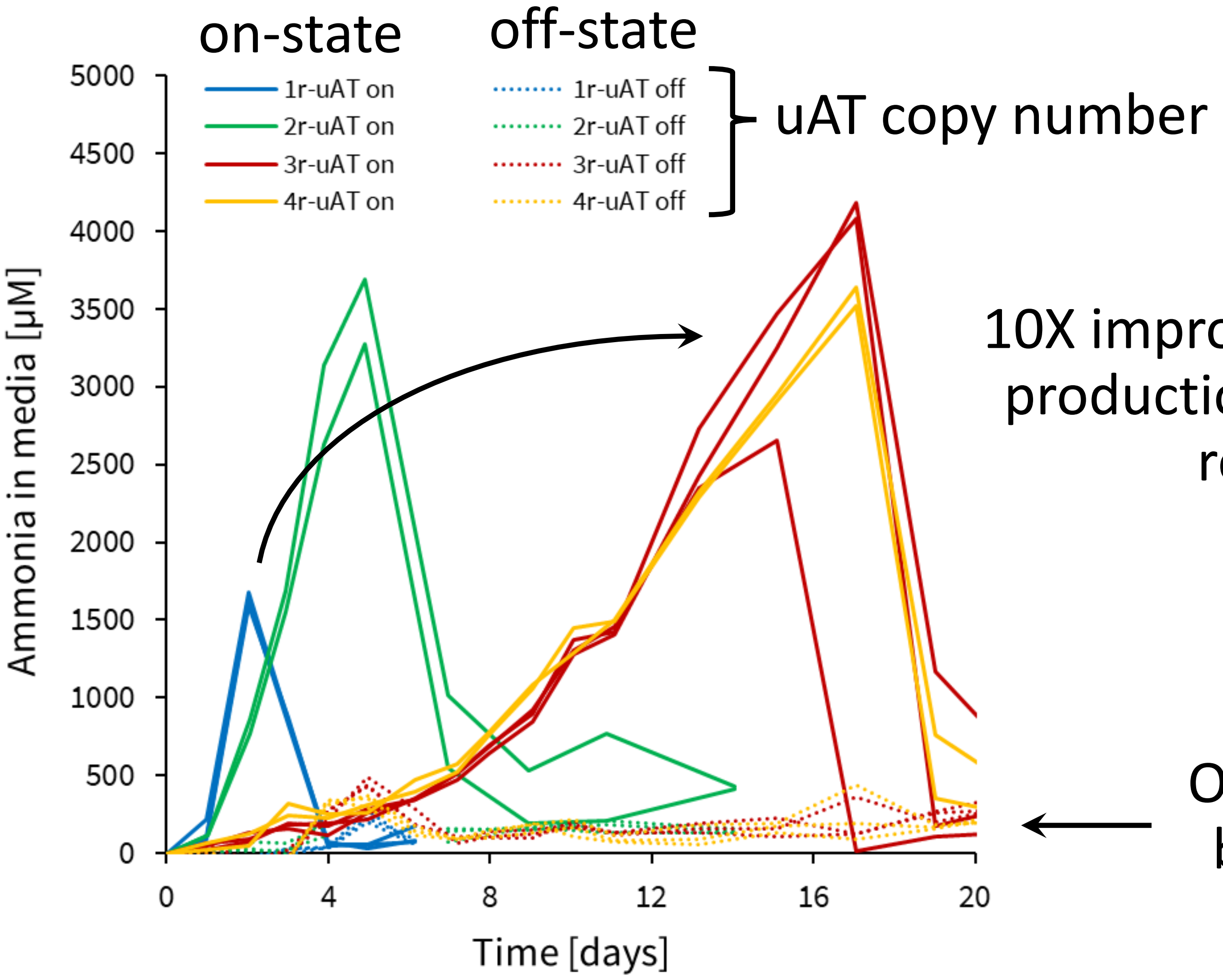
Part description	Part reference	Purpose	Origin
Promoters	pTetR (BBa_R0040), pΩ2	Precise transcription of uATs and rTetR	pTetR is from *E. coli* (BioBrick Registry of Standard Biological Parts), and pΩ2 is synthetic (assembled from multiple promoters from *E. coli* and *Delftia acidovorans*)
Ribosome binding sites and riboswitches	BBa_B0030, BBa_B0034, BCD2, riboJ	Precise translation of uATs and rTetR	Synthetic (BioBrick Registry of Standard Biological Parts, Mutalik et al 2013 and Lou et al 2012)
Terminators	BBa_B1002, BBa_B1006, BBa_B0015	Insulate synthetic circuits	Synthetic (BioBrick Registry of Standard Biological Parts)
Reverse Tet Repressor (rTetR)	BBa_C0040	Repress ammonia excretion in presence of anhydrotetracycline	Based on *E. coli*, modified with point mutations as described in Roney et al 2016, codon refactored
uATs	uAT10, uAT10r3, uAT23, uAT23r2	Ammonia excretion	*A. brasilense* Sp245 or *E. coli* K12 partial glnE genes and codon refactored versions thereof
Spacers	Various 2-30 bp length segments	Separate components, useful in DNA assembly process	Synthetic non-coding sequences, some containing restriction sites

All parts were scarlessly inserted into non-coding parts of the *A. brasilense ΔglnE* chromosome using pTS9 (Schnabel and Sattely 2021) as a delivery chassis in triparental mating with donor strain *E. coli* DH5alpha and helper strain *E. coli* HB101 pRK600. No markers remain.

