NCSU PEANUT BREEDING PROGRAM PROPOSAL

Marker-Assisted Selection in Virginia-Type Peanut for Multiple Disease Resistance

2020-0236

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Marker-Assisted Selection Report

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- COVID-19 Update on Field Program
- Marker-Assisted Selection Methodology
  1. Seed Chipper/Corer
  2. Crude DNA Isolation
  3. Multiplexing Marker Technology
- Impacts of Methodology
- Greenhouse Speed Breeding

Peanut Breeding Program – Marker Use

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1. Verify all parental materials used in crossing block
2. Confirm all crosses (F_1 Plants)
3. Genotype all early generation materials (F_2 through F_5) at large effect markers for marker-assisted selection

Genome-Wide Association Analysis

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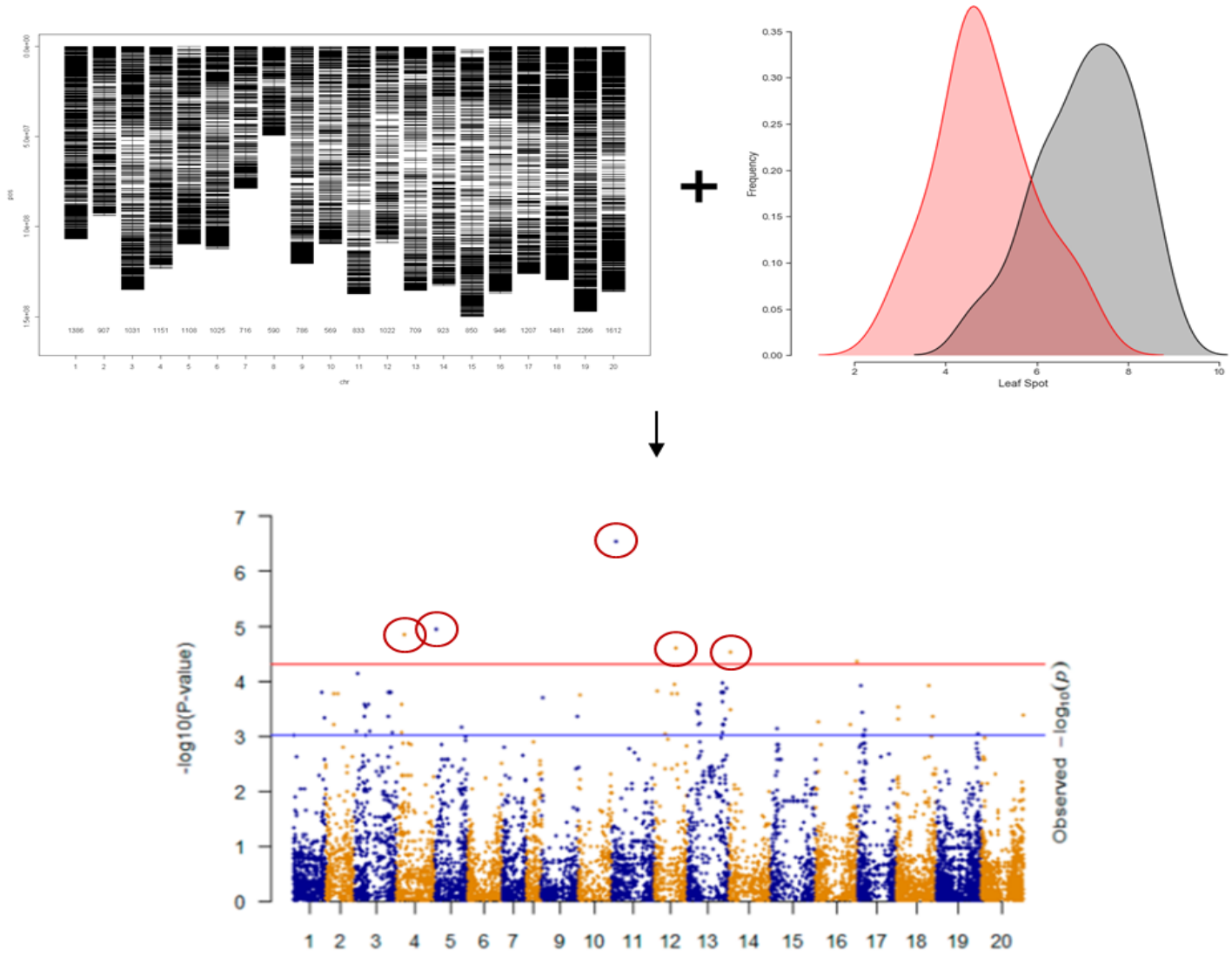

Marker-Assisted Selection in Peanuts

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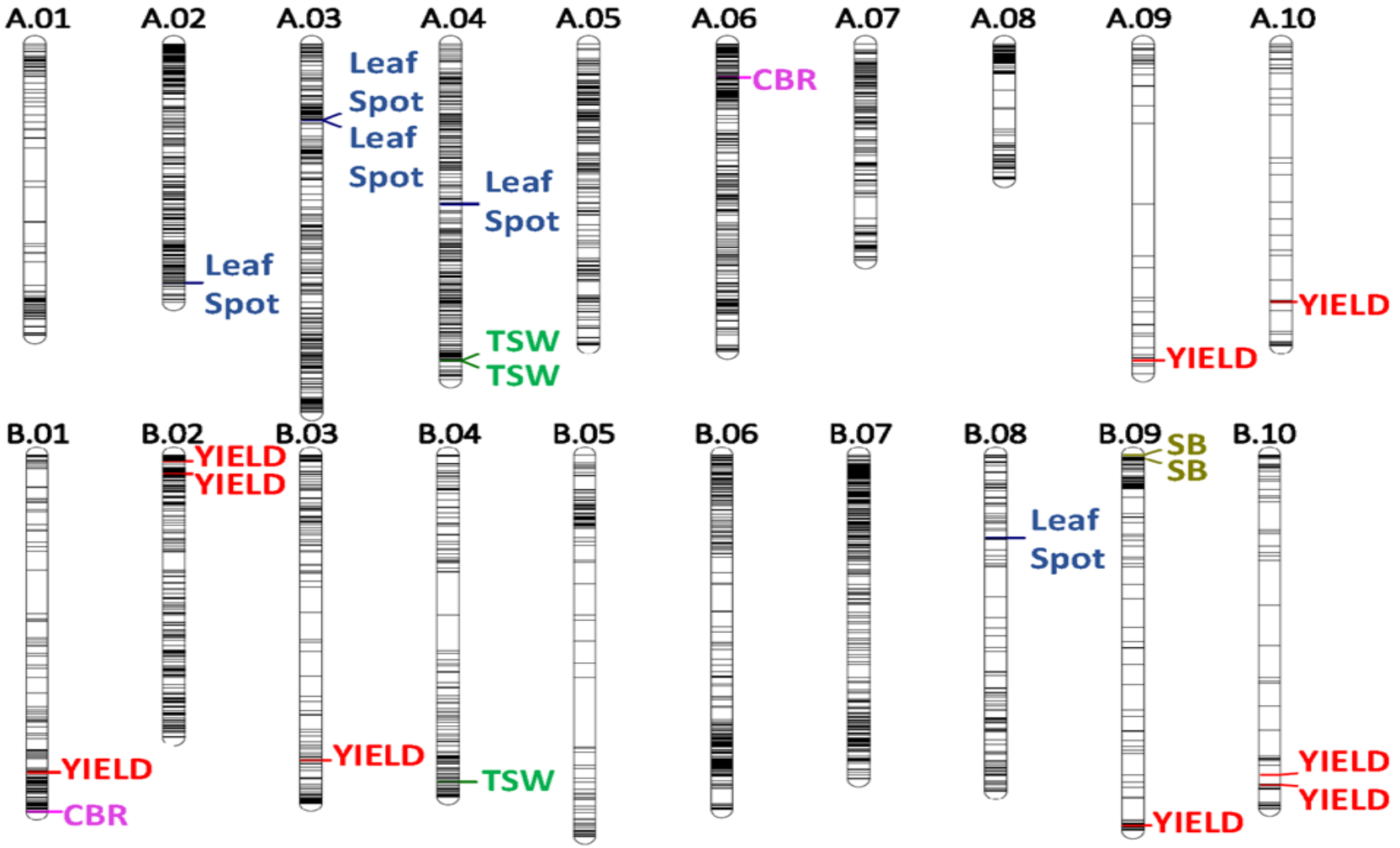

Identify Marker Trait Associations

GWAS or QTL Analysis Results

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Trait*	A. Hypogaea Chromosome	A. Hypogaea Physical Position (bp)	GWAS Adjusted r2	Frequency of Desirable Allele	Frequency of Undesirable Allele
PY	10	101,147,237	0.07	0.9	0.1
PY	19	153,897,034	0.07	0.61	0.39
PY	8	34,876,837	0.08	0.79	0.21
LS	2	94,810,239	0.1	0.93	0.07
LS	3	30,614,262	0.02	0.97	0.03
LS	18	32,865,575	0.13	0.06	0.94
CBR	13	123,937	0.07	0.5	0.5
CBR	11	148,970,476	0.07	0.2	0.8
SB	19	985,995	0.12	0.5	0.5
TSWV	14	135,149,720	0.06	0.09	0.91
FAD2B	19	154,049,242	N/A	0.89	0.11

* PY - Pod Yield, LS - Leaf Spot, CBR - Cylindrocladium black rot, SB - Sclerotinia blight, TSWV - Tomato spotted wilt virus, and FAD2B - Gene providing the high-oleic trait

Other Traits? Flavor, Folate, etc.

Greenhouse Crossing Program

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Spring (F_1)

Disease Resistant
Parent ♂

Agronomic Trait
Parent ♀

Summer (BC_1F_1)

F1 Cross ♂

Agronomic Trait
Parent ♀

Winter (Puerto Rico - Winter Nursery)

Marker-Assisted Selection in Peanuts

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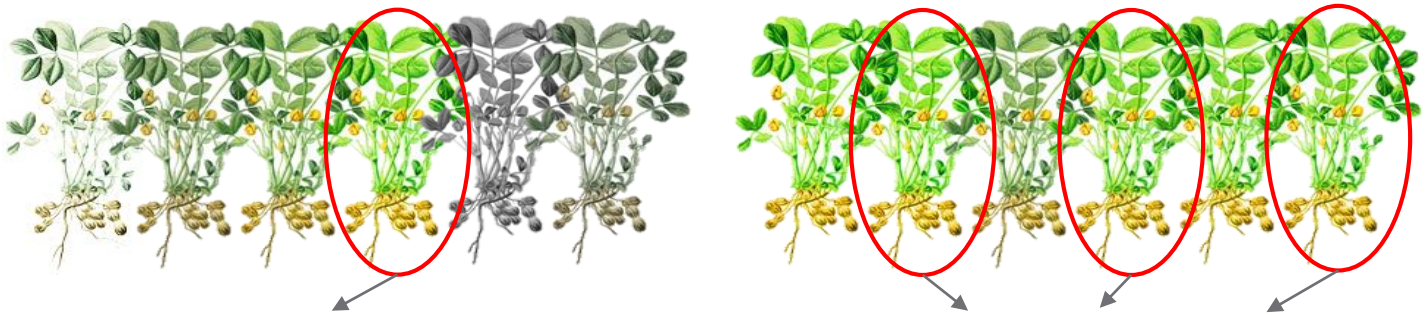
F2 Among and Within Family Selection



Generational Pipeline

Advanced Disease Pipeline

~ 200 Family Lines — F3 Single Plant Selections

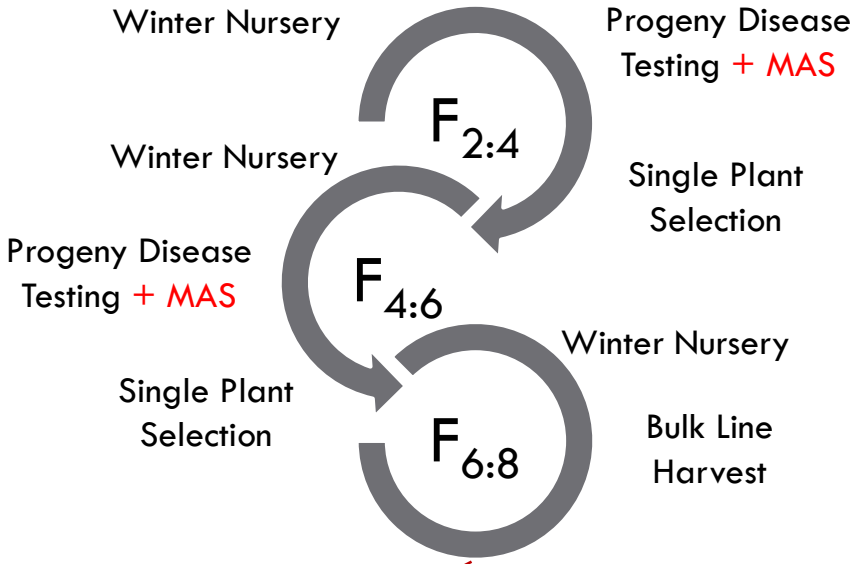
~ 350 Family Lines — F4 Single Plant Selections

~ 500 Family Lines — F5 Single Plant Selections

~ 400 Family Lines — F6 Single Plant Selections

~ 250 Family Lines — F7 Bulk Line Selections

~ 150 Family Lines — Preliminary Yield & Disease Screening



Program Testing Network

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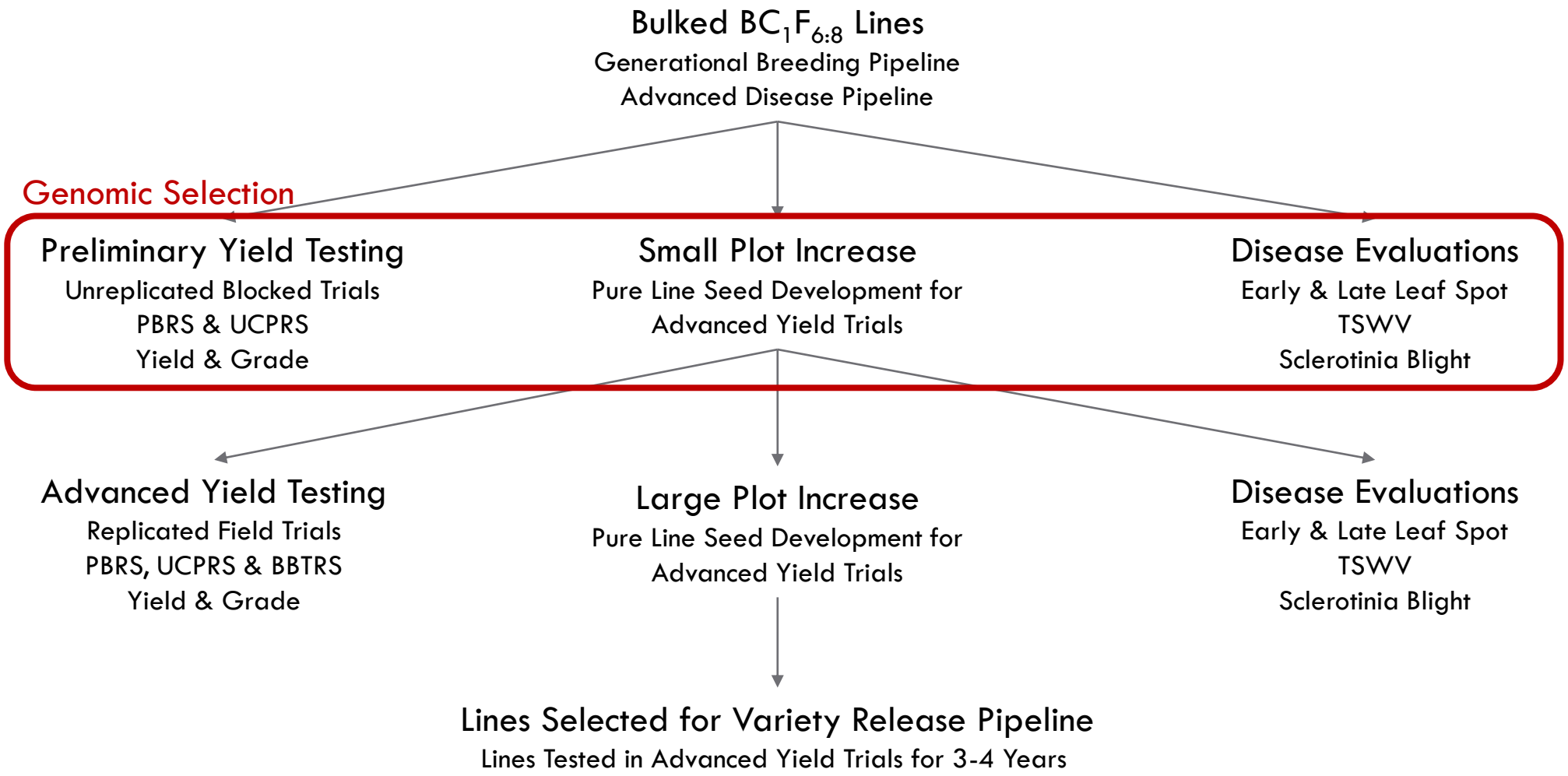

Marker Assisted Selection Methodology

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1. Manual Seed Chipper > Sample 384 seeds rapidly
2. Standard “crude” DNA extraction from 384 samples in one hour at minimal costs
3. Custom multiplex SNP genotyping

Seed Chipper – Monsanto Patent Avoided

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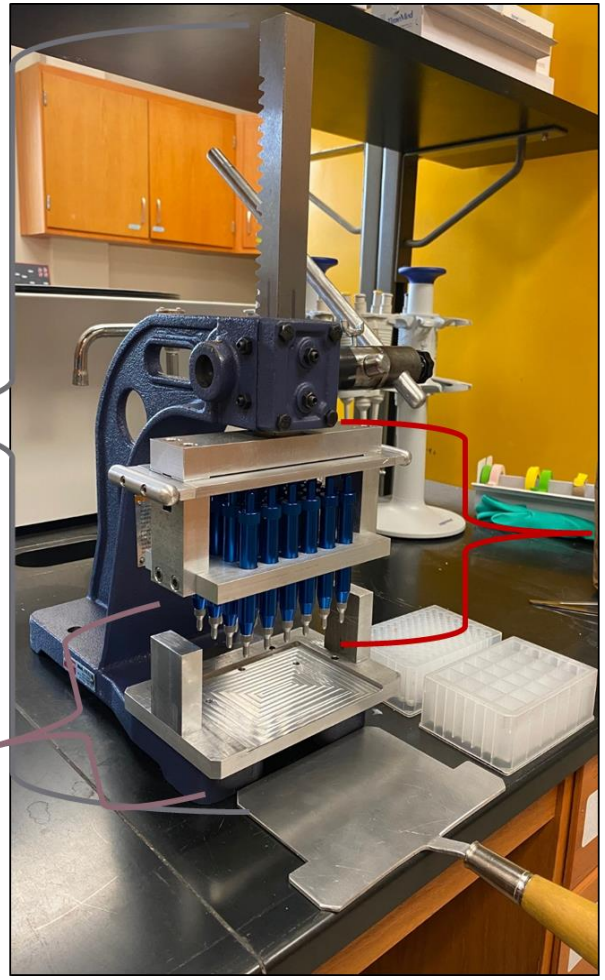
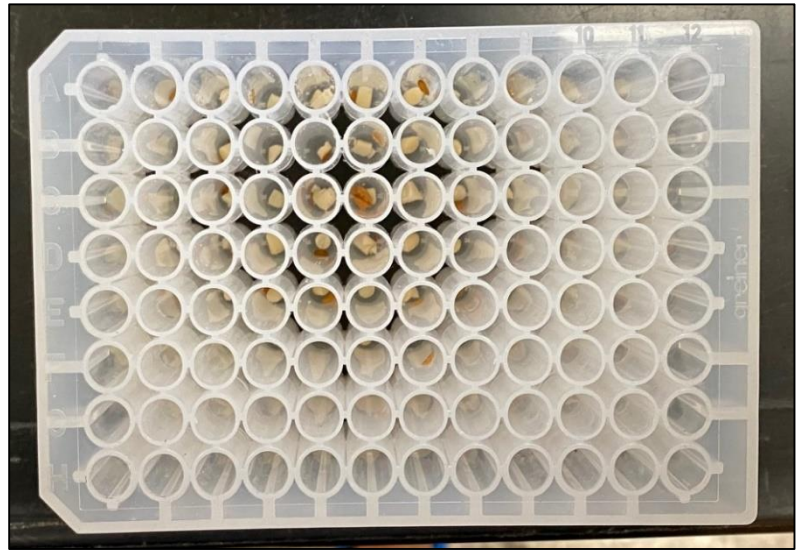
1. Standard Arbor Press



3. Custom-built base

2. 24 modified biopsy punches in fixed positions (4 x 6 layout) with a custom-built single ejector



Crude DNA Isolation

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1. Spin briefly in plate centrifuge.
2. Add 50µL Buffer A to each well and mix by inversion.
3. Heat at 65oC for 10 minutes.
4. Add 200µL Buffer B and mix by inversion
5. Spin briefly and transfer 60µL of supernatant to a new plate
6. Use 2µL in PCR reaction, no need to quantify DNA

Buffer A:

Stock	Volume	Final Concentration
10 M NaOH	200uL	100mM
Tween 20	400uL	2%
Ultrapure H2O	19.4mL	N/A

Buffer B:

Stock	Volume	Final Concentration
1 M Tris-HCl#	8mL	100mM
0.5 M EDTA	320uL	2mM
Ultrapure H2O	72mL	N/A

Zheng *et al.* 2015. Non-destructive high-throughput DNA extraction and genotyping methods for cotton seeds and seedlings. *BioTechniques* 58: 234-243.

KASP (or PACE) Assay

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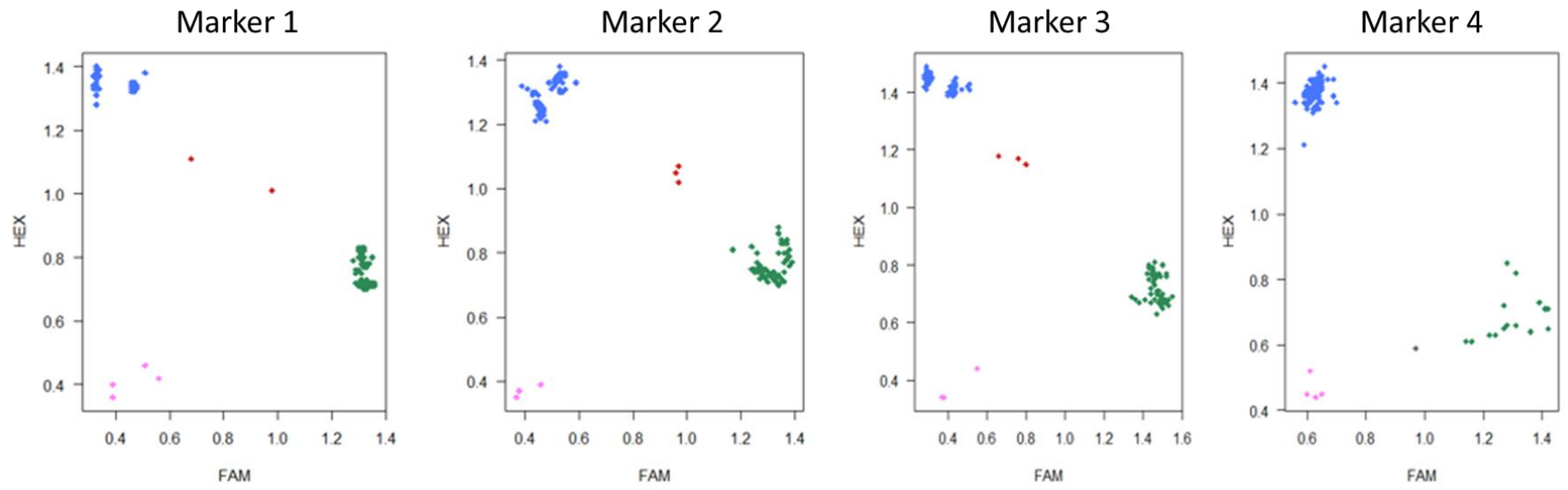
Most Marker Behave Similarly…

- Markers can often be grouped based on their fluorescence pattern
- What would happen if you arranged the beneficial allele on the same axis across all assays and then pooled the beneficial markers together?



Marker-Assisted Selection

Inbreds w/ high-quality DNA isolated with Qiagen kit, quantified and normalized

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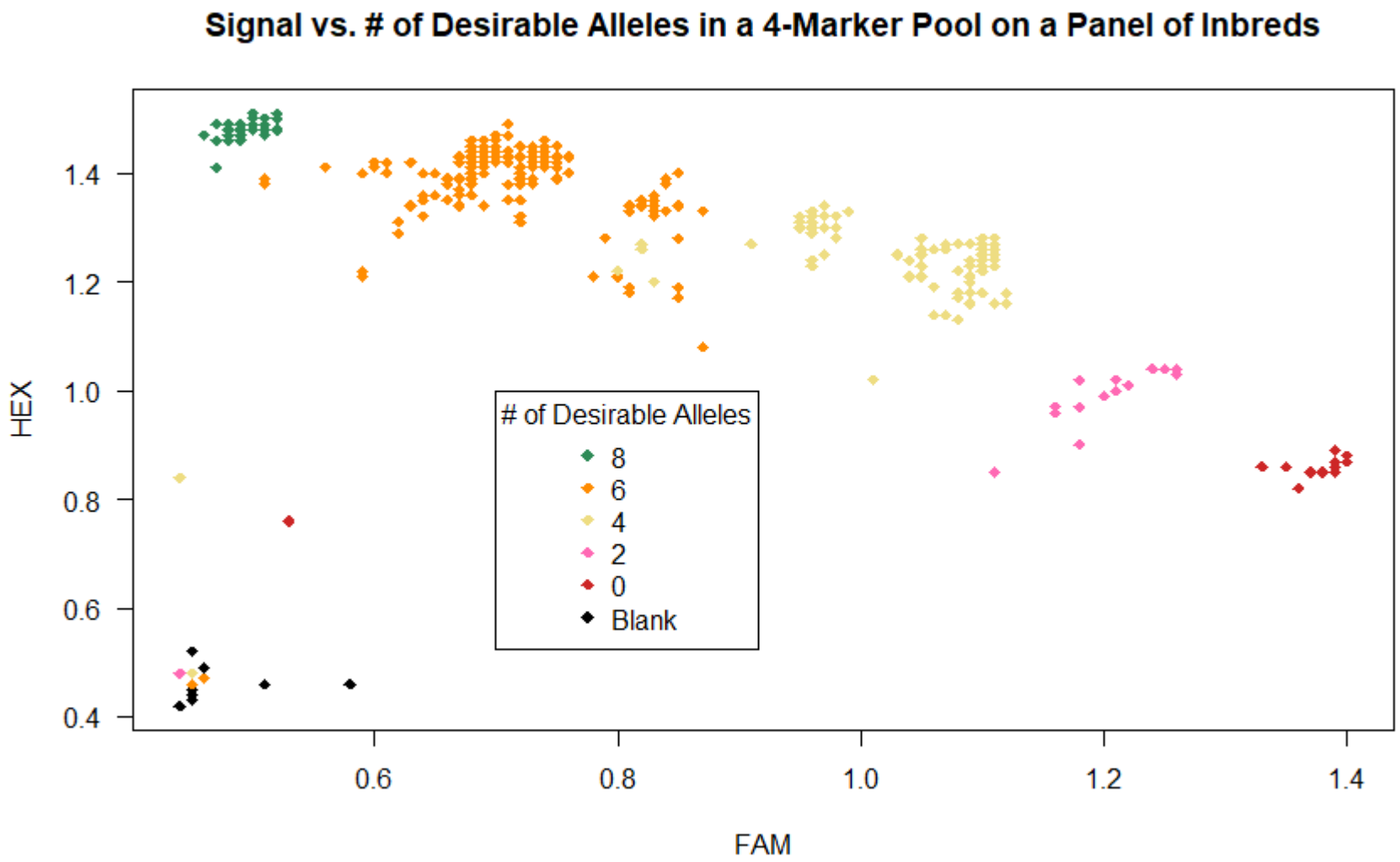

Marker-Assisted Selection

Inbreds w/ crude DNA isolation – not quantified or normalized

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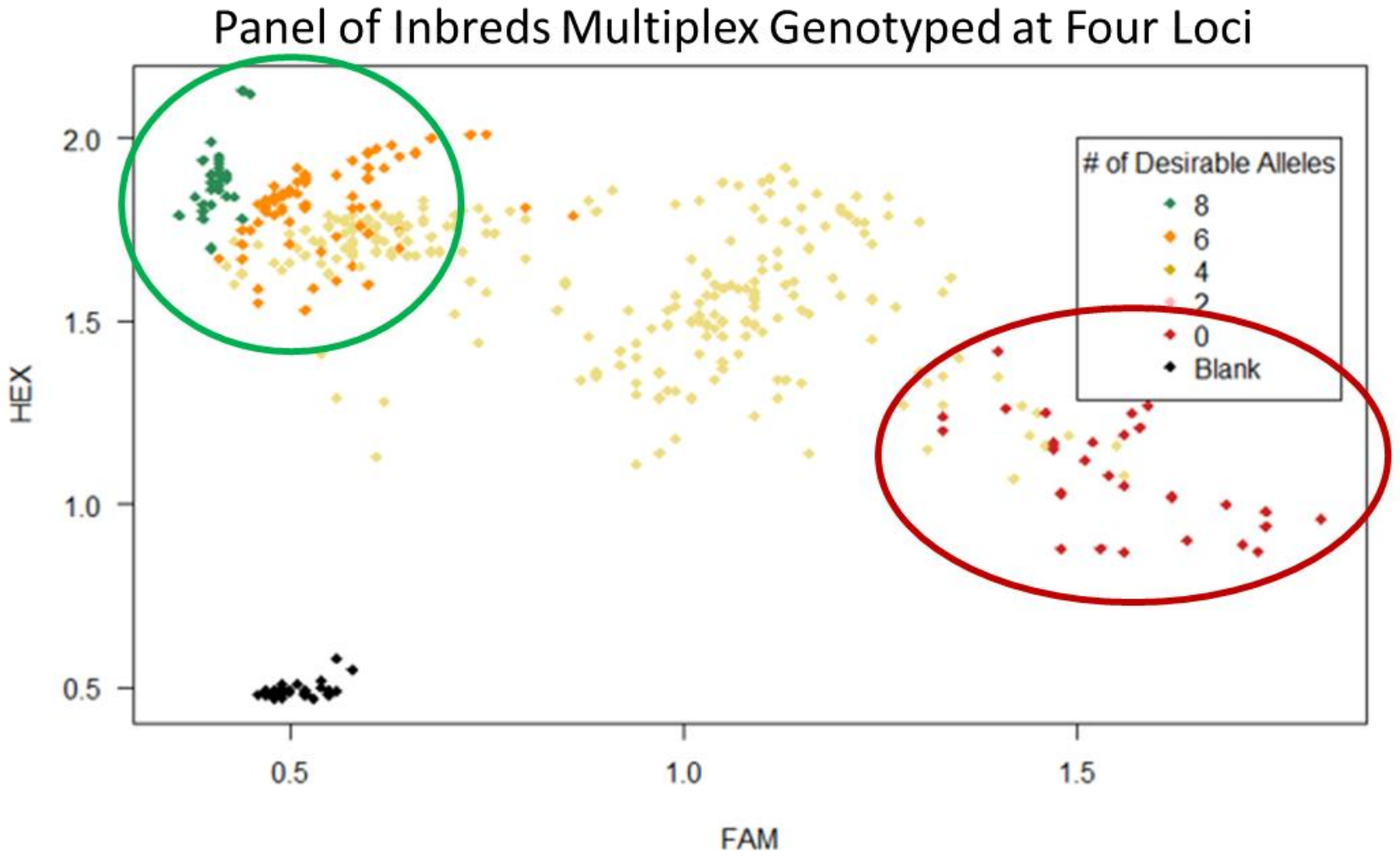

Impact of Methodology

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Pros:

- Reduced genotyping costs
- Increased speed
- Simplified data analysis and selection – selection index just HEX/FAM ratio

Verify all parental materials used in crossing block

Confirm all crosses (F_1 Plants)

Genotype all early generation materials (F_2 through F_5) at large effect markers for marker-assisted selection

- ❖ Ideally adopt a speed breeding approach to turn 3+ generations per year in the greenhouse
- ❖ Concentrate field resources on genomic selection and testing later generation materials

Greenhouse Speed Breeding w/ MAS

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Spring (F_1)

Disease Resistant
Parent ♂

Agronomic Trait
Parent ♀

Summer (BC_1F_1)

F1 Cross ♂

Agronomic Trait
Parent ♀

~~Winter (Puerto Rico - Winter Nursery)~~

Greenhouse Speed Breeding w/ MAS

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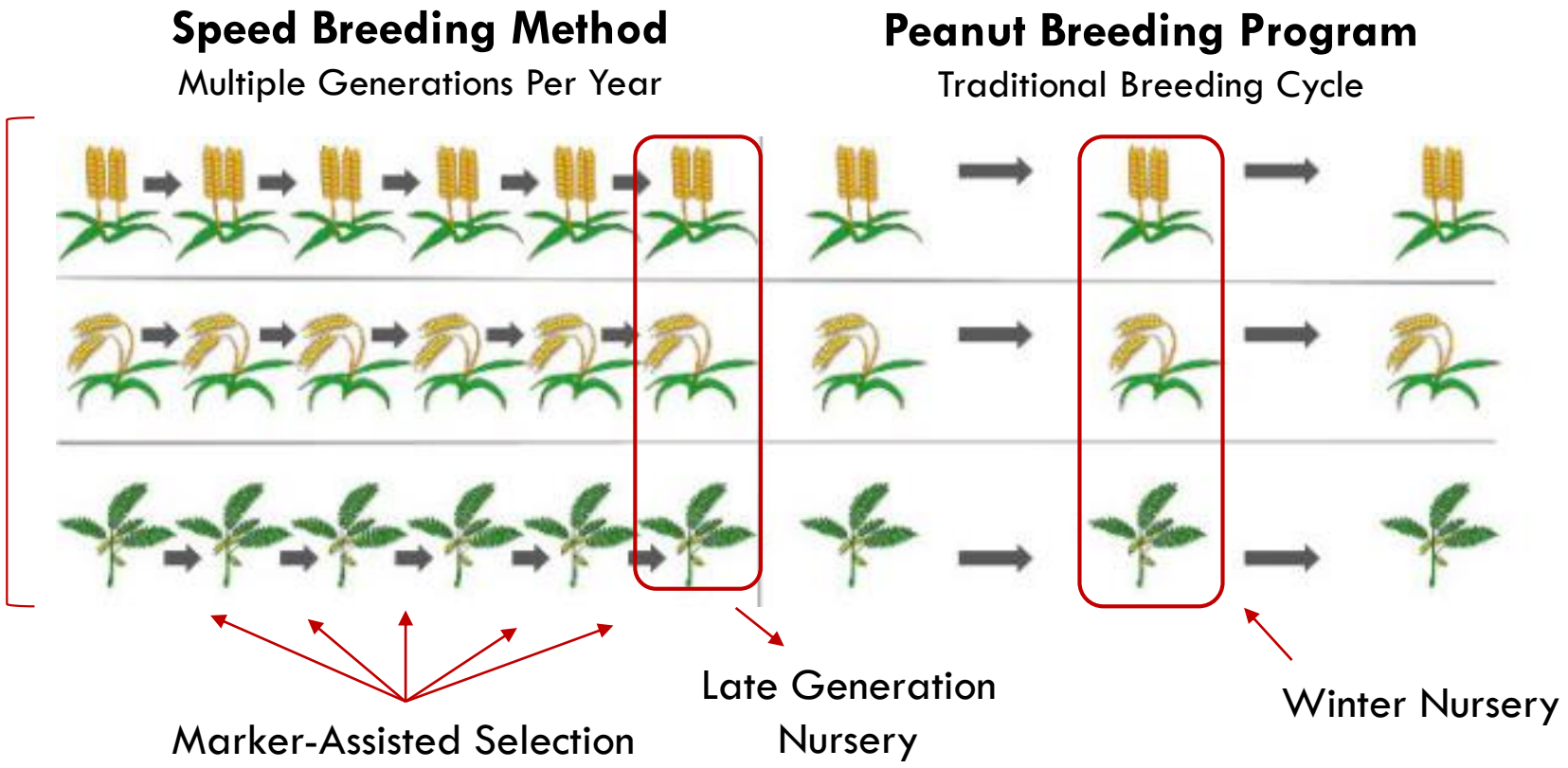
BC_1F_1 and F_1 Progeny in Greenhouse



Greenhouse Speed Breeding w/ MAS

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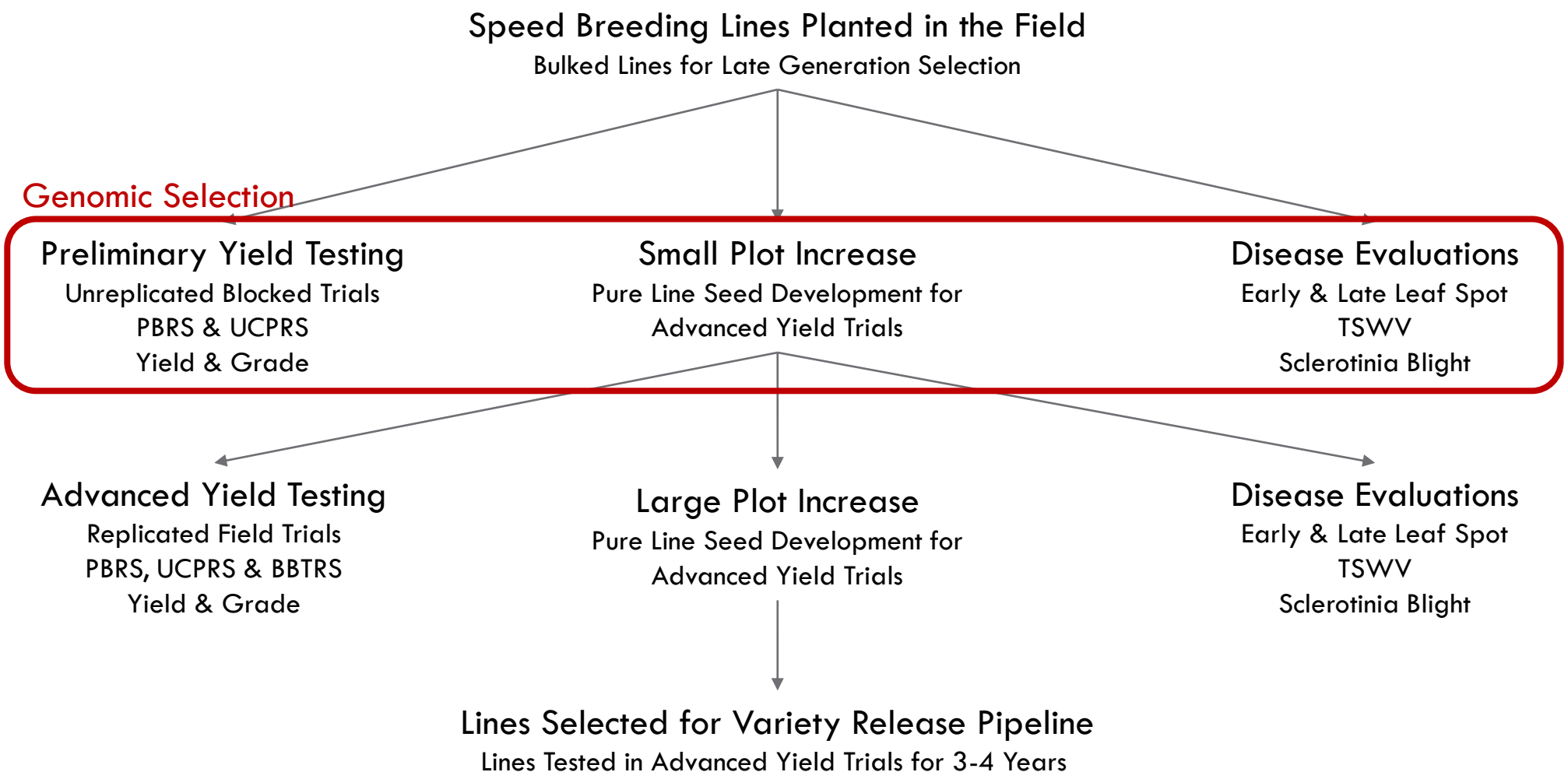

2021 Budget

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FUNDS REQUESTED:		2021
EPA Salaries and fringe benefits[1]		
Graduate Student Stipends and Support[2]		$21,000
SPA Salaries and fringe benefits[1]		
Part-time Labor	(including: 8.60% fringe benefits, and add $142/year health insurance for hourly / part-time workers who will work ≥ 30hrs/wk. for longer than 3 months)	$5,000
Supplies	(such as: lab and field consumables, fuel, etc.)	$2,500
Equipment	(greater than $500)	
Travel	(for airfare, overnight lodging and meals associated with project tasks)	
Other	(such as: motor pool rental, lab analysis, contracted services, greenhouse space, page/publication charges)	
TOTAL in Dollars		$28,500

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Marker Development through Next-generation Sequencing (NGS) for Late Generation Selection

NC-42

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Next-Generation Sequencing Report

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Protocol Development Steps

1. Development of internal reference *A. hypogaea* cv. 'Bailey II'
2. Whole-genome sequencing of diverse breeding lines in NCSU peanut breeding program
3. Align whole-genome sequences to internal reference for single nucleotide polymorphism (SNP) discovery
4. *In silico* digest of *Arachis hypogaea* cv. 'Bailey II' reference genome
5. Use *in silico* site information (alignments) to maximize SNP recovery

Bailey II – Internal Reference Genome

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Table 1. Comparison of genome assemblies based on summary statistics compiled for Bailey II, Tifrunner (both versions), Fuhuasheng and Shitoqi for use as internal reference genome for the North Carolina State University peanut breeding and genetics program.

Assembly	Scaffold Total	Contig Total	Max Scaffold Length	Max Contig Length	% Main Genome in Scaffolds	BUSCO*
Bailey II (CANU)	89	1,334	90,278,000	61,544,000	100.00%	5,192
Tifrunner (v.1)	384	4,039	160,880,000	9,488,000	99.79%	5,182
Tifrunner (v.2)	442	4,139	160,028,000	9,488,000	99.78%	5,183
Fuhuasheng (v.1)	20	31,747	168,161,000	1,735,000	100.00%	5,078
Shitoqi (v.1)	21	7,747	159,155,000	8,551,000	100.00%	5,178

* Measure of completeness of genome

Whole-Genome Sequencing

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Table 2. List of 48 lines subjected to whole-genome sequencing (10x coverage) and SNP discovery using the Bailey II internal reference genome for alignment.

(EV) Bailey*	(EV) Perry*	(GL) GP-NC 343*	(MC) GP-NC WS 3	(MC) GP-NC WS 14	(MD) HTS IL-028*
(EV) Bailey II*	(EV) Sugg*	(GL) PI 121067	(MC) GP-NC WS 4	(MC) GP-NC WS 15	(MD) HTS IL-029*
(EV) Emery*	(EV) Sullivan*	(GL) PI 269685	(MC) GP-NC WS 5	(MC) GP-NC WS 16	(MD) HTS IL-047*
(EV) N05006	(EV) Wynne*	(GL) PI 270806	(MC) GP-NC WS 6	(MC) HTS 16-03*	(MD) HTS IL-049*
(EV) N11055B*	(EV) Gregory*	(GL) PI 576636*	(MC) GP-NC WS 7	(MC) HTS 16-04*	(MD) HTS IL-051*
(EV) N13042ol*	(FV) NC 5	(GL) PI 665000	(MC) GP-NC WS 11	(MC) HTS 16-06*	(MD) HTS IL-052*
(EV) N16021*	(FV) NC 6	(MC) GP-NC WS 1	(MC) GP-NC WS 12	(MC) SPT 07-01*	(MD) HTS IL-058*
(EV) N96076L*	(GL) Carolina Runner	(MC) GP-NC WS 2	(MC) GP-NC WS 13	(MD) HTS IL-002*	(MD) HTS IL-067*

* Denotes Whole-Genome Sequencing in Leaf Spot Trial

EV - Elite Virgina-type Cultivar or Breeding Line

FV - Virginia-type Founder Cultivar or Breeding line

GL - Germplasm Line

MC - Multiple Disease Resistant Lines with Introgressions from *A. cardenasii*

MD - Multiple Disease Resistant Lines with Introgressions from *A. diogoi*

Result: > 4 million SNP markers identified – Maximize SNP recovery with Genotype-By-Sequencing

Genotype-By-Sequencing (GBS)

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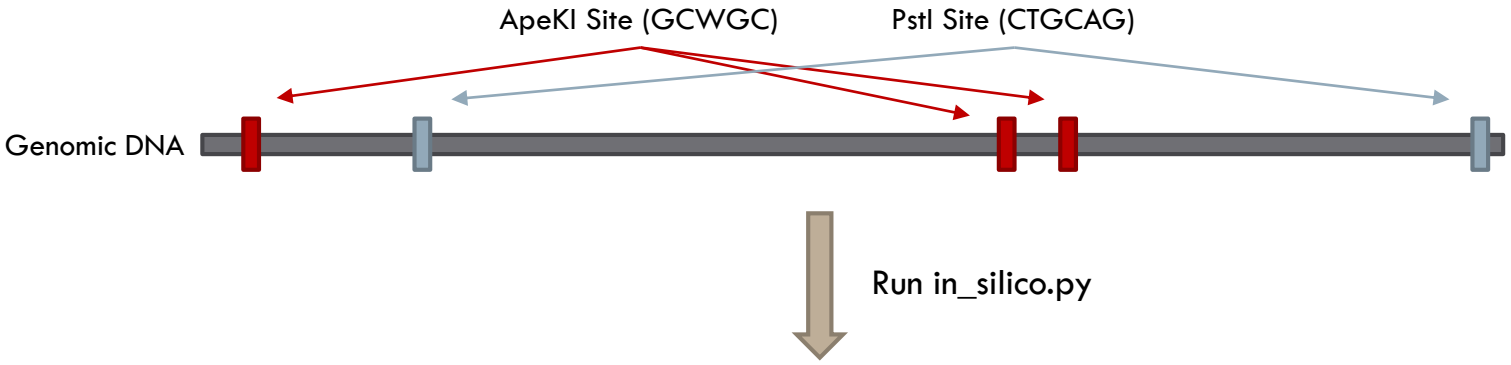
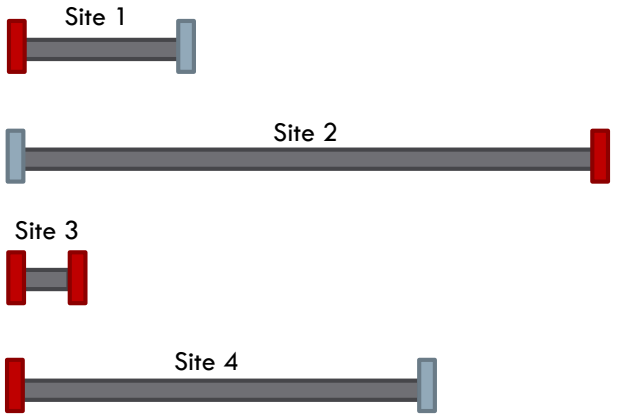
In silico, construct reduced representation libraries (RRLs) by digesting each DNA sample with a restriction enzyme pair (example: ApeKI – PstI)





Results from in_silico.py

Enz_Start	Cut	Source	Del	Couple	Size
15912.0 R	Freq	No	No	0.0	
16497.0 R	Rare	No	No	585.0	
19495.0 R	Freq	No	No	2998.0	
26735.0 R	Freq	No	No	7240.0	
27246.0 R	Rare	No	No	511.0	
28152.0 R	Freq	No	No	906.0	
29719.0 R	Freq	No	No	1567.0	
29986.0 R	Rare	No	No	267.0	
31478.0 R	Freq	No	No	1492.0	
31622.0 R	Freq	No	No	144.0	
32802.0 R	Rare	No	No	1180.0	
32950.0 R	Rare	No	No	148.0	
34667.0 R	Freq	No	No	1717.0	
52086.0 R	Freq	No	No	17419.0	
52251.0 R	Rare	No	No	165.0	
52332.0 R	Freq	No	No	81.0	
54382.0 R	Freq	No	No	2050.0	
54612.0 R	Rare	No	No	230.0	
54658.0 R	Freq	No	No	46.0	

Genotype-By-Sequencing (GBS)

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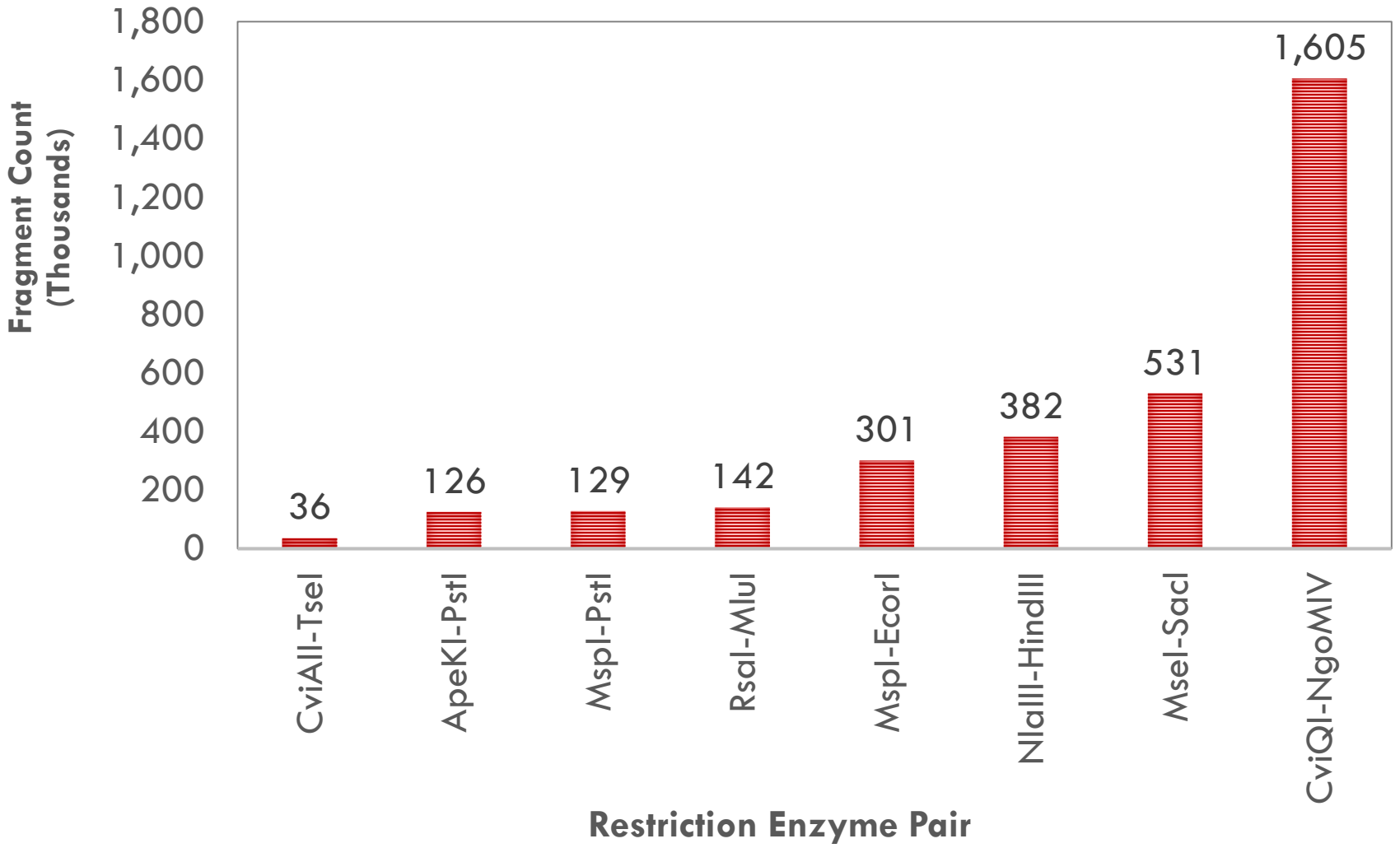

Genotype-By-Sequencing (GBS)

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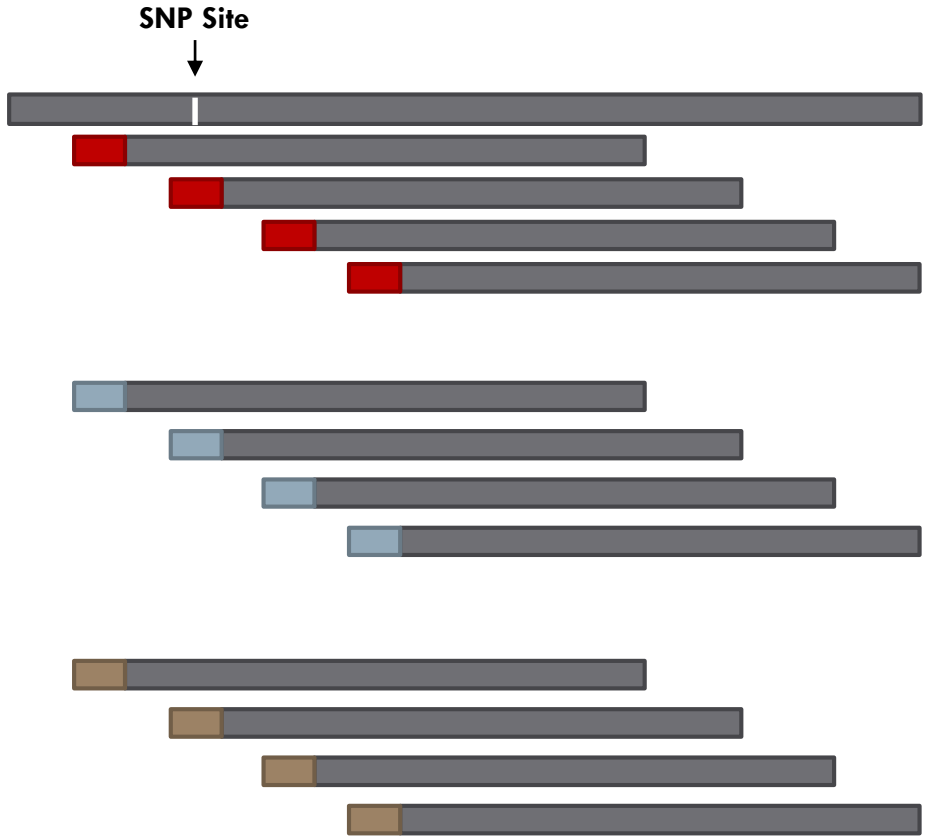
Align whole-genome sequences to internal reference 'Bailey II' for SNP discovery



Genotype-By-Sequencing (GBS)

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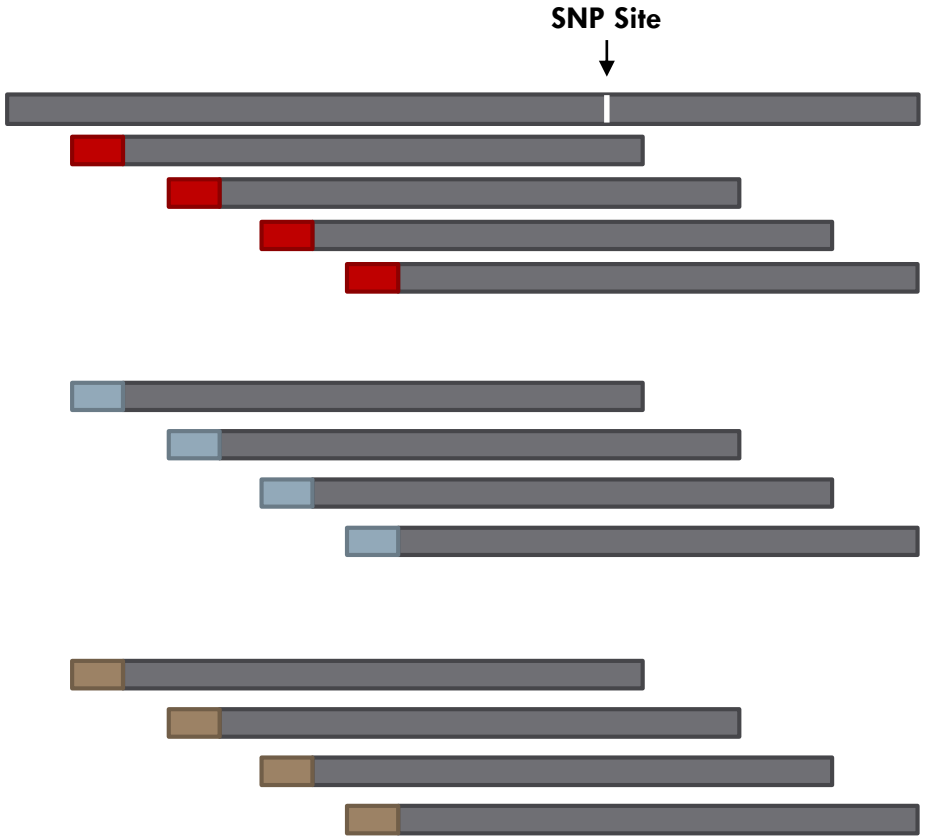

Genotype-By-Sequencing (GBS)

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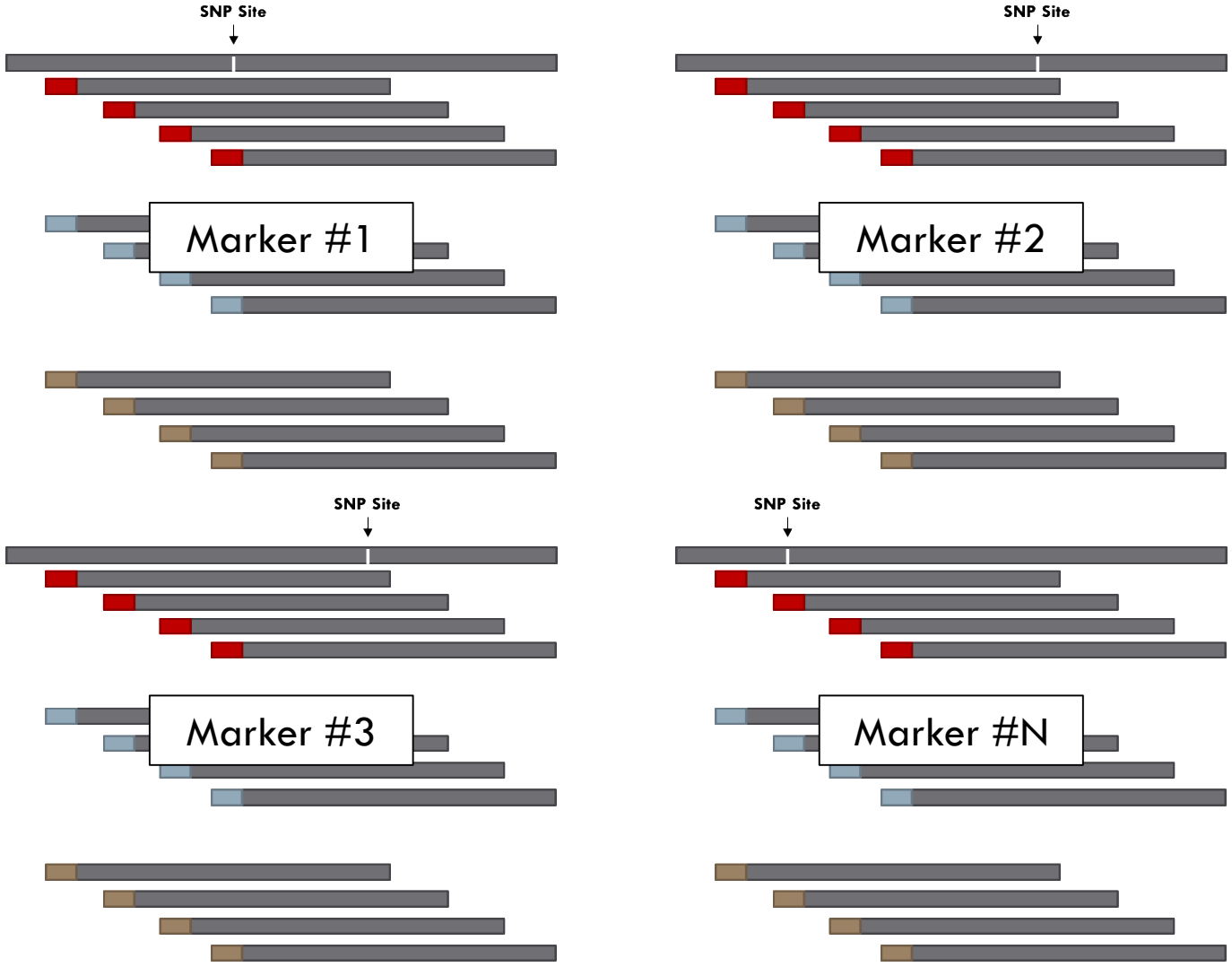

Genotype-By-Sequencing (GBS)

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Use results from *in silico* digest to count potential recovery sites of SNPs discovered from whole-genome alignment

Results from in_silico.py

Enz_Start	Cut	Source	Del	Couple	Size
15912.0 R	Freq	No	No	0.0	
16497.0 R	Rare	No	No	585.0	
19495.0 R	Freq	No	No	2998.0	
26735.0 R	Freq	No	No	7240.0	
27246.0 R	Rare	No	No	511.0	
28152.0 R	Freq	No	No	906.0	
29719.0 R	Freq	No	No	1567.0	
29986.0 R	Rare	No	No	267.0	
31478.0 R	Freq	No	No	1492.0	
31622.0 R	Freq	No	No	144.0	
32802.0 R	Rare	No	No	1180.0	
32950.0 R	Rare	No	No	148.0	
34667.0 R	Freq	No	No	1717.0	
52086.0 R	Freq	No	No	17419.0	
52251.0 R	Rare	No	No	165.0	
52332.0 R	Freq	No	No	81.0	
54382.0 R	Freq	No	No	2050.0	
54612.0 R	Rare	No	No	230.0	
54658.0 R	Freq	No	No	46.0	

Filtering Results (in_silico.py)

- Sequence fragment size selection (150-500 bp)
- **Frequent** – **Rare** sticky ends
- Sites identified from whole-genomes sequencing falling between starting positions

Validation: Run GBS protocol with selected enzyme pairs for read depth evaluation and SNP count recovery post alignment

Genotype-By-Sequencing (GBS)

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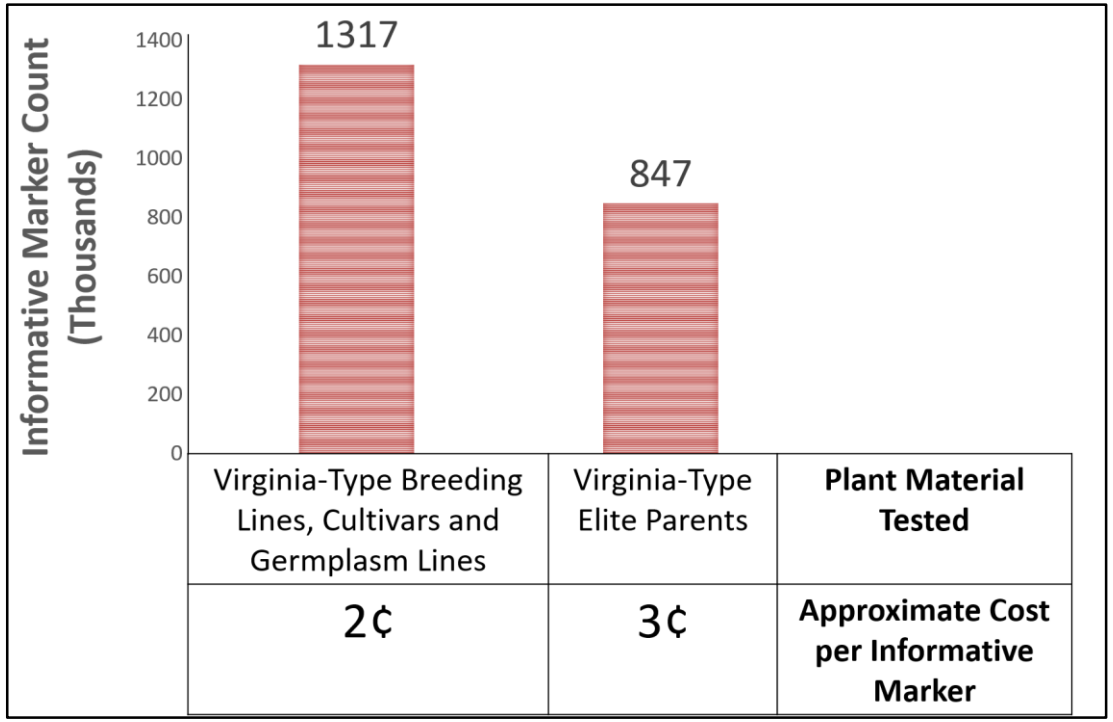
