Optimizing Plant-microbe Interactions for Sustainable Supply of Nitrogen for Bioenergy Crops

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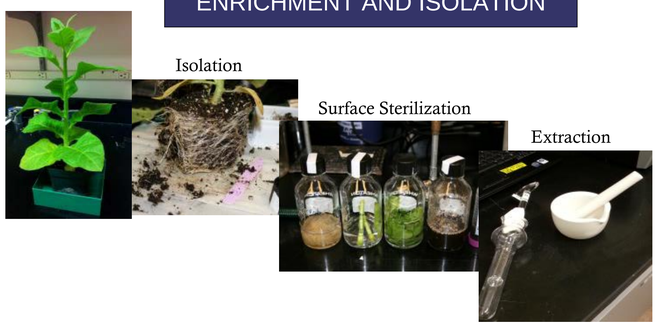
ABSTRACT

Nitrogen (N) is an essential component of DNA and proteins. It is also a key element of life that is often limited in plants, which negatively affects their growth. In natural ecosystems, plants are strongly affected by their associated micro-biome. Plants have developed strong, symbiotic relationships with microbes to cope with the low availability of nitrogen in the soil. Optimizing the relationship between plants and diazotrophic bacteria (nitrogen-fixing bacteria) could provide adequate amounts of nitrogen to the host-plant and thus eliminate the need of fertilizer use in energy crop cultivation. Therefore, we investigated the diversity of microbes in Tobacco (*Nicotiana tabacum*), considered as potential energy crop for bioenergy production. Several bacterial isolates from the phylogenic order *Rhizobiales* were obtained from the rhizoplane and roots of this plant using several different N-deficient media. Majority of these isolates grew best with simple sugars and small organic acids. Further, we were able to identify isolates capable of fixing molecular nitrogen, as observed from PCR amplification targeting the *nifH* gene. Together these results help to understand the impact of plant associated microbes on the growth and survival of these biofuel plants. This understanding is necessary for the develop eco-friendly economically sustainable energy crops by decreasing their dependency on fertilizer.

ENRICHMENT AND ISOLATION

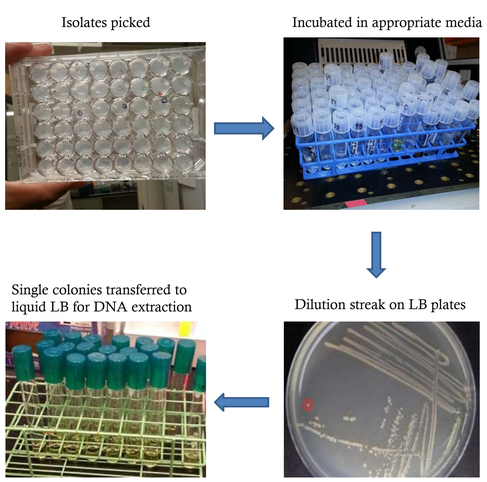


Serial Dilution Plate

	Glucose	Fructose	Citrate	Butyrate	Malate	Lactate	Acetate	Glycerol
	1	2	3	4	5	6	7	8
A								
B								
C								
D								
E								
F								

Serial Dilution

Using *Nicotiana tabacum* as a model plant, we isolated bacteria from five sources: leaves, stems, roots, rhizosphere, and rhizoplane. The leaves, stems, and roots were surface sterilized using a series of washings with 1.5% bleach and water. Extraction of bacteria was done incubating samples in 5 mM sodium pyrophosphate with subsequent mechanical break down of plant material. Three, 48-well plates, per extraction source were setup using three different N-deficient media and 8 different carbon sources. A serial dilution method was used in attempt to obtain single colonies. Four separate growth stages were utilized in order to ensure a pure culture for DNA extraction and phylogenetic characterization.



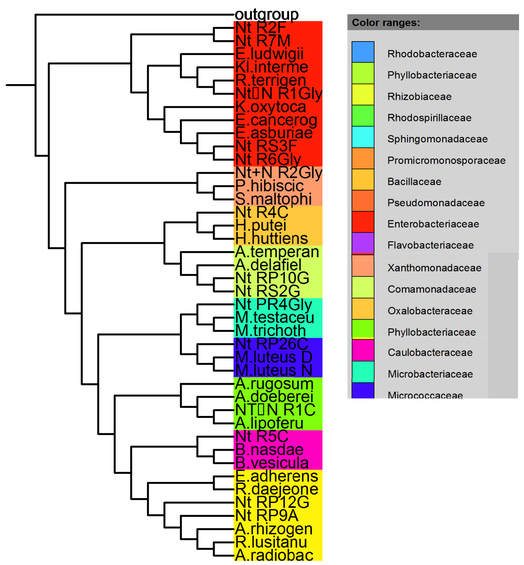
RESULTS

Isolates Identified from Tobacco (*Nicotiana tabacum*)

Isolate	Closest Relative by 16S rDNA Sequencing in NCBI Database	Similarity
NtR2F	*Enterobacter ludwigii*	99%
NtR3C	*Herbaspirillum huttiense*	99%
NtR4C	*Herbaspirillum huttiense*	99%
NtR5C	*Brevundimonas nasdae strain W1-2B*	99%
NtR6Gly	*Enterobacter cancerogenus*	99%
NtRP4Gly	*Microbacterium testaceum strain DSM 20166*	99%
NtRS2G	*Acidovorax delafieldii strain 133*	99%
NtRS3F	*Enterobacter asburia*	99%
NtR7M	*Enterobacter ludwigii*	99%
NtRP6C	*Herbaspirillum huttiense*	99%
NtRP8B	*Rhizobium etli*	98%
NtRP9A	*Agrobacterium radiobacter K84*	99%
NtRP10G	*Acidovorax delafieldii strain 133*	99%
NtRP12G	*Ensifer adhaerens*	97%
NtR9Gly	*Enterobacter ludwigii*	98%
NtR13F	*Enterobacter ludwigii*	99%
NtR20G	*Enterobacter ludwigii*	99%
NtRP27F	*Ensifer adhaerens*	97%
NtRP28F	*Micrococcus leteus*	99%
NtR22G	*Rhizobium tropici*	98%
NtR23C	*Rhizobium tropici*	98%
NtNR1C	*Azospirillum doebereinerae*	99%
NtNR1Gly	*Raultella terrigena*	99%

Diazotrophic Isolates from Tobacco (*Nicotiana tabacum*)

Isolate	Closest Relative by 16S rDNA Sequencing in NCBI Database	Similarity
NtRP8B	*Rhizobium etli*	98%
NtRP12G	*Ensifer adhaerens*	97%
NtRP27F	*Ensifer adhaerens*	97%
NtR22G	*Rhizobium tropici*	98%
NtR23C	*Rhizobium tropici*	98%
NtNR1C	*Azospirillum doebereinerae*	99%
NtNR1Gly	*Raultella terrigena*	99%



Approximately 1250 bp sequences of the 16s rDNA gene of all isolates obtained were compared with sequences of the reference organisms by Blast search using NCBI database. Multiple alignments of the sequences based on phenetic procedures were done with the ClustalX version 2.1 (*Bioinformatics*, 23, 2947-2948).

The jModelTest2 program (*Nature Methods* 9(8), 772) was used to select the best nucleotide substitution model. The color code indicates clades clustering to the same bacterial family. Phylogenetic relationship of isolates is shown for *Nicotiana tabacum*.

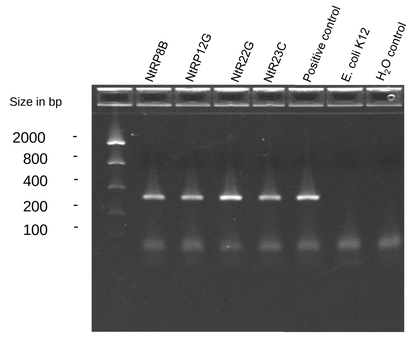
NITROGEN FIXATION

PolFR primer were used to amplify *nifH*. The *nifH* gene is considered to be fairly conserved, and is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria. The amplified product is visualized on the gel as the 300 bp fragment. The primer sequences are listed below.

PolF: 5´ TGCGAYCCSAARGCBGACTC 3´

PolR: 5´ ATSGCCATCATYTCRCCGGA 3´

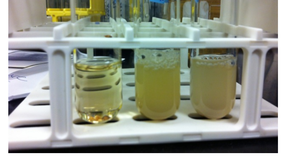
Several isolates belonging *to Rhizobium spp, Azospirillum spp., Raultella spp., and Ensifer spp.* tested positive for *nifH (*see list of isolates). We are in the process of confirming nitrogen fixing capability by these strains using physiology based assays.



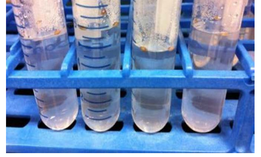
REISOLATION OF BACTERIA FROM SEEDS

To follow up on the endophytic character of isolated diazotrophic bacteria, sterilized tobacco seeds were incubated overnight with the *nifH* positive strain NtRP8B. After incubation, seeds were removed from the media and surface sterilized using a series of washings with 1.5% bleach and sterilized water. Crushed seeds were used as inoculum for new LB media. After noticeable growth was observed, DNA extraction and 16S gene amplification were performed. Positive PCR results were purified and submitted for phylotyping. Sequence analysis revealed the re-isolation of strain NtRP8B, and thus the capability of this bacteria to re-infect seeds. In another approach, inoculated seeds are also being used in attempt to promote plant growth. This is work in progress.

Seeds in NtRP8B cultures



Invaded seeds growing in plant media



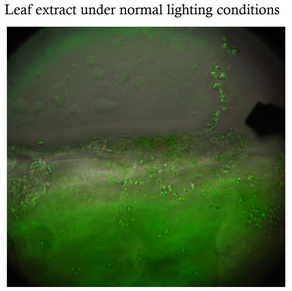
CONCLUSION

We successfully identified and isolated microbes closely associated with *Nicotiana tabacum*. Several of these isolates are potential nitrogen fixing bacteria as shown by PCR amplification of *nifH*. In accordance to Koch's´ postulates, we were also able to re-isolate nitrogen-fixing bacteria from surface sterilized seeds after bacterial re-infection. This also confirms their endophytic character.

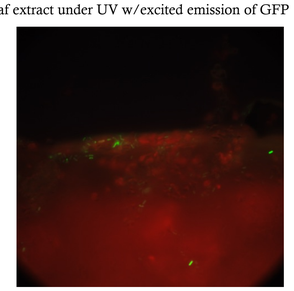
FUTURE PROJECTIONS

- Measure and compare plant growth after germination
- Transform and track diazotrophic bacteria and nitrogen-fixation inside of plant using GFP plasmid
- Identify cross-talk between plants and endophytic bacteria
- Perform N-fixation assay (Acetylene reduction assay)
- Genetically modify energy crops to attract diazotrophic endophytic bacteria

Leaf extract under normal lighting conditions



Leaf extract under UV w/excited emission of GFP



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