Addendum to OTRF: November 17, 2020

Directed evolution of endophytic microbes to promote nitrogen use efficiency (NUE) and drought tolerance in turfgrass

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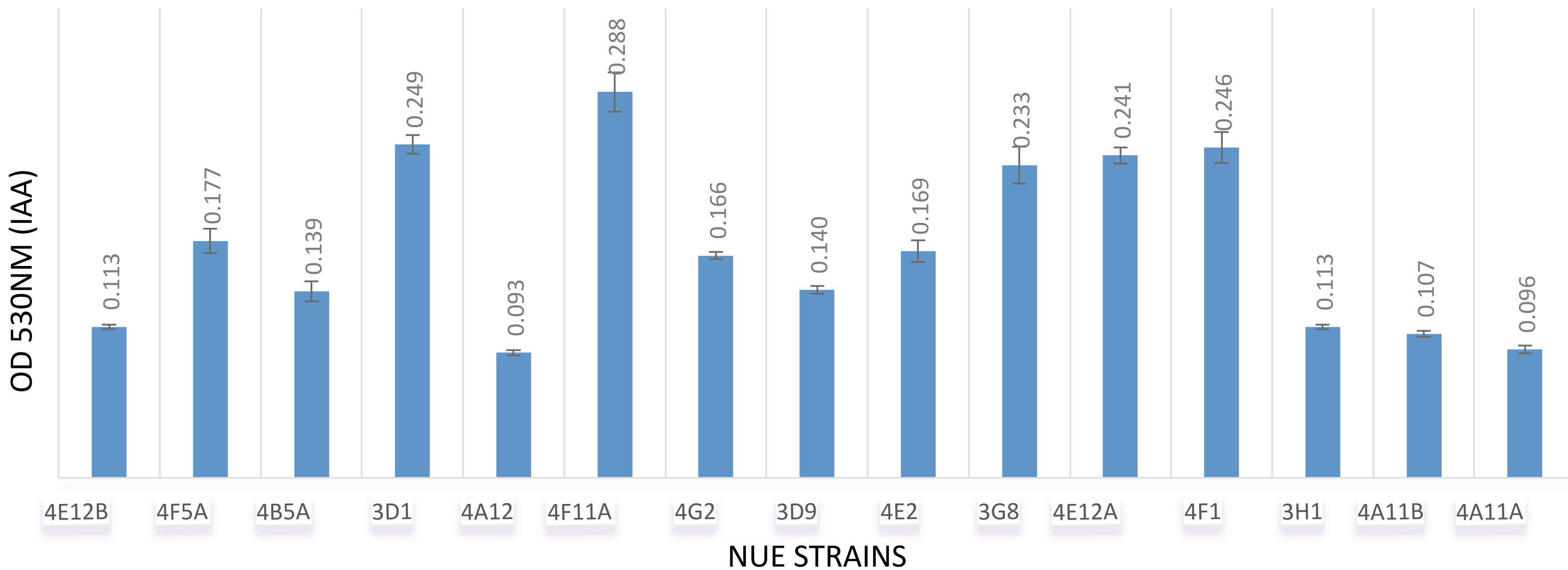
Testing bioinoculant physiology *in vitro*

-Investigating potential mechanisms by which bioinoculants might confer growth promotion under low N conditions
-Previously showed that the bacterial strains could grow on N-free media, could fix N in vitro and/or secrete the amino acid glutamine

Relative IAA (Auxin) secretion *in vitro*



- IAA, indole-3 acetic acid (auxin) – promotes root growth
- 3H1 has low IAA secretion……

