Evaluation of Biological Control Rhizobacteria for Reduction of Angular Leaf Spot in Dark Tobacco

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Angular Leaf Spot

- ***Pseudomonas syringae* pv. *tabaci*** (tox-)
- **Foliar** Disease
- Increasing in Kentucky since 2016
- Bacteria are spread in **water droplets**
- Favored by cloudy wet weather
  - We obtained *Pst.* isolate #455 from Dr. Emily Pfeufer
- **Streptomycin** currently used
  - Environmental Antibiotic Resistance

Biological Control with beneficial bacteria

- Control insects, mites, weeds, or **plant pathogens**
- Direct: by antimicrobial metabolites produced by bacteria
- Indirect: by inducing systemic resistance - ISR (eliciting resistance pathways to generate PR proteins, jasmonates, chitinases, glucanases, etc.)

- **Other advantages**
  - Many bacteria have **growth promotion** effects in seedlings
  - No **environmental** contamination with harmful chemicals
  - Minimized **safety** concerns
  - In many cases, **less expensive** than chemicals

Bacterial Selection

- Chose list of **32 bacterial strains** to be tested against *Pseudomonas syringae* pv. *tabaci*

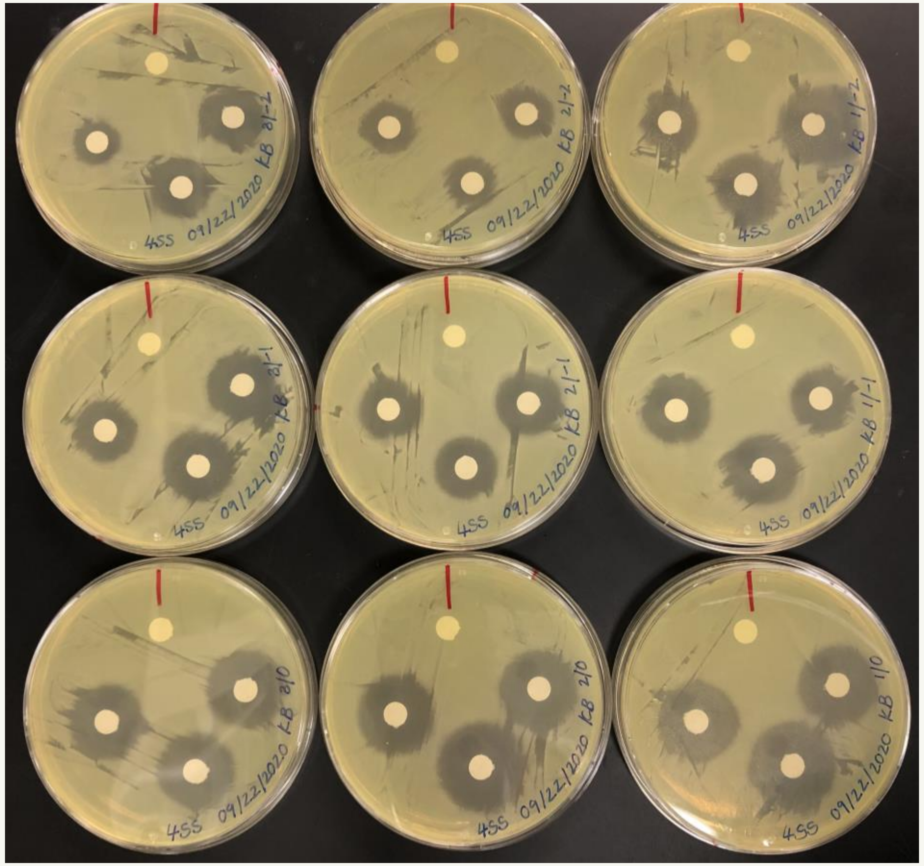
  - Collection of rhizobacteria from Dr. Joseph W. Kloepper at Auburn University (by Material Transfer Agreement with the University of Kentucky).

    These have shown to have ISR in several crops (cotton, cucumber, pepper, tomato)

  - Most of genus ***Bacillus*** -commonly used for biological control
    - Other genera include:
      - ***Serratia***
      - ***Burkholderia***
      - ***Strenotrophomas***
      - *Microbacterium*

Overview of Methods

In-vitro antibiosis

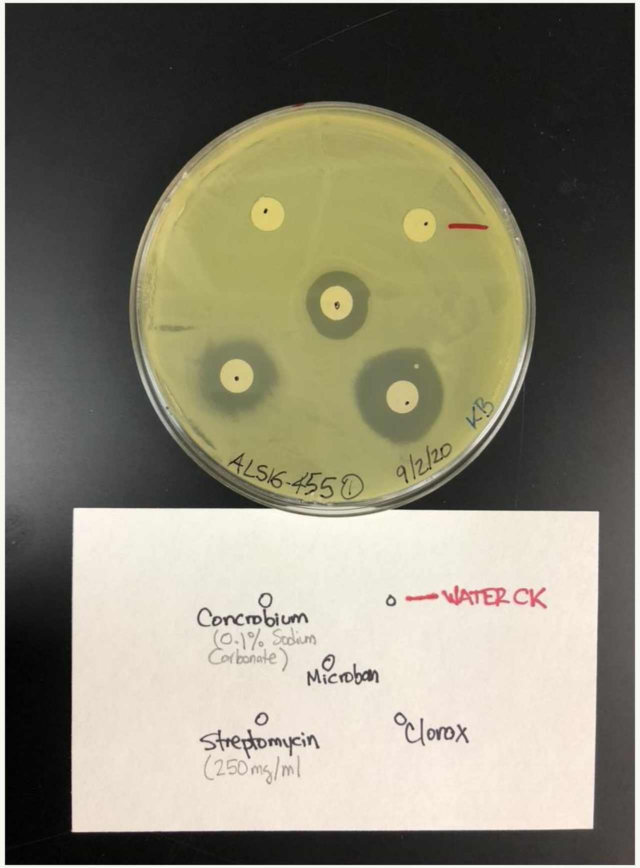


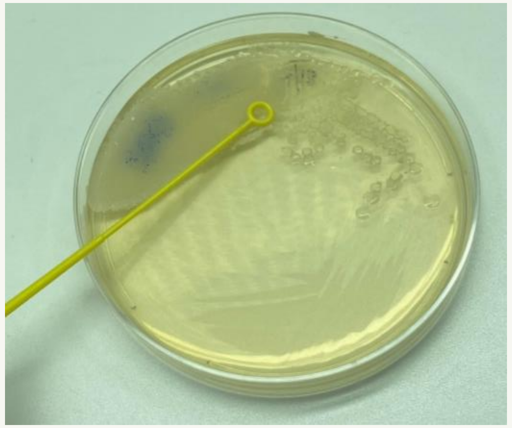
Greenhouse Trials

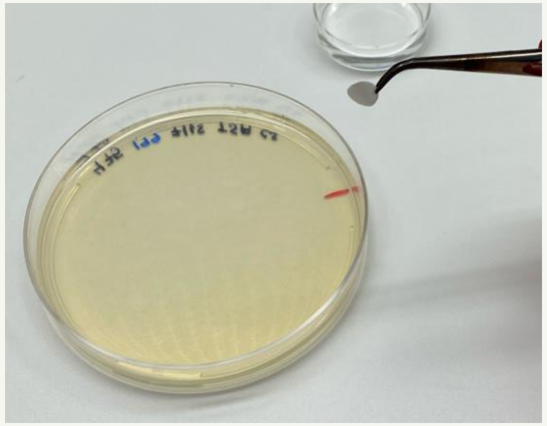
In-vitro Antibiosis

METHOD: Disk diffusion on solid media

- **Dilute *Pst.***
  - 2 half loopfuls in 20 mL sterile DI water, vortex
- **Plate *Pst.***
  - Spread as bacterial lawn
- **Plate DI water control disk**
- **Rhizobacteria Suspension**
  - 5 colonies in 5mL DI water
- **Plate 3 filter paper disks imbedded with rhizobacteria**
- **Incubate**
  - 28°C in dark incubator for 24 hours







In-vitro Antibiosis Experimental Design

Pathogen: Angular Leaf Spot (*Pseudomonas syringae* pv. *tabaci*)

Test 3 pathogen concentrations: 0, -1, -2

Treatments: Rhizobacterial Strains

Media: Trypticase Soy Agar (TSA)

Experimental Unit: One TSA plate with 3 disks of rhizobacteria and 1 water control disk

Replications: 3

Arrangement: Completely randomized

Evaluation: Measure inhibition zone areas in mm

In-vitro Antibiosis Results

Table 1

In-vitro antibiosis of AU rhizobacteria vs. *Pseudomonas syringae* pv. *tabaci* on TSA

	Average Inhibition Zone (mm^2)		
Strains/dilutions	**0**	**-1**	**-2**
Bacillus cereus/proteolyticus (AP-94)	0	0	1.72
Bacillus safensis (AP-110)	1.78	3.08	4.78

Other strains chosen for greenhouse testing based on history of biocontrol with other bacterial pathogens in tomato:

- *Serratia marcescens* (AP-4)
- *Bacillus altitudinis* (AP-281)

Hypothesis

One or more of the selected rhizobacteria will reduce the incidence of Angular Leaf Spot in dark tobacco

Greenhouse Experimental Design

- **Tobacco Variety:** Dark Tobacco KT D8 (most susceptible to ALS)
- **Environment:** Greenhouse at 80℉ and 50-70% relative humidity
- **Treatments:**
  - Healthy Check (Untreated Control)
  - Disease Check (Pathogen Only)
  - Rhizobacterial Strains
- **Experimental Unit:** One Plant
- **Replications:** 5
- **Arrangement:** Randomized Complete Block (RCB)

Rhizobacteria Inoculation Methods

Spray Method

- 2 TSA plates of rhizobacteria diluted in 1 L DI water
- 20 µl of **Silwet** (adjuvant)
- Spray 35-50 ml per plant
- Arrange according to RCB labels

Soil Drench Method

- 2 TSA plates of Rhizobacteria diluted in 500 mL DI water
- 100 mL poured per plant

Challenging with *Pseudomonas syringae* pv. *tabaci*

- **Spray Inoculation**: challenged with *Pst* after **24 hours**
- **Soil Drench Inoculation**: challenged with ALS after **7 days**
- Light mist applied first
- **Solution**: 2 KB plates of pathogen per Liter of DI water + 20 µl of Silwet 35-50 mL **sprayed** per plant
- Arranged in trays using RCB

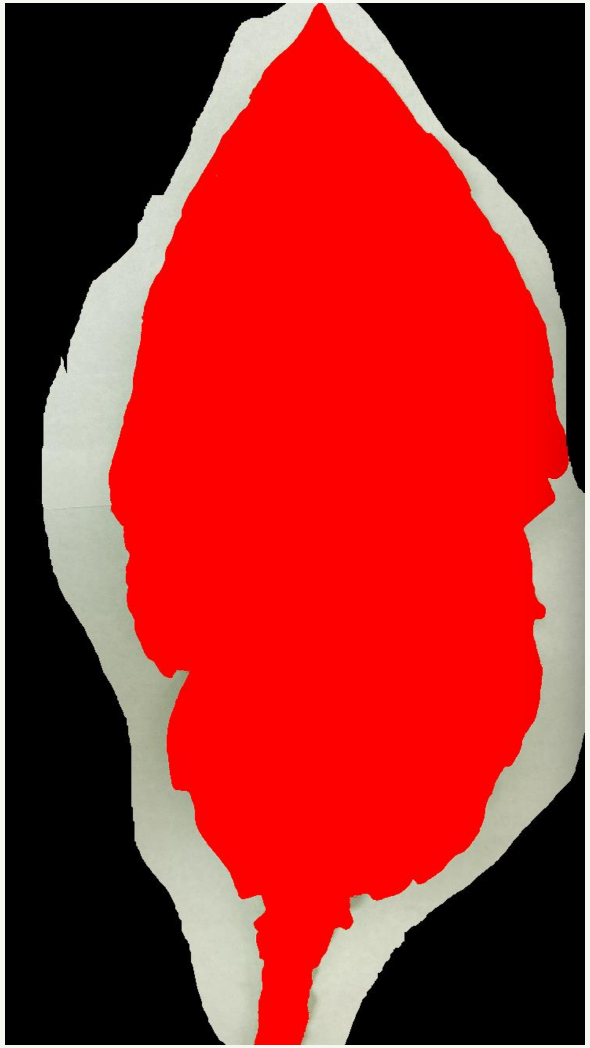
Greenhouse evaluations

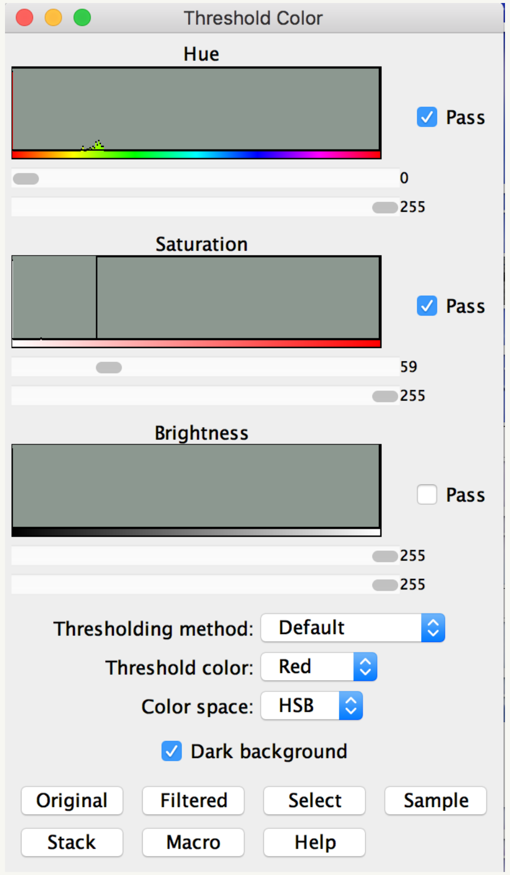
- Counting number of lesions after **7 days**

- Determine total disease area in each leaf after **14 days**

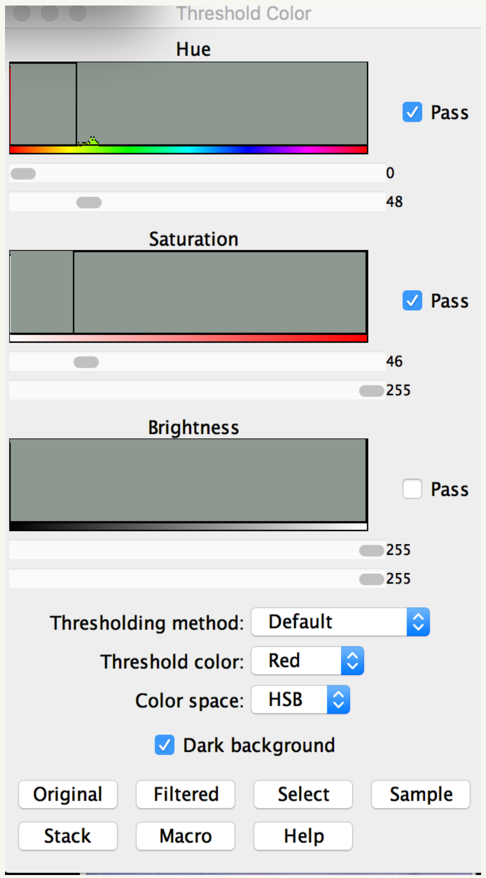
ImageJ

Total Leaf Area





Diseased Leaf Area



Statistical Analysis

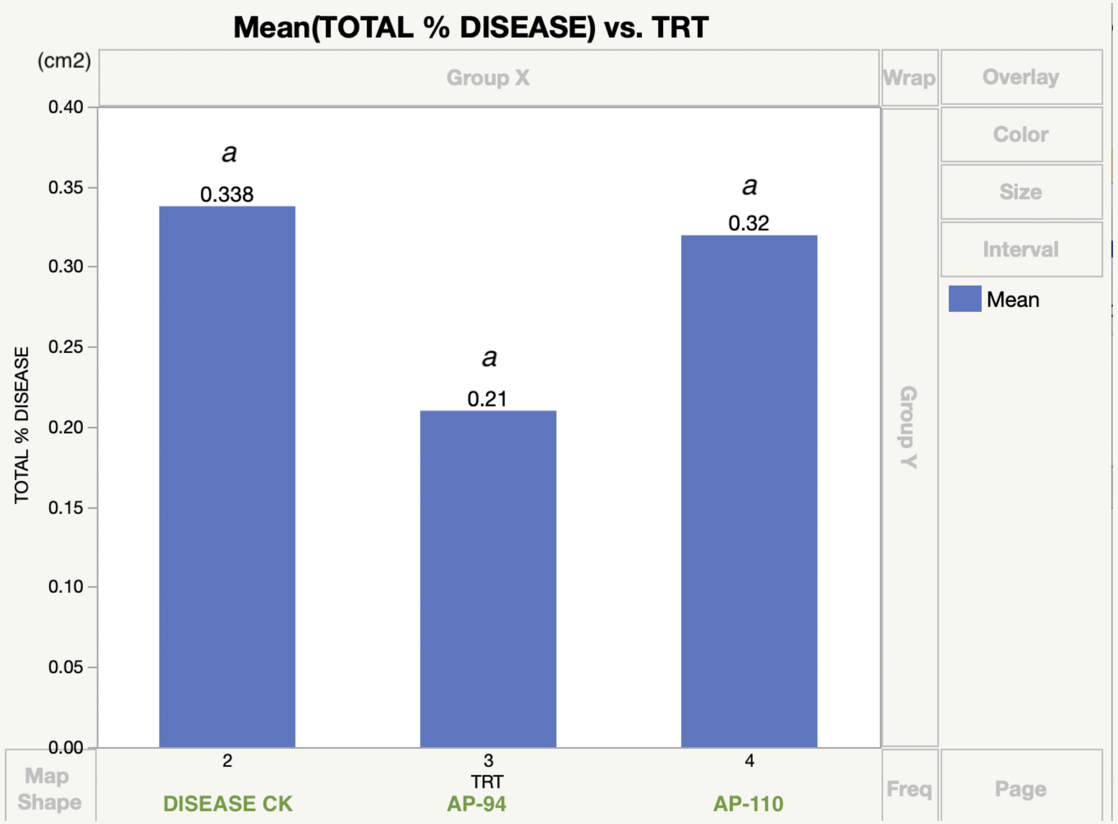
- ANOVA

- Student’s t-test

  - Consulted with a statistician

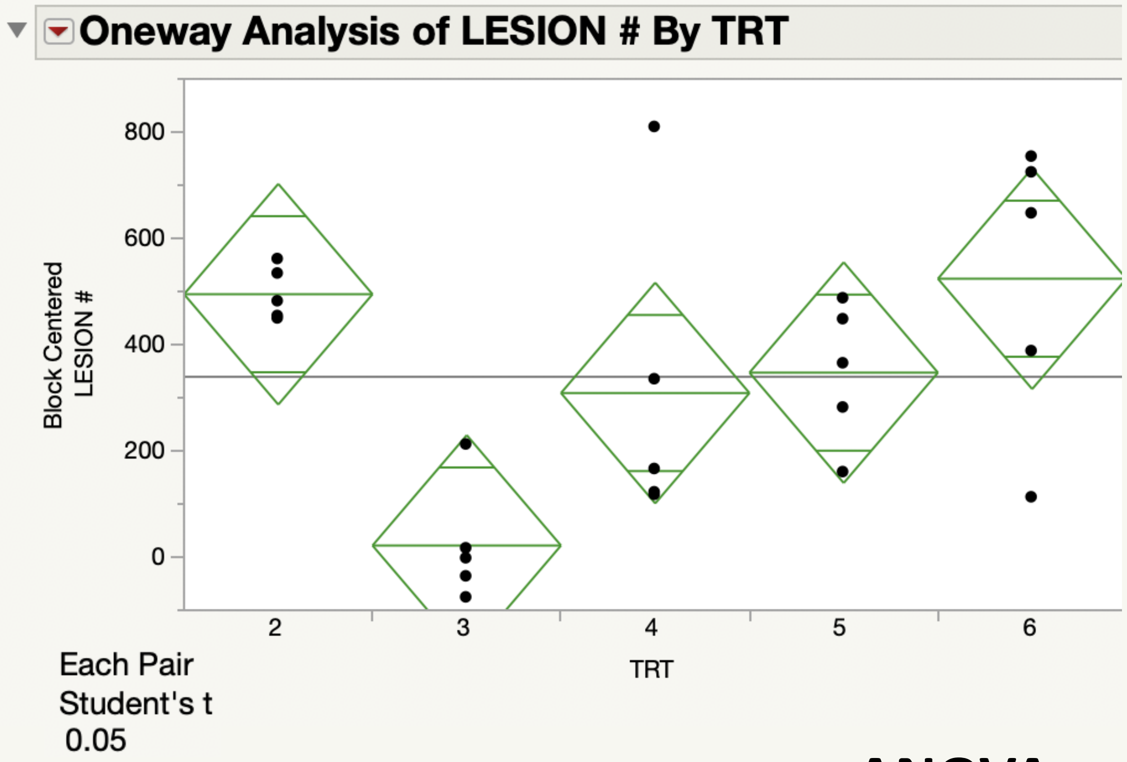
jmp Statistical Discovery.™ From SAS.

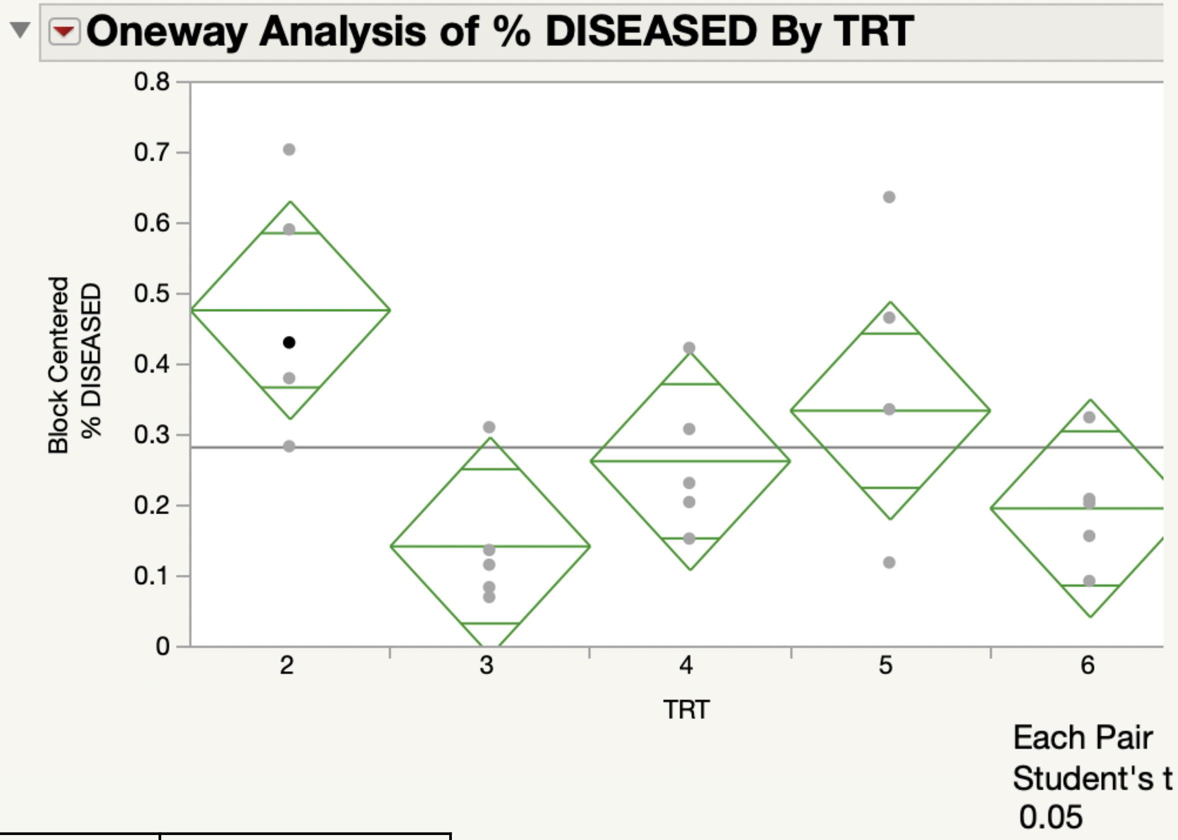
Experiment 4 (Spray)



Treatment	Average Total # of Lesions	Average total % disease
1: Healthy Check	-	-
2: Disease Check	99	0.34
3: *B. cereus/proteolyticus* (AP-94)	63	0.32
4: *B. safensis* (AP-110)	104	0.21

Experiment 10 (Soil drench)

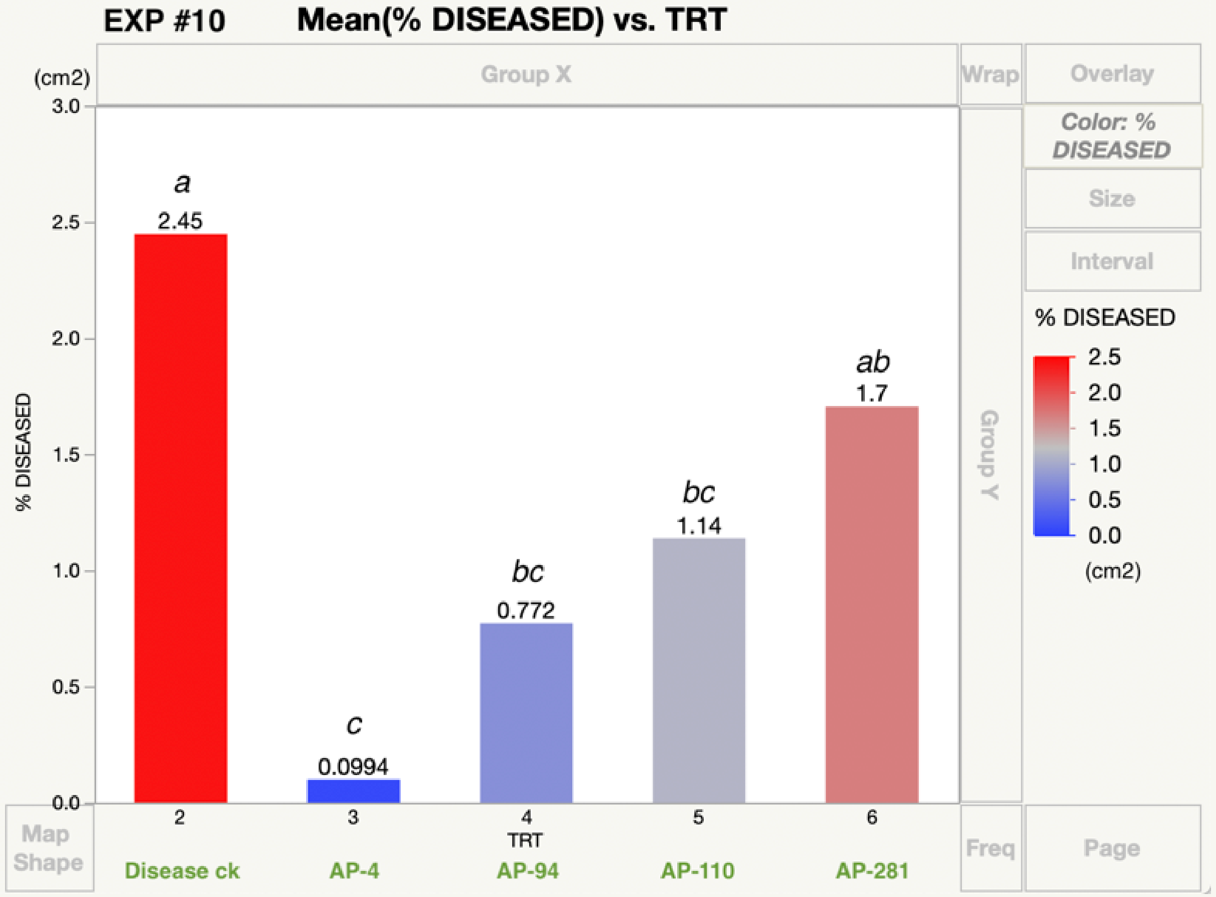




ANOVA

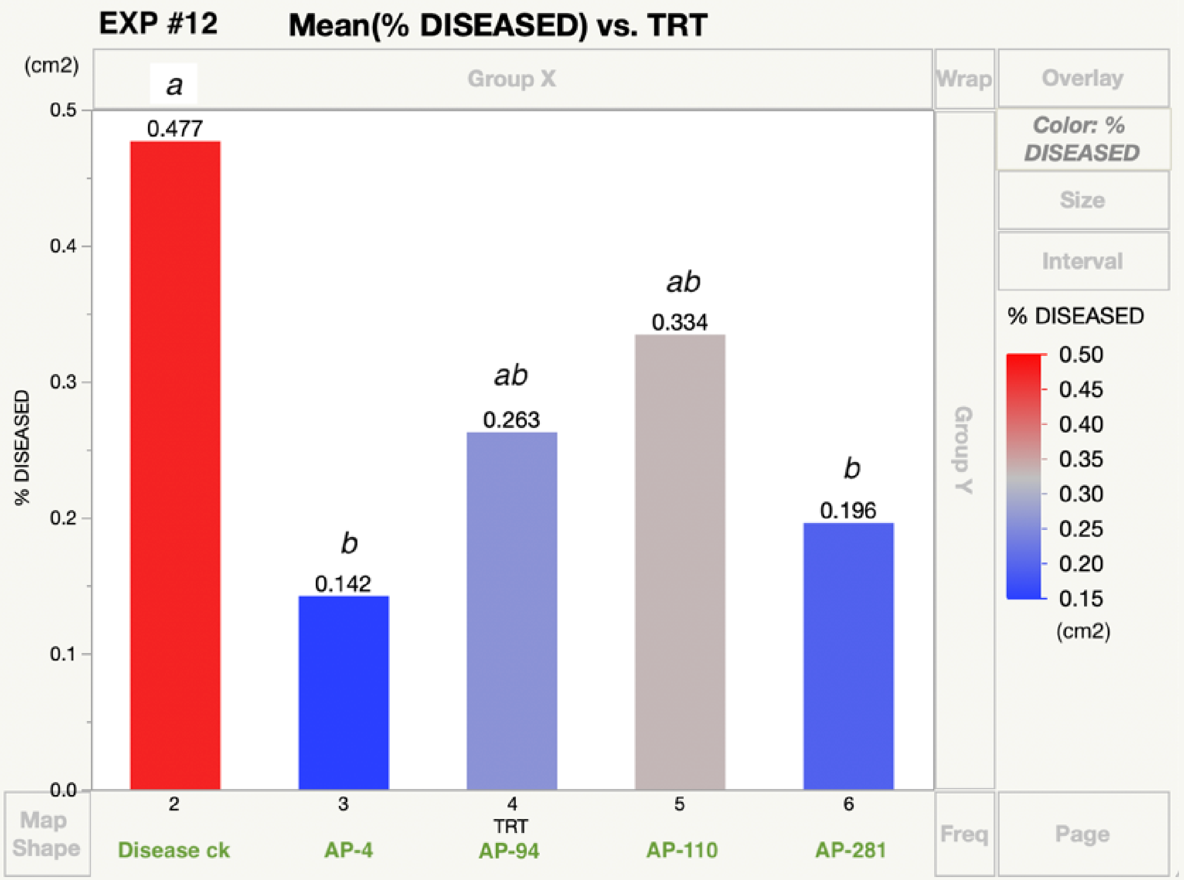
	# Lesions	% Disease
Rsquare	0.544	0.563
Prob>F	0.0168	0.0128

Experiment 10 (Soil drench)



Treatment	Average Total # of Lesions	Average total % disease
1: Healthy Check	-	-
2: Disease Check	495 a	2.45 a
3: *S. marcescens* (AP-4)	**22 b***	**0.10 c***
4: *B. cereus/proteolyticus* (AP-94)	309 ab	**0.77 bc***
5:*B. safensis* (AP-110)	347 a	**1.14 bc***
6: *B. altitudinis* (AP-281)	524 a	1.70 ab

Experiment 12 (Soil drench)



Treatment	Average Total # of Lesions	Average total % disease
1: Healthy Check	-	-
2: Disease Check	135 a	0.48 a
3: *S. marcescens* (AP-4)	**49 b***	**0.14 b***
4: *B. cereus/proteolyticus* (AP-94)	99 ab	0.26 ab
5:*B. safensis* (AP-110)	106 ab	0.33 ab
6: *B. altitudinis* (AP-281)	71 ab	**0.20 b***

Conclusions - I

***Serratia marcescens* (AP-4)**

- The **most promising** of the 4 rhizobacterial strains analyzed
- Showed reduction in disease in **experiments 10 and 12** both with in lesion count **and** % diseased leaf area

***Bacillus cereus/proteolyticus* (AP-94)**

- Inhibited growth of *Pst in-vitro*
- Showed reduction in disease in **experiment 10** % diseased leaf area **only**

***Bacillus safensis* (AP-110)**

- Inhibited growth of *Pst in-vitro*
- Showed reduction in disease in **experiment 10** % diseased leaf area **only**

***Bacillus altitudinis* (AP-281)**

- Showed reduction in disease in **experiment 12** % diseased leaf area **only**

Conclusions - II

- Inoculation via **soil drench** worked better than the spray method, confirming the findings in Kloepper's lab, that induced systemic resistance could be involved in reducing ALS with rhizobacteria inoculations

- Switch from counting lesions to a **visual disease rating scale** (0-10)

- Increase **humidity** in the greenhouse to have consistent disease

- *Serratia marcescens* (AP-4) showed **potential** to be used as a biological control for treating angular leaf spot in dark tobacco, and could be included in **preliminary field trials** in 2022

Future Directions

- Try different **time** periods between spray inoculation and challenging
  - **48 hours** or **7 days** spray rhizobacteria
- Simultaneous **spray and drench** inoculations
- **Mixes** of 2 or 3 rhizobacteria
- **Seed** and **float bed** treatments

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