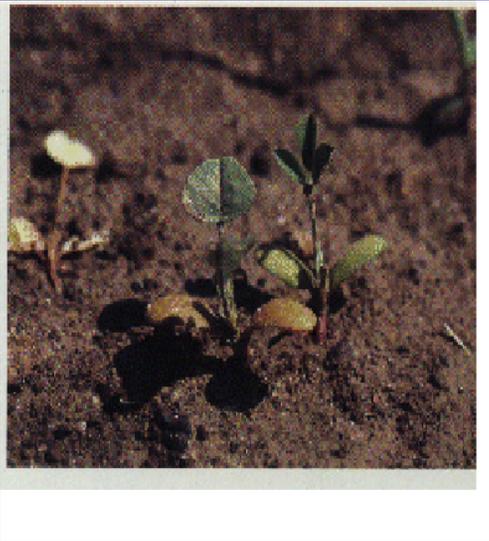


Quantifying soilborne pathogens in alfalfa with fluorescent PCR

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Symptoms of Soilborne Disease



Seedling diseases

Crown Rots



Winter Kill

Stand Decline



Standard tests¹ and check
populations are used for several
soilborne diseases

Aphanomyces Root Rot

Phytophthora Root Rot

Fusarium Wilt

Verticillium Wilt

¹National Alfalfa Variety Review Board

Alfalfa Cultivar Classification* for Disease Resistance

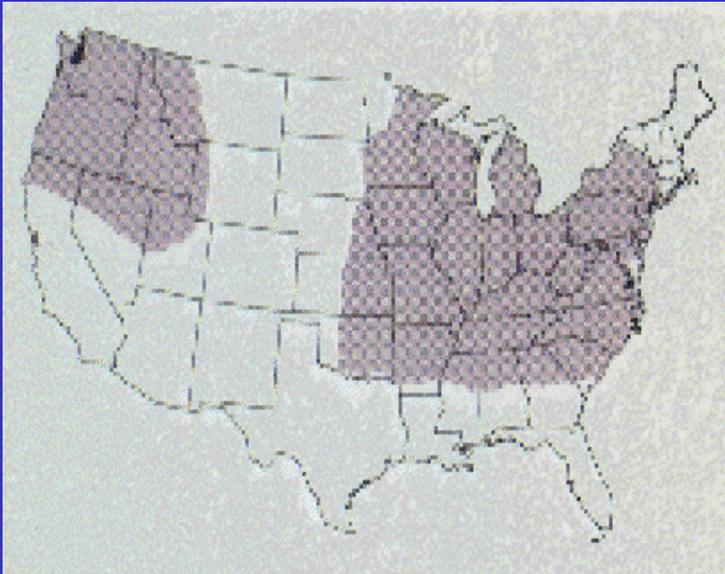
Class	Percent Resistant Plants
High Resistance	>50
Resistance	31 – 50
Moderate Resistance	15 – 30
Low Resistance	6 – 14
Susceptible	< 6