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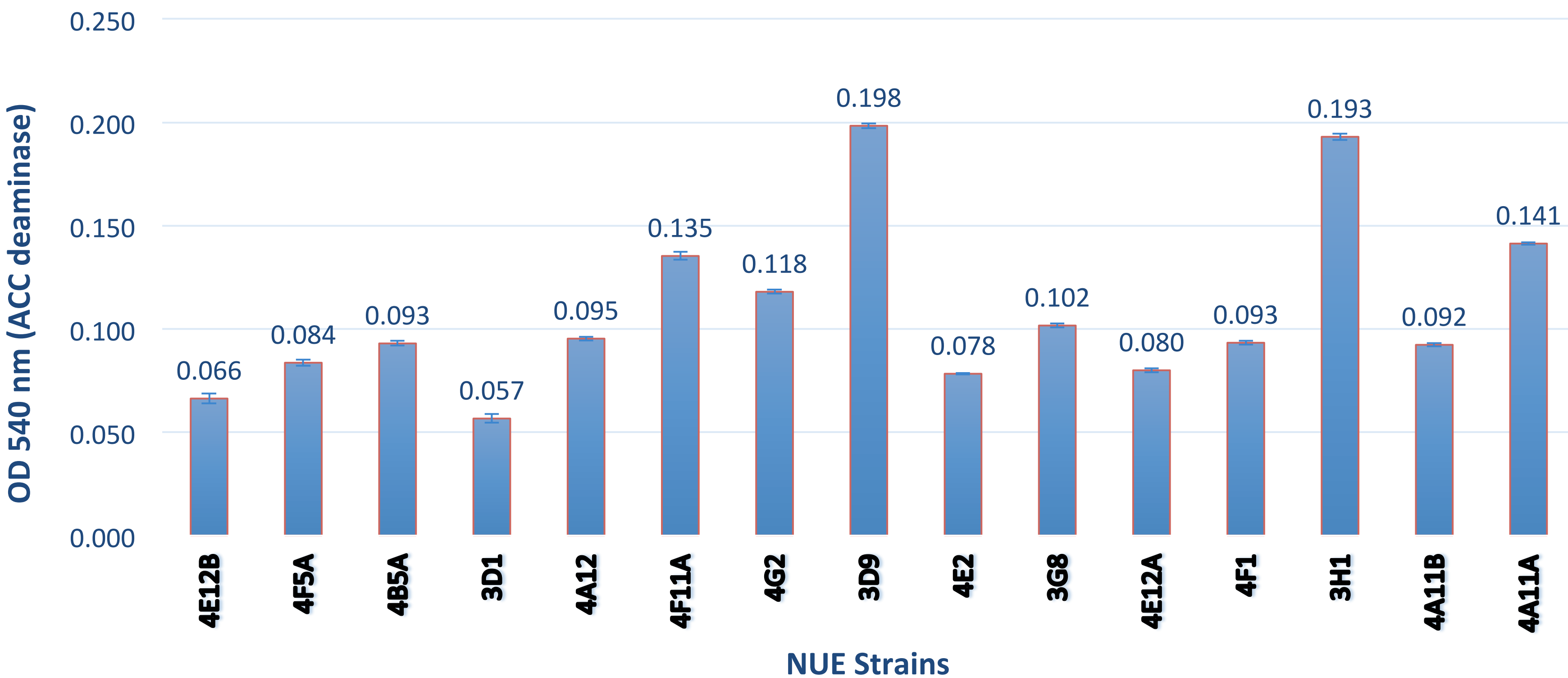
Relative concentration of ACC deaminase

[degrades the precursor of the hormone ethylene (which blocks root growth), and results in the production of ammonia as an N-source]



- ACC deaminase – reduces the level of "stress ethylene" in plants
- 3H1 has high ACC deaminase activity (direct N source or blocks ethylene which could otherwise inhibit root growth)

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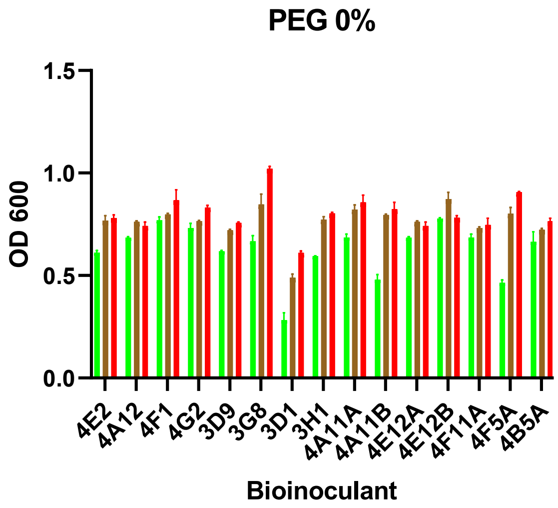
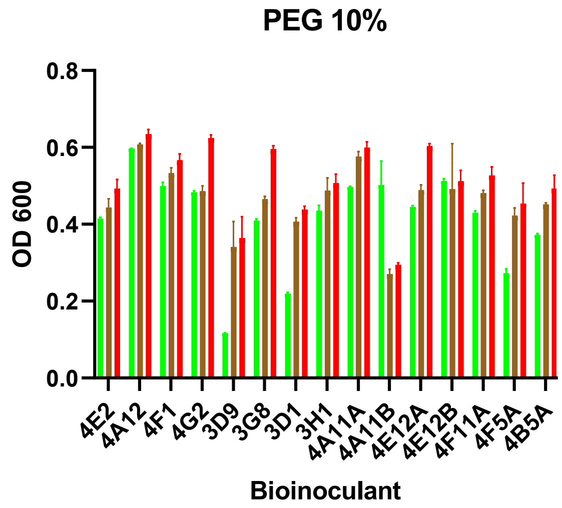
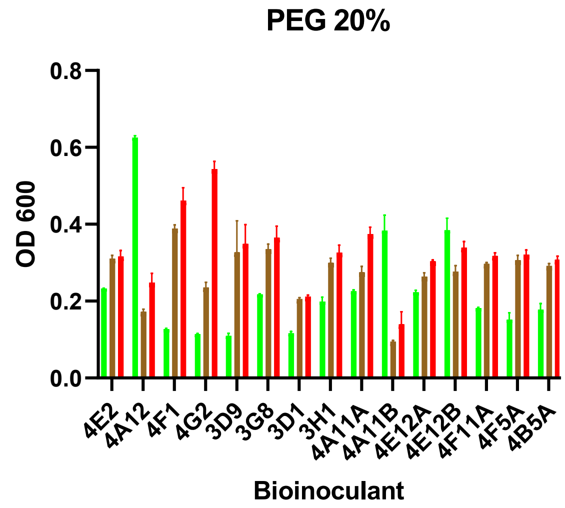
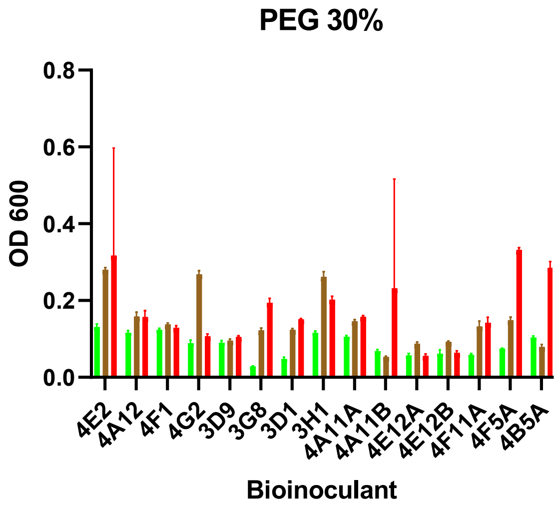
Drought stress tolerance *in vitro*

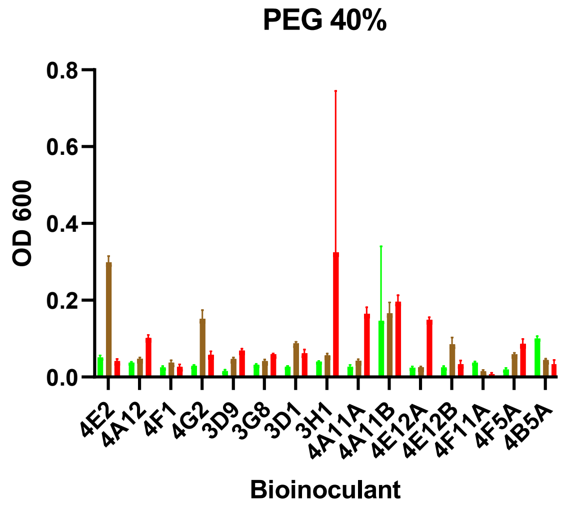
(PEG tolerance)

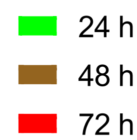












- Needs further research

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Major challenges + promise of inoculants

>Good results in the lab but highly variable results under field conditions:
--Variation within a farm (patchy)
--Year to year variation
--Variation between turf species and cultivars

>Delivery of a viable inoculant can be logistically challenging
>Emerging intellectual property constraints
>Safety (environmental, human, livestock)

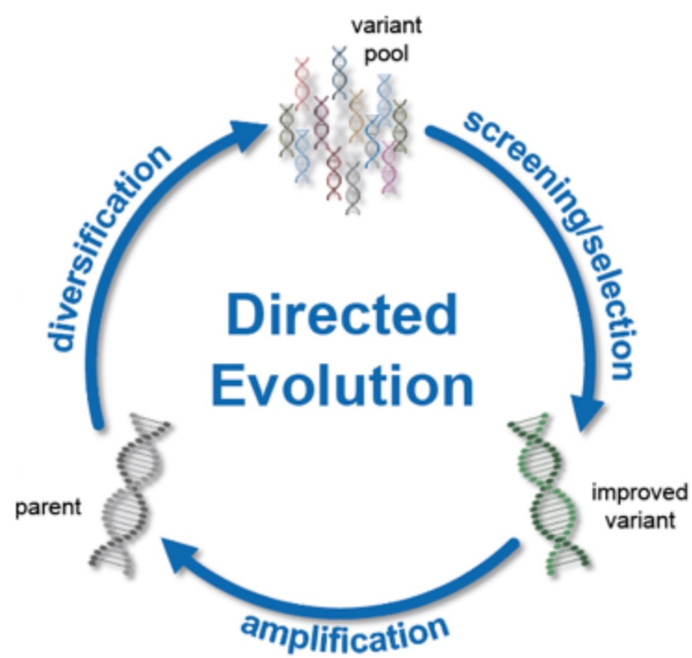
>As a result, farmers strongly prefer chemicals for their consistency and track record.

>However, inoculants may offer multi-function benefits (yield, multiple stress tolerances including disease), meet organic certification, may be less polluting and not contribute to climate change.

This project aims to address these issues directly.

Next phase: Rapid directed evolution

Overall objective: increase cereal crop NUE provided by the best bioinoculant



Problem: Bioinoculants may colonize plants poorly/inconsistently, especially in the field

- Reasons
  - Microbes: Competition with other microbes (soil, endogenous plant microbiome)
  - Environment: e.g. Temperature fluctuations
  - Plant: Host variety genetic variability (e.g. plant defence)
  - These factors may interact with one another (M x E x P)

- **Solution – improve microbe colonization ability via rapid directed evolution**

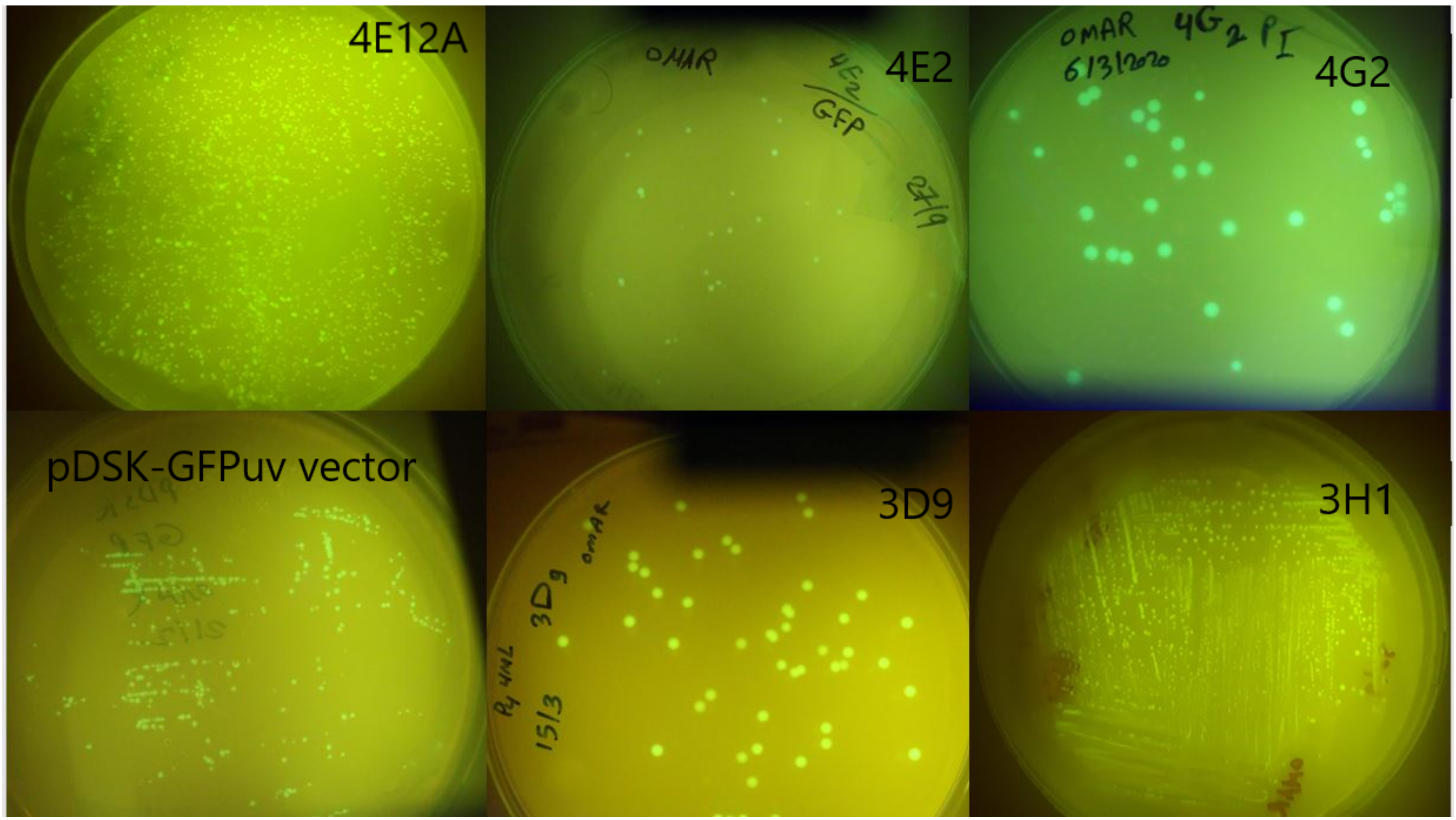
Hypothesis

- The ability of endophytic bioinoculants to promote NUE in turf can be improved with directed evolution

- Objective: Increase "success rate" of endophytic colonization using directed evolution in the presence of: soil microbes X unstable environment X variable turf species

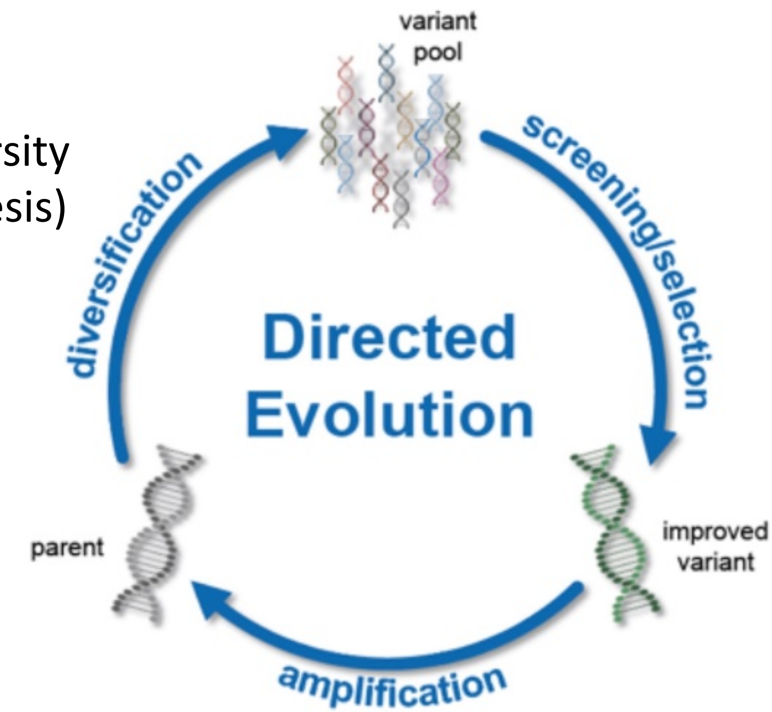
Methodology - GFP tagged-bioinoculants

- Bioinoculants have been tagged with plasmid GFP, and antibiotic resistance
- **Allows for visual confirmation of colonization inside plants**
- GFP/resistance tag will later be removed (non-GMO)



Methodology: 10-15 cycles of selection

1) Optional:
Introduce
bacterial diversity
(UV mutagenesis)

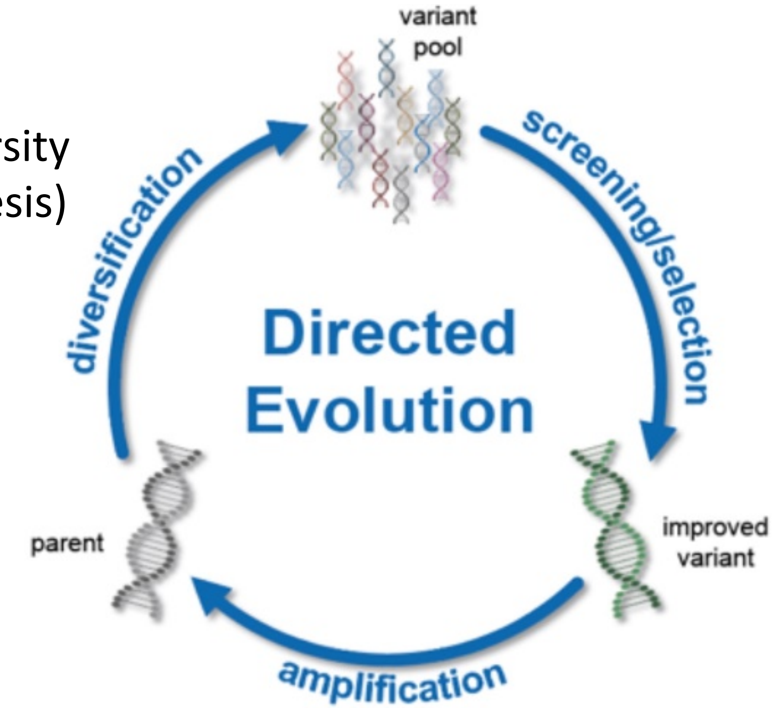


Methodology: 10-15 cycles of selection

2) Inoculate 5-10 different turf species

3) Grow with living field soil

1) Optional: Introduce bacterial diversity (UV mutagenesis)



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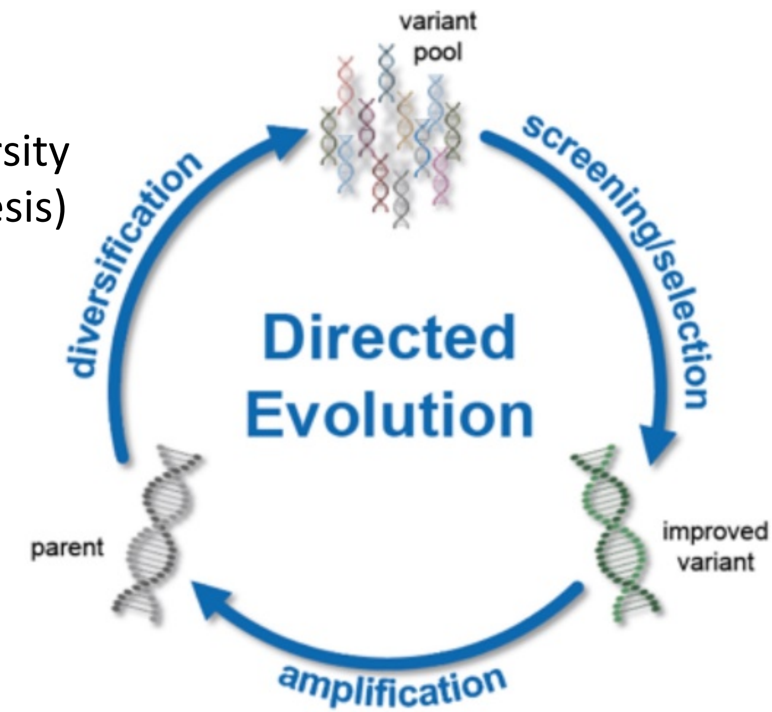

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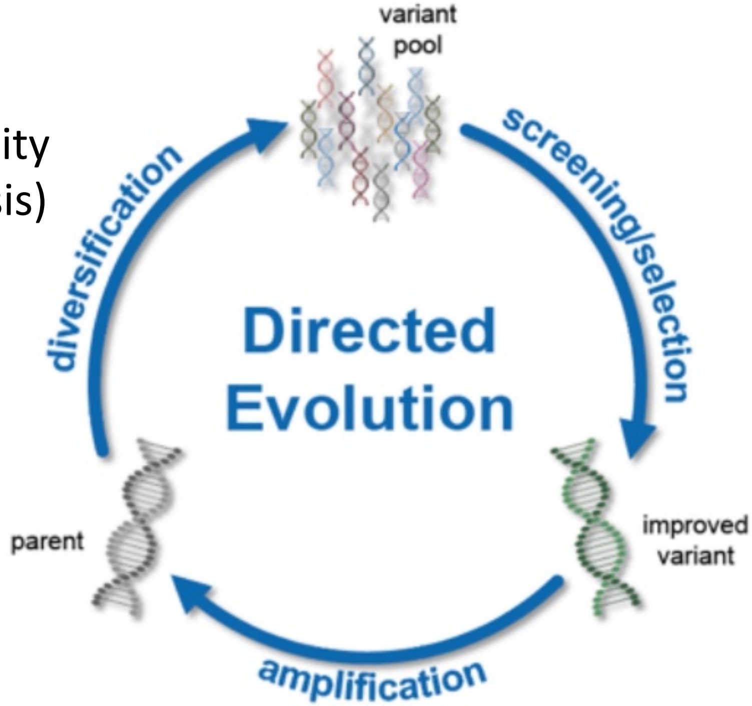

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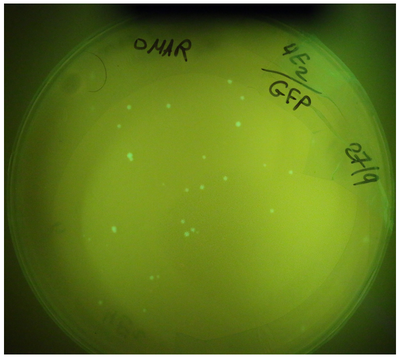
5) Isolate and select successful colonizers



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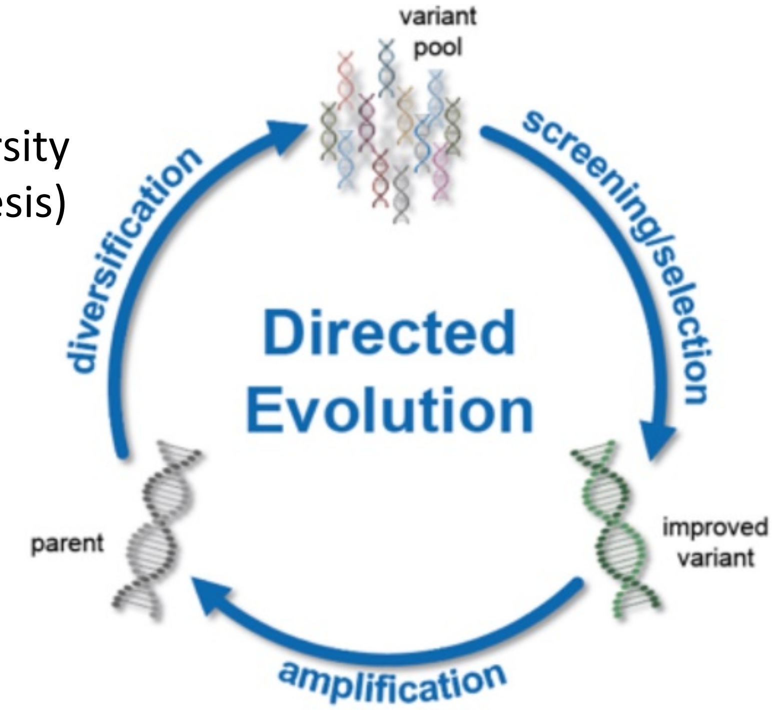

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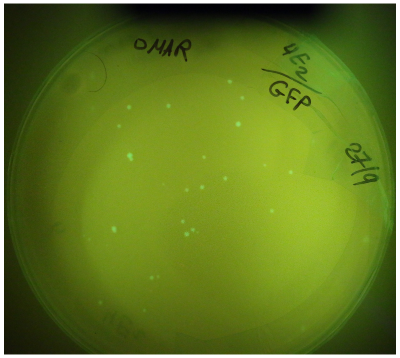

6) Amplify successful colonizers (liquid culture)

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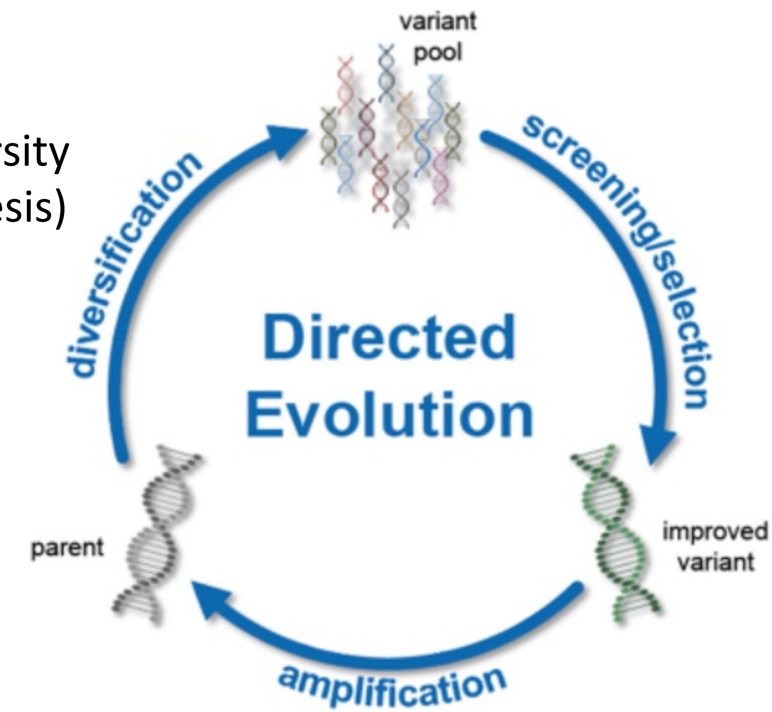

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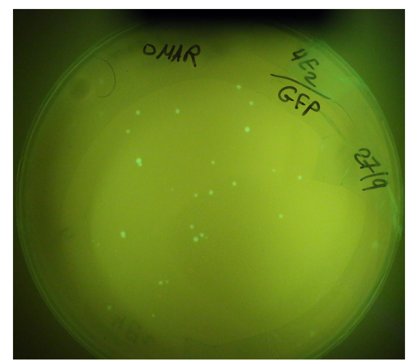
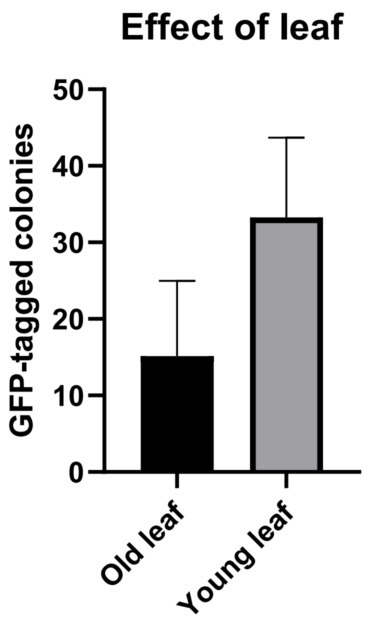


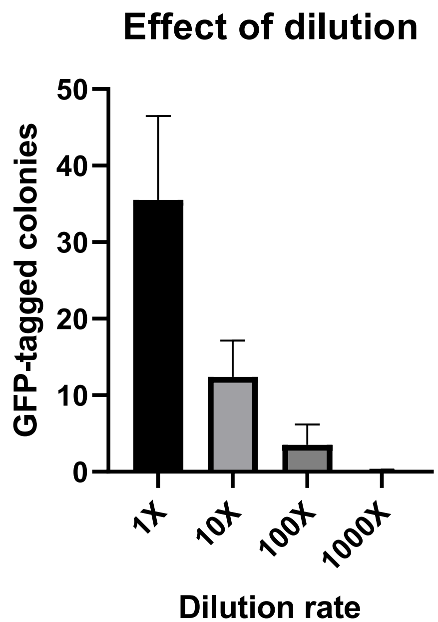
7) Repeat cycle

6) Amplify successful colonizers (liquid culture)

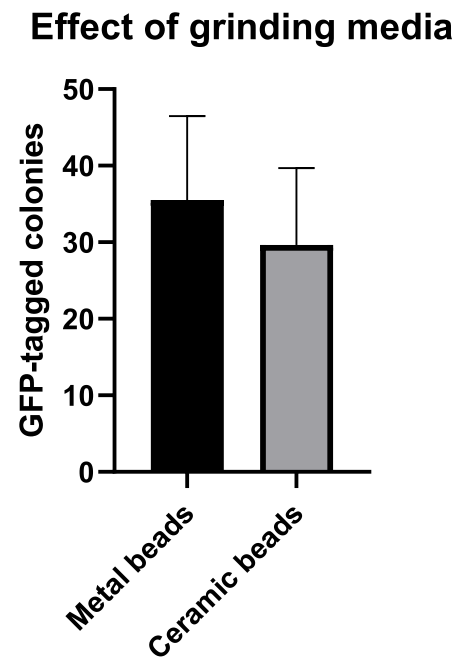
Pre-evolution optimation: What is the ideal colonizer isolation method?



(homogenized tissue not diluted)



(young leaf)



(young leaf)

- For colonizer isolation, will select young leaf, grind with metal beads, and dilute 100-fold
- Kanamycin concentration of 25 ug/ml in selection media is ideal
- Purchased bead mill, as vortex grinding produced no colonies
- Bead mill speed of 3 m/s, for 30 sec found to be ideal

Summary of directed evolution methodology

Objective : Increase success rate of endophytic colonization

1. Inoculate 5-10 turf species with identified NUE promoting bioinoculants
2. Grind young plant tissue, screen for symbiosis establishment via GFP
3. Introduce diversity via mutagenesis
4. Re-inoculate, screen for increased colonization

Following each objective, re-inoculate, screen for increased biomass under low N and/or drought conditions, and sequence genome of top bioinoculants