Modification summary

- Symbiotic host, widely used, no negative effects on plants.
- Only primary metabolic modifications have been made, no secondary (e.g. toxin biosynthesis) modifications are present.
- The only coding modifications are uATs and rTetR.
- All other synbio parts are non-coding and commonly used.
- All modifications are scarless and in precise locations.

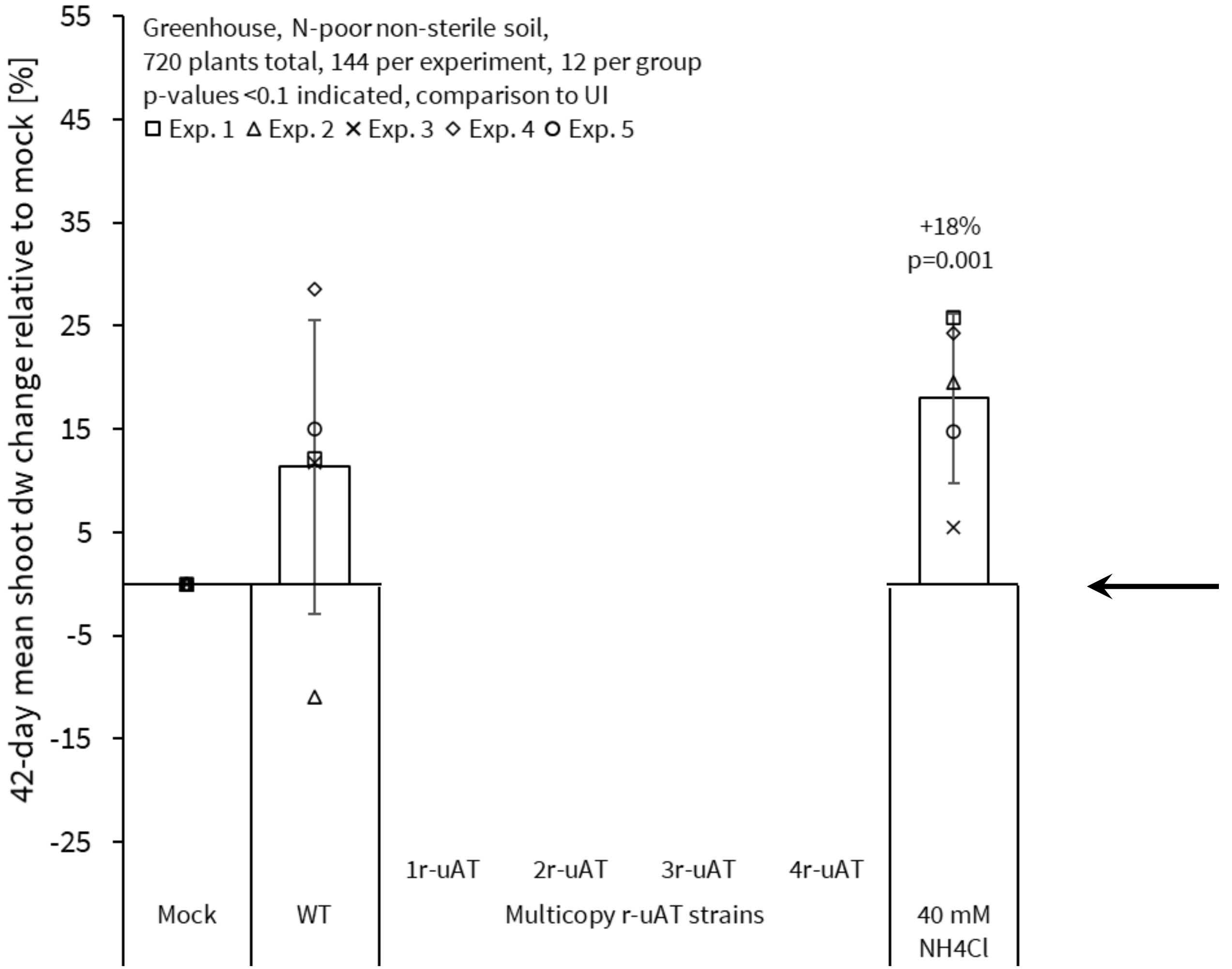
Ammonia production of uAT strains



10X improved lifetime of NH_3 production through genetic redundancy

Off-state strains behave as WT

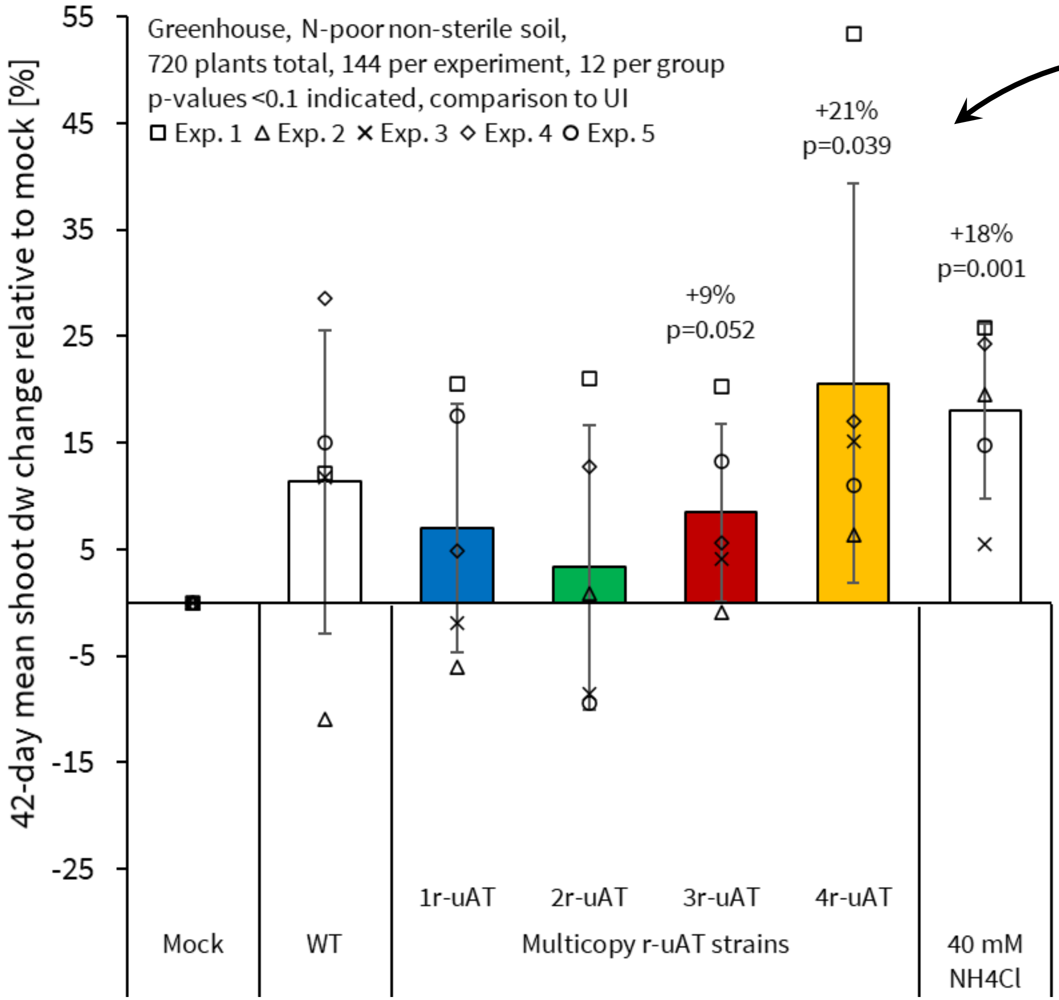
Plant growth promotion of *Z. mays*



Growth promotion was quantified in 5 independent replicate experiments of 144 plants each.

Here plotting the mean from each experiment, normalized to mock inoculation.

Plant growth promotion of *Z. mays*



4r-uAT (quadruple copy) strain has **+21%** growth promotion compared to mock.

We are working on additional strains with improved performance.

Towards field testing & future improvements

Advantages of uAT strains:

- The bottom-up modular design → performance can be tuned
- Control of NH_3 production → can be rewired to respond to other stimuli
- Multicopy redundancy → improved stability
- Conserved mechanism → can be adapted to other microbes

Question: GEMs are increasingly using synbio parts. How will their regulation be streamlined?

Commercial and Regulatory Implications

indigo

Indigo's go-to-market strategy for biological products is through channel partners

biotrinsic™ by indigo

Go-To-Market Channels	Description
(A) Partnership, Out-Licensing	Companies that sell Indigo products under their own brand
(B) Retail	Ag Stores that sell farm inputs directly to growers
(C) Distribution	Companies that supply retailers with farm inputs

Note: Retail partners includes partners that serve in both retail and distribution functions

Commercial strategy for GEM product

- Indigo will launch this *seed applied product* for use by *corn* growers in the *United States* for (1) nitrogen fertilizer reduction at yield parity and (2) yield increase under 100% nitrogen fertilizer application
- Product will be developed as a flowable powder (FP) for on-farm seed treatment and water dispersion (WD) for upstream treatment of corn

Field trialing strategy for GEM product

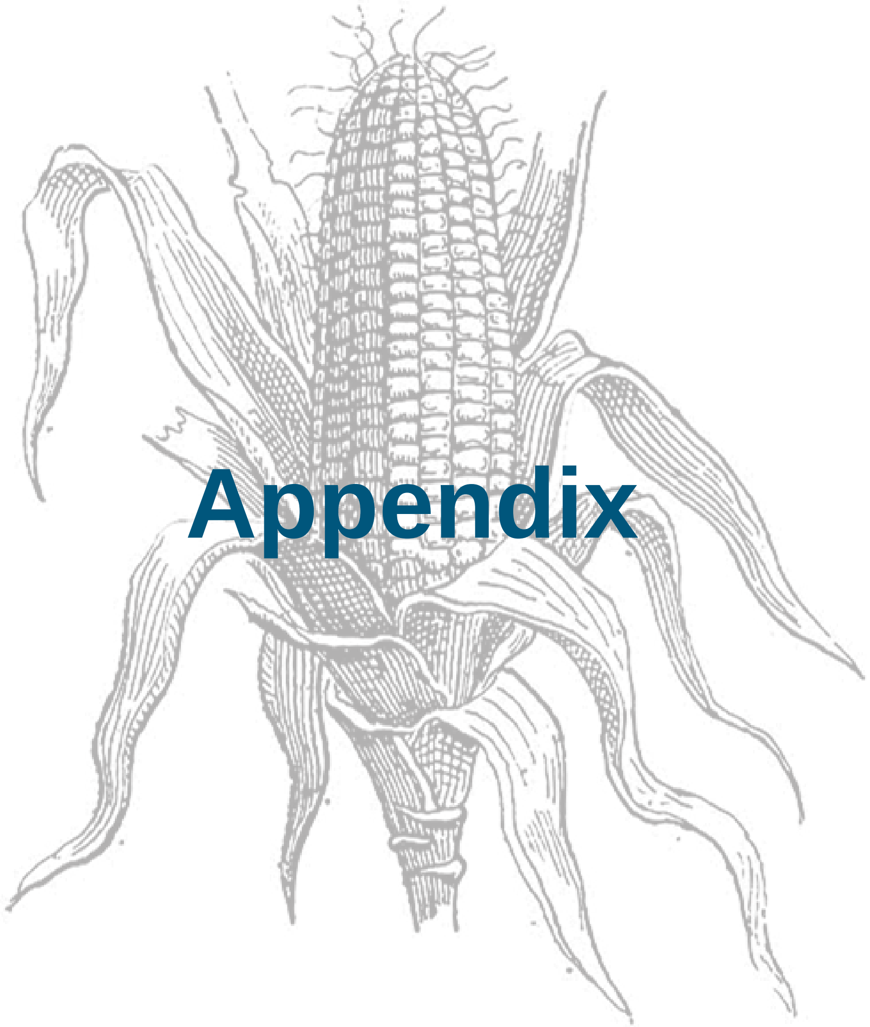
Trialing Methodology

- We run a minimum of two seasons for field testing of products for nitrogen use efficiency:
  - Season One consists of small, replicated field trials with universities and private CROs with a demonstrated high level of expertise in nutrient management field testing. The season one cohort is based on demonstrated efficacy in *in-vitro* and *in-planta* assays.
  - Season Two expands to large plot field trials in order to understand performance on larger acreage, in more regions, and across more broad environmental conditions and soil textures. The season two cohort is based on in-field performance in season one.

Trial Design

- Season One trials include a minimum of four replications per nitrogen rate per site. Nitrogen is applied at four rates per location on a gradient from 0 to 100% in order to understand performance across a gradient of nitrogen rates.
  - Regression analysis is used to determine the N rate needed with microbial-treated plots to match the yield of non-treated plots at 100% N application.
- Season Two trials include a minimum of two replications per site. Treatments are planted in 1-acre blocks at different nitrogen application rates and spatial planting and harvest data is obtained. Spatial modeling and machine learning are used to determine where, when, and how our products perform.

indigo



Appendix

Regulatory framework of GEM products for Agricultural use

Lab/Greenhouse Evaluation

Governing Bodies:
- USDA Biotechnology Regulatory Services (BRS)

Field Release

Governing Bodies:
- EPA BPPD - Emerging Technologies Branch
- USDA BRS

Commercialization

Governing Bodies:
- USDA BRS
- US States Departments of Ag
- EPA Biopesticides and Pollution Prevention Division (BPPD)