* National Alfalfa Variety Review Board

Aphanomyces euteiches

Plant pathogen that causes severe root rot disease in alfalfa, peas, and beans

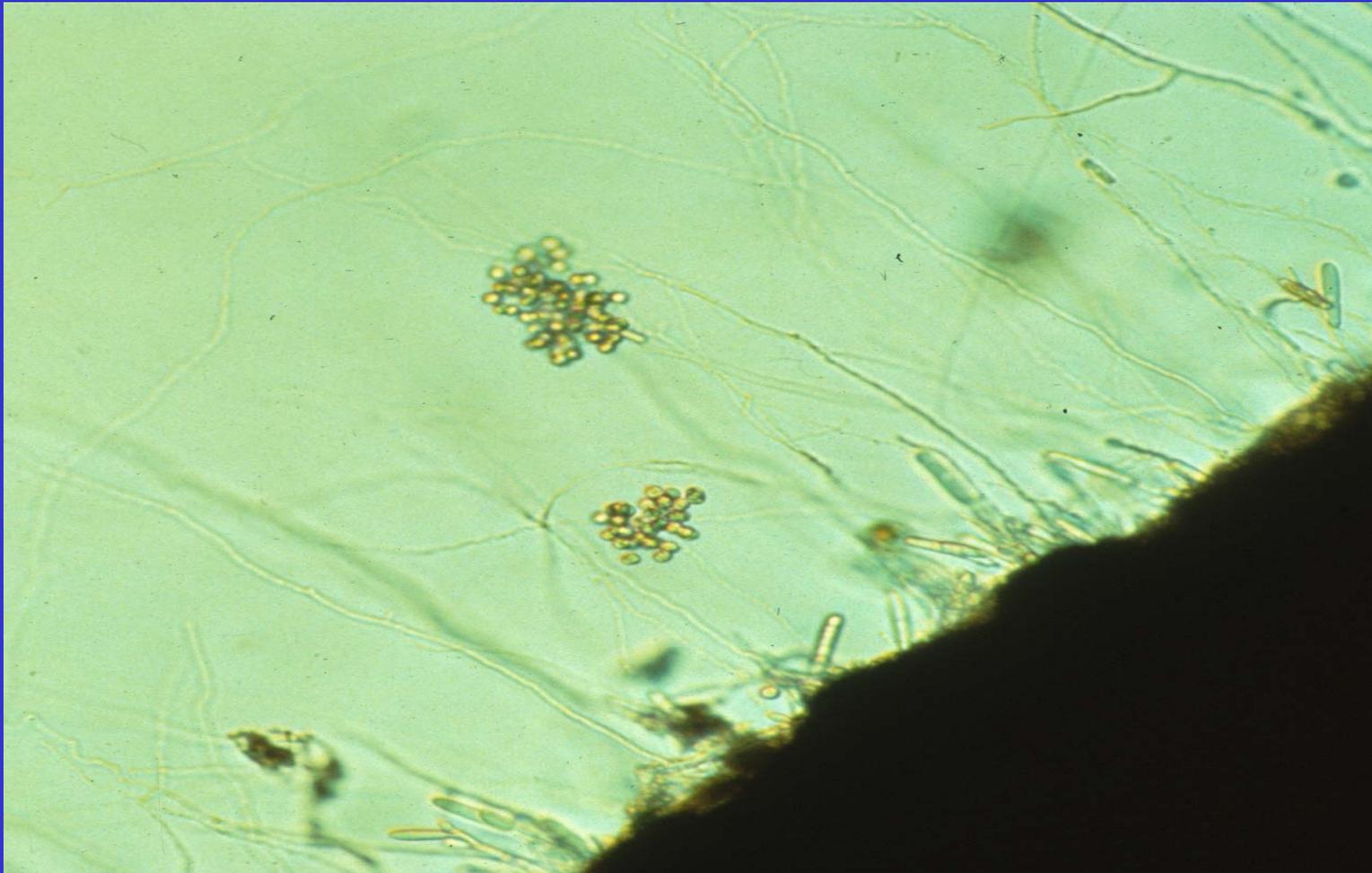
Affected



Infected alfalfa



Aphanomyces Sporangia on Alfalfa Root



Aph^{res}

Aph^{sus}



Rating¹ Alfalfa for Aphanomyces Resistance

1= healthy (R)

2= slight necrosis of roots and hypocotyl (R)

3 = moderate necrosis and stunting (S)

4 = extensive necrosis and stunting (S)

5= dead seedling (S)

¹National Alfalfa Variety Review Board

Phytophthora Root Rot

- Close 'relative' of *Aphanomyces*.
- Global distribution
- Causes seedling disease in wet cool soils, and also disease of mature plants.

Phytophthora Root Rot

- Produces a survival spore (oospore) that can survive for decades in soil.
- Tap root rots off at level where water drainage is impeded by soil compaction.
- Causes disease 'complex' with *Aphanomyces* and nematodes.

Phytophthora root rot of alfalfa seedlings

Infected

Healthy



Phytophthora root rot *in situ*



Rating¹ Alfalfa for Phytophthora Resistance

Two Classes:

1. Resistant = Vigorous plant, at most slight chlorosis and necrosis.
2. Susceptible = stunted, chlorotic, necrotic plant

¹National Alfalfa Variety Review Board

Can methods for evaluating resistance be improved?

- Disease rating scales are subject to within and between-evaluator variation.
- Rating systems have limited discriminatory power due to use of a semi-continuous scale.

Problem: How to discriminate
among plants that are
phenotypically similar based on
visual assessment of disease
severity??

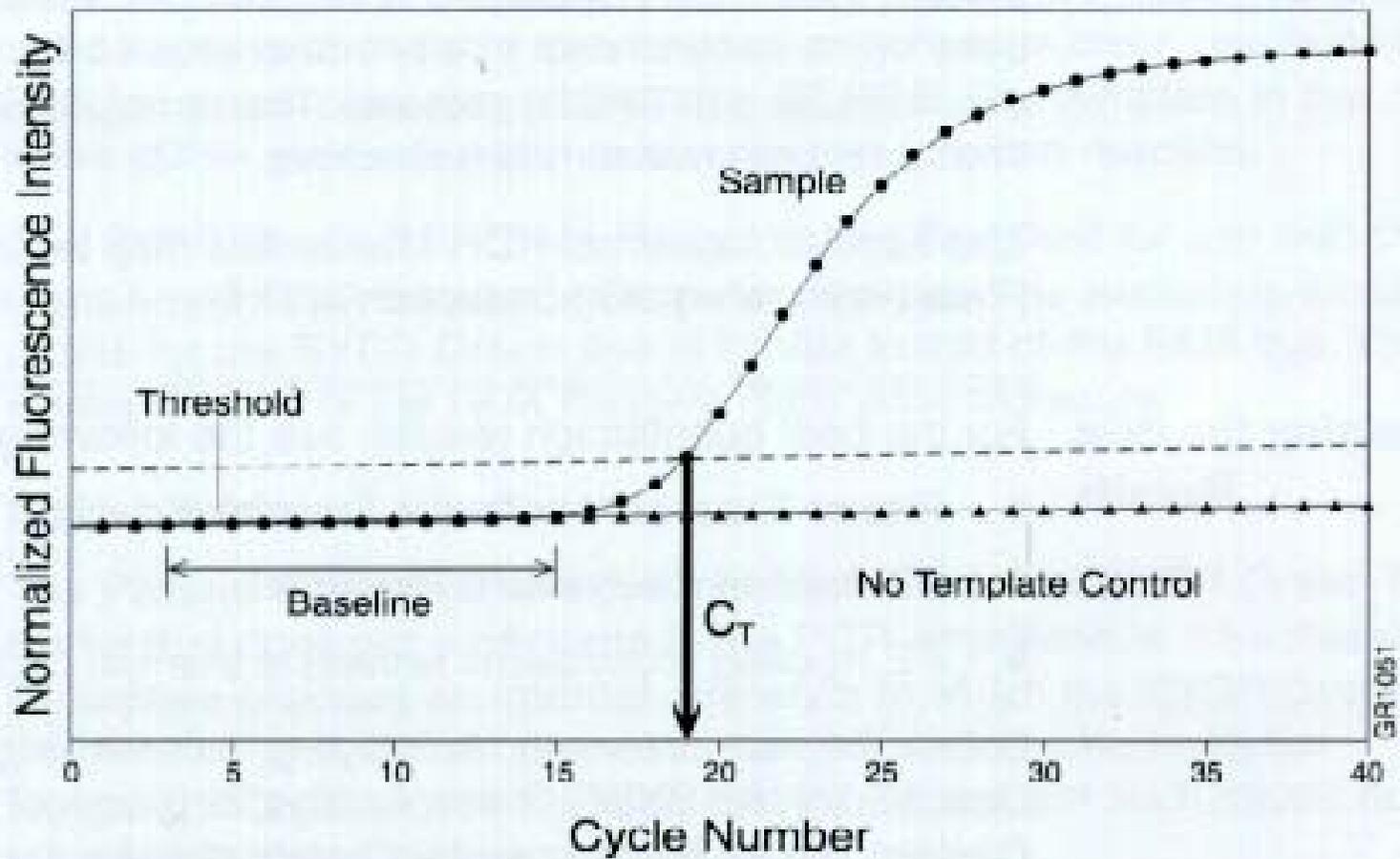
Possible Solution..... Use quantitative PCR to examine differences among plants in the amount of pathogen present in infected tissue.



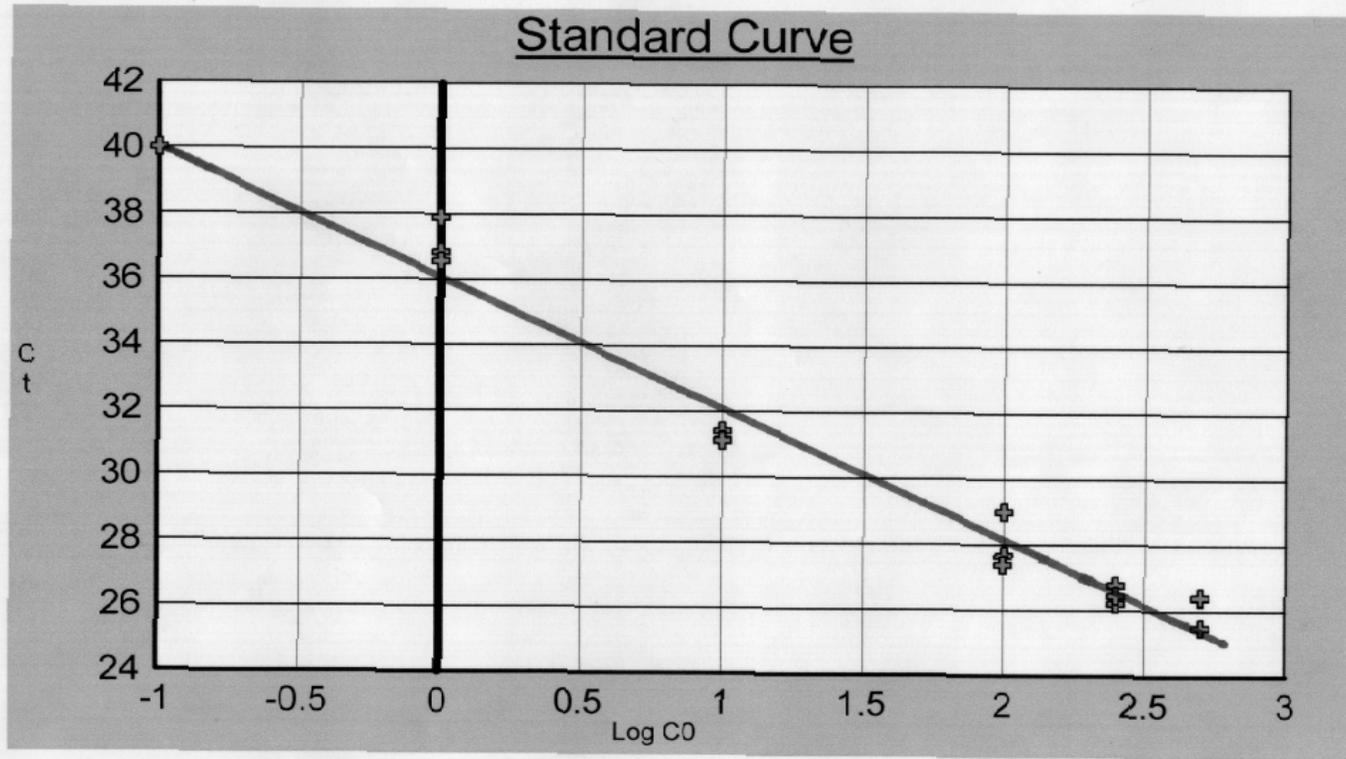
Real-time Quantitative PCR

- “Electrophoresis-free” PCR
- Based on the detection of fluorochrome relative to standards using known quantities of DNA
- Amplicon size range 50 bp-150 bp

Amplification Plot



More DNA = Lower C_T

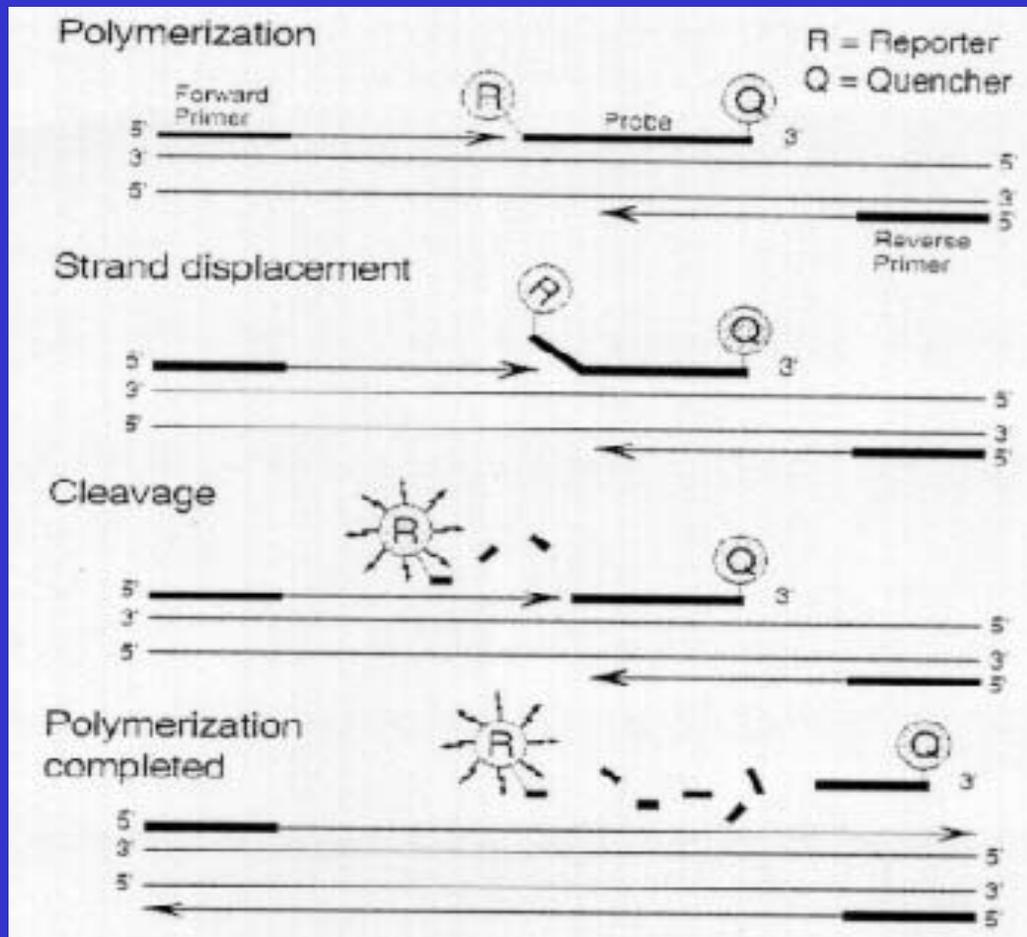


Slope: -3.972257

Intercept: 36.183788

Correlation: -0.989962

Taqman Chemistry: Separation of Reporter Dye and Quencher Dye Increases Fluorescence



How to make a pathogen-specific quantitative PCR assay

1. Identify a DNA sequence that is unique to the pathogen (such as a SCAR).
2. Use DNA sequence to identify candidate primer/Taqman probe sets.
3. Confirm specificity and sensitivity of assay using purified pathogen and plant DNA

OBJECTIVES

- Use quantitative PCR to investigate the relationship between resistance and quantity of *Aphanomyces* and *P. medicaginis* DNA in individual plants.
- Discriminate between resistant and susceptible check alfalfa populations using qPCR.
- Discriminate between commercial cultivars using qPCR.

MATERIALS and METHODS

Pathogen isolates

A. euteiches MF-1* and MW5-43 (Race 1)

A. euteiches NC 1* and WI-98(Race 2)

*Type isolates for races 1 and 2

P. medicaginis WI301

Alfalfa Check Varieties

Aphanomyces

race 1: Saranac (S), WAPH- 1 (R)

race 2: Saranac (S), WAPH-1 (S), WAPH-5 (R)

Phytophthora

Saranac (S), WAPH-1 (R)

Plant Inoculations

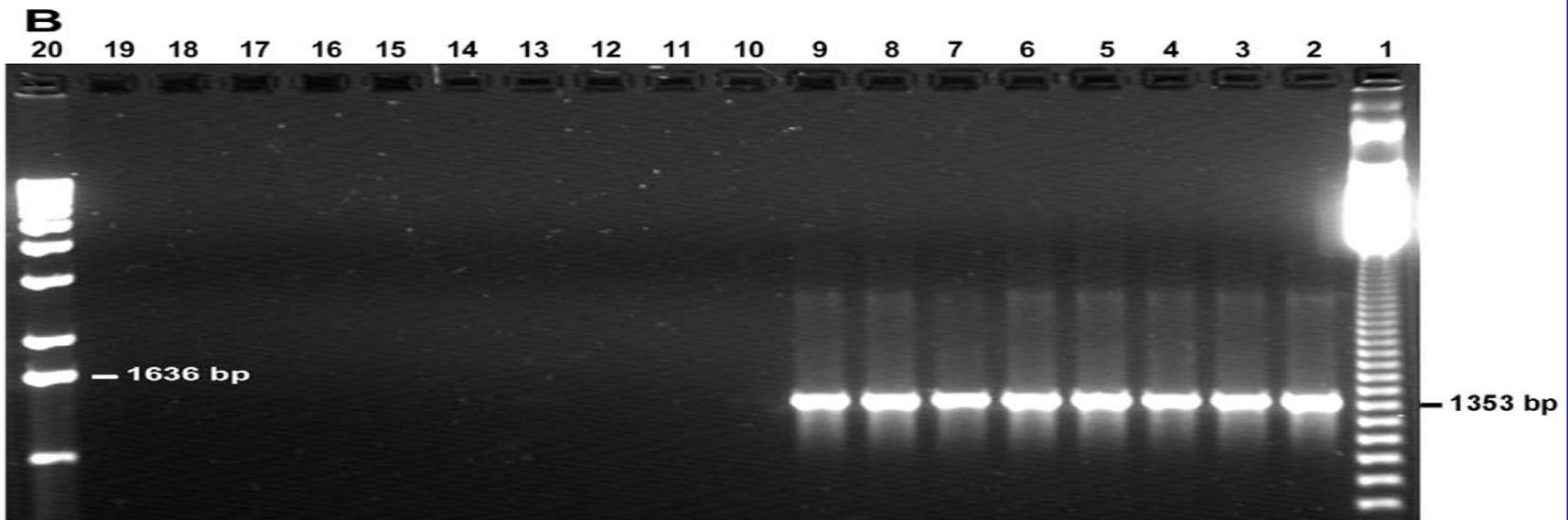
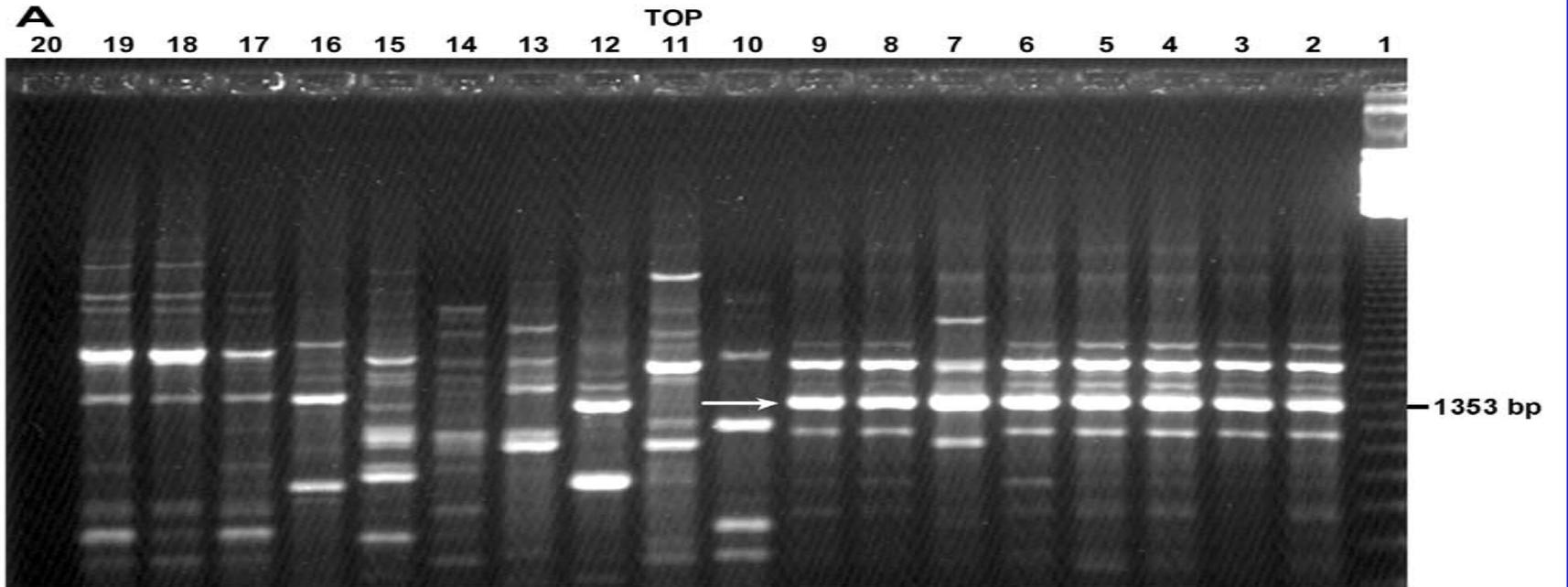
- NAAIC standardized test (1000 zoospores/plant).
- Plants were scored for DSI and individually harvested or randomly bulked (15 plants/bulk).
- DNA was extracted from roots.
- qPCR, with 3 repetitions DNA sample.

Alfalfa cultivars tested for resistance to *A. euteiches* MF-1 (Race 1)

- 15 cultivars tested, 3 for each resistance class (HR,R,MR,LR,S). WAPH-1 and Saranac included as controls.
- Plants scored for DSI, then bulked.
- 6 bulks (15 plants)/cultivar/experiment

RESULTS

SCAR Specific for *A. euteiches*



BOTTOM

Primer-probe Set for Quantitative PCR¹ of *A. euteiches*

100-TGCGACGCTGAGCTTGACCTTGTCGAATGCCTCTTG**GAC**

TGCAATGTCGTCCAAGACTTTGCAACCACCGAGCGAGCC

Forward Primer 136F

Taqman Probe

GCGCACTGCGTCGATCTCTTCATCTCAGCTTTGT-211

Reverse Primer 211R

¹Amplifies a 76 bp fragment

RESULTS: Standard checks (disease free)



Saranac

Waph-1

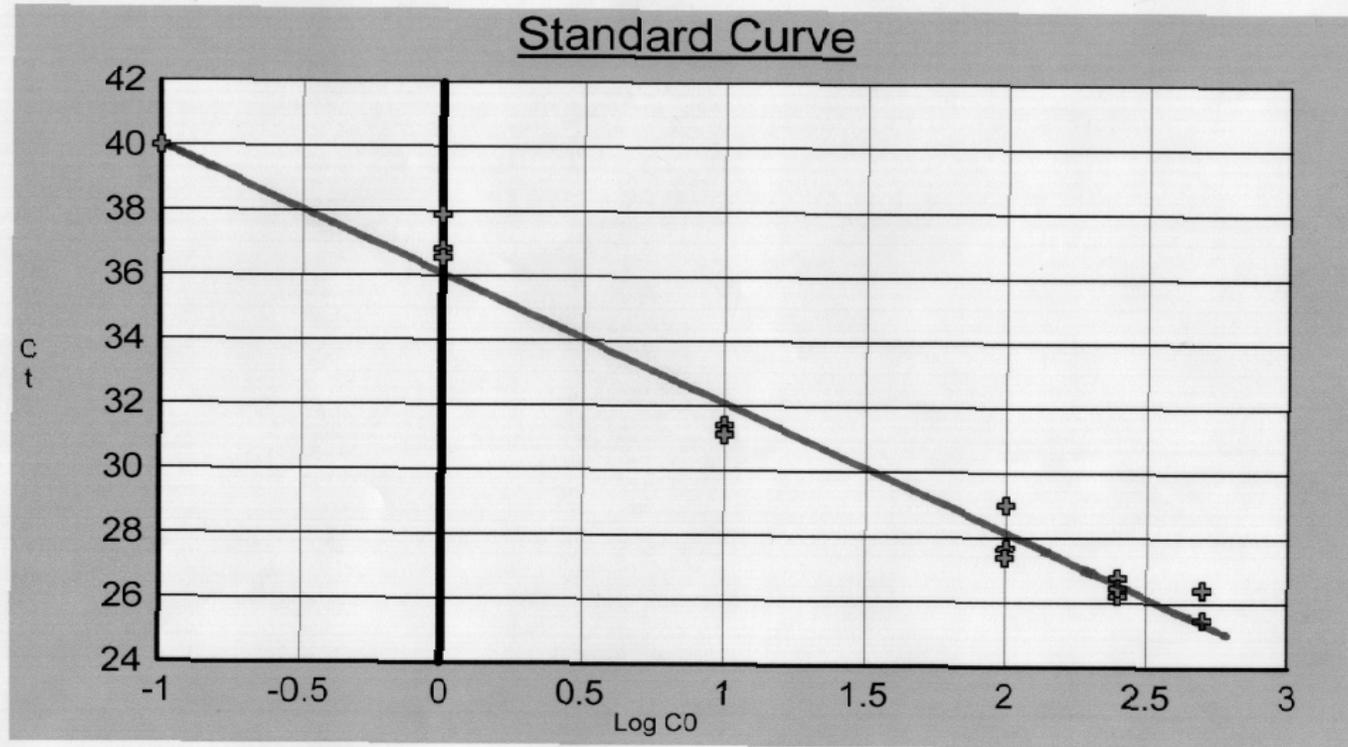
RESULTS: Standard checks (*A. euteiches* MF-1)



Saranac (S)

Waph-1 (R)

Detection of *A. euteiches* with Quantitative PCR

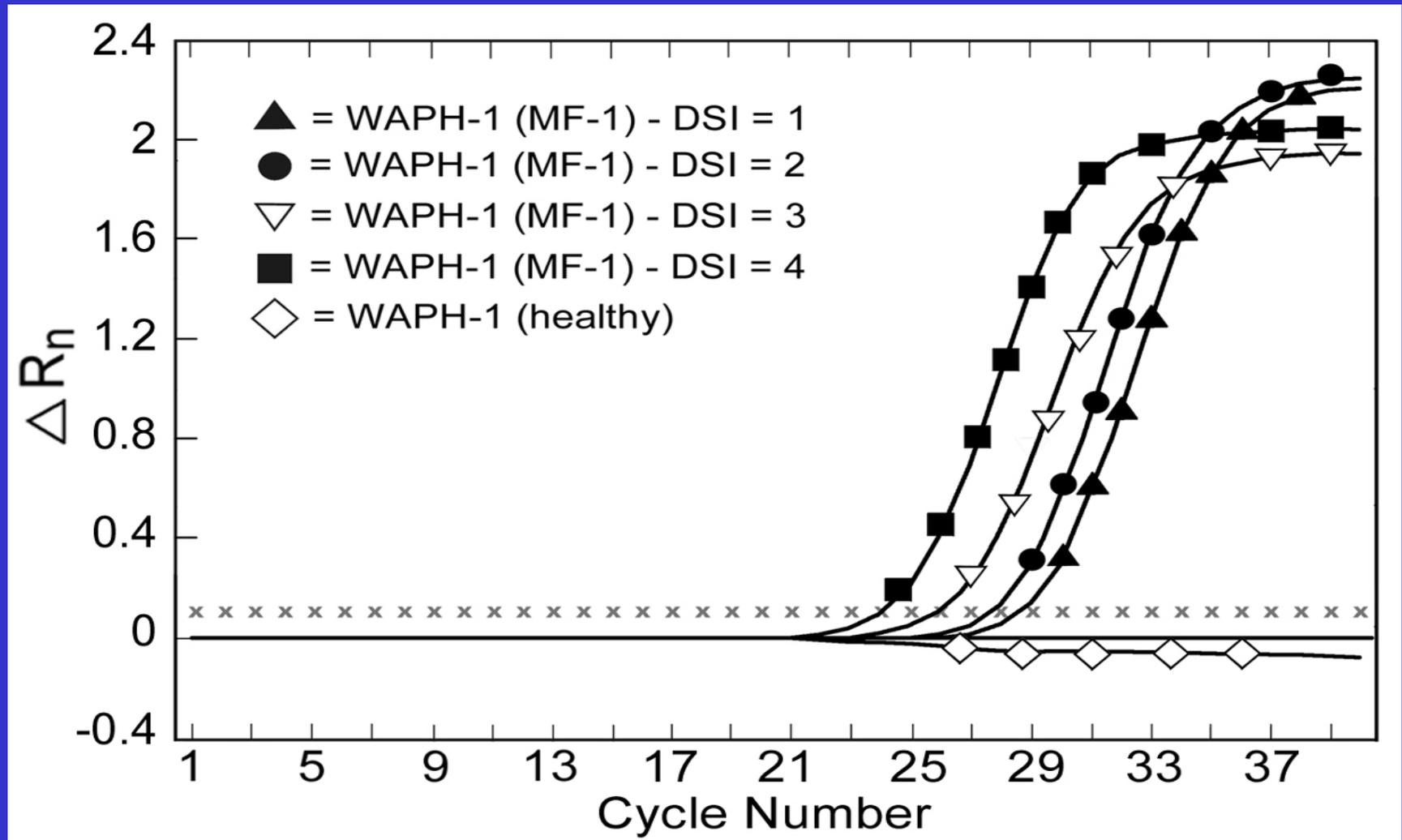


Slope: -3.972257

Intercept: 36.183788

Correlation: -0.989962

Detection of *A. euteiches* in Single Plants



Detection of *A. euteiches* in Single Plants

DSI*	WAPH-1 (MF-1)	WAPH-5 (NC-1)
1	1.27 a	4.11 a
2	1.79 a	5.74 b
3	3.98 b	16.13 c
4	12.86 c	15.47 c
LSD ($\alpha = 0.05$)	1.97	1.46
ρ (Prob > $ \rho $)	0.85 (<0.0001)	0.83 (<0.0001)

* N = 12 plants/class, 100 ng root DNA/PCR rxn

Detection of *A. euteiches* in Single Plants

DSI*	WAPH-1(MW5-43)	WAPH-5(WI-98)
1	1.45 a	1.60 a
2	4.00 b	1.69 b
3	12.59 c	2.39 c
4	12.40 c	3.81 c
LSD ($\alpha = 0.05$)	2.35	0.68
ρ (Prob > $ \rho $)	0.82 (<0.0001)	0.61 (<0.0001)

* N = 12 plants/class, 100 ng root DNA/PCR rxn

Bulk Analysis*: Standard Check Populations

	<i>A. euteiches</i> MF-1		<i>A. euteiches</i> NC-1	
Population	ng DNA	DSI	ng DNA	DSI
WAPH-1	2.12a	2.79a	13.58a	3.99a
Saranac	8.75b	3.92b	12.66a	3.96a
WAPH-5	-	-	2.63b	2.66b
LSD($\alpha=0.05$)	1.60	0.13	1.23	0.13
$\rho(P > \rho)$ (DSI vs DNA)	0.78 (0.0004)		0.79 (<0.0001)	

*15 plants/bulk, 4 bulks/pop/exp, 100 ng root DNA/PCR rxn

Bulk Analysis*: Standard Check Populations

Population	<i>A. euteiches</i> MW5-43		<i>A. euteiches</i> WI-98	
	ng DNA	DSI	ng DNA	DSI
WAPH-1	1.62 a	2.59a	9.81 a	4.00 a
Saranac	8.03 b	3.81b	11.79 b	3.99 a
WAPH-5	-	-	2.62 c	3.00 b
LSD($\alpha=0.05$)	1.65	0.13	1.83	0.19
$\rho(P > \rho)$ (DSI vs DNA)	0.86 (<0.0001)		0.74 (<0.0001)	

*15 plants/bulk, 4 bulks/pop/exp, 100 ng root DNA/PCR rxn

Bulk Analysis*: Commercial Cultivars

Variety	NAVRB Rating	ng DNA	DSI
WAPH-1	HR(✓)	1.08 (1)a	2.87 (3)ab
Winterking	R	2.25 (2)ab	2.70 (1)a
Ranier	HR	2.34 (3)ab	2.99 (6)b
WL 232HQ	HR	2.36 (4)ab	2.83 (2)ab
Ultralac	HR	2.71 (5) bc	2.88 (4)ab
WL 325HQ	R	3.72 (6)cd	2.98 (5)b
5347 LH	R	3.79 (7)cd	3.49 (8)cd
Saranac	S(✓)	7.29 (17) f	3.84 (17)g
LSD($\alpha = 0.05$)		1.31	0.22
ρ ($P > \rho $)	0.54 (< 0.0001)		

15 plants/ bulk, 6 bulks/cultivar/exp, 100 ng root DNA/PRC rxn

Quantifying *P. medicaginis* in Bulked* Alfalfa Samples

Population	ng DNA
WAPH – 1 (HR)	0.31 a
Agate (Res)	1.31 b
Saranac (Sus)	2.29 c
LSD ($\alpha = 0.05$)	0.13
ρ (Prob > $ \rho $) ng DNA vs # sus. plants	0.90 (< 0.0001)
ρ (Prob > $ \rho $) ng DNA vs # res. Plants	-0.89(< 0.0001)

*15 plants/bulk, 8 bulks/population, 100 ng root DNA/rxn

Quantifying *P. medicaginis* in Single Plants*

Class	ng DNA/plant	Range (ng DNA)
Resistant (N = 12)	0.05 a	0 – 0.27
Susceptible (N=12)	2.04 b	1.11 – 2.71
LSD ($\alpha = 0.05$)	0.11	

* 100 ng root DNA/rxn

Summary of Results

- PCR primer/probe sets selectively amplified pathogen DNA and not host DNA.
- Quantification of DNA based on PCR assay was very precise ($R^2 \geq 0.97$).

Summary of Results (*A. euteiches*)

- Correlation between amount of pathogen DNA and disease severity was positive and highly significant for single plants and bulked plant samples.
- Separation of commercial varieties for resistance based on qPCR closely approximated published classification based on results of standard test.

Summary of Results (*P. medicaginis*)

- Correlation between amount of pathogen DNA and number of resistant plants in a bulked sample was negative and highly significant.
- Correlation between amount of pathogen DNA and number of susceptible plants was negative and highly significant.
- WAPH-1 (HR), Agate (Res), and Saranac (S) could be clearly separated based on qPCR.

Future Research Objectives

- Develop quantitative PCR assays for other alfalfa pathogens (*Fusarium* and *Verticillium*).
- Use qPCR assays to develop alfalfa germplasm with extreme resistance to multiple soilborne pathogens.
- Use qPCR assays to study population dynamics in plants infected with multiple pathogens.

Contributors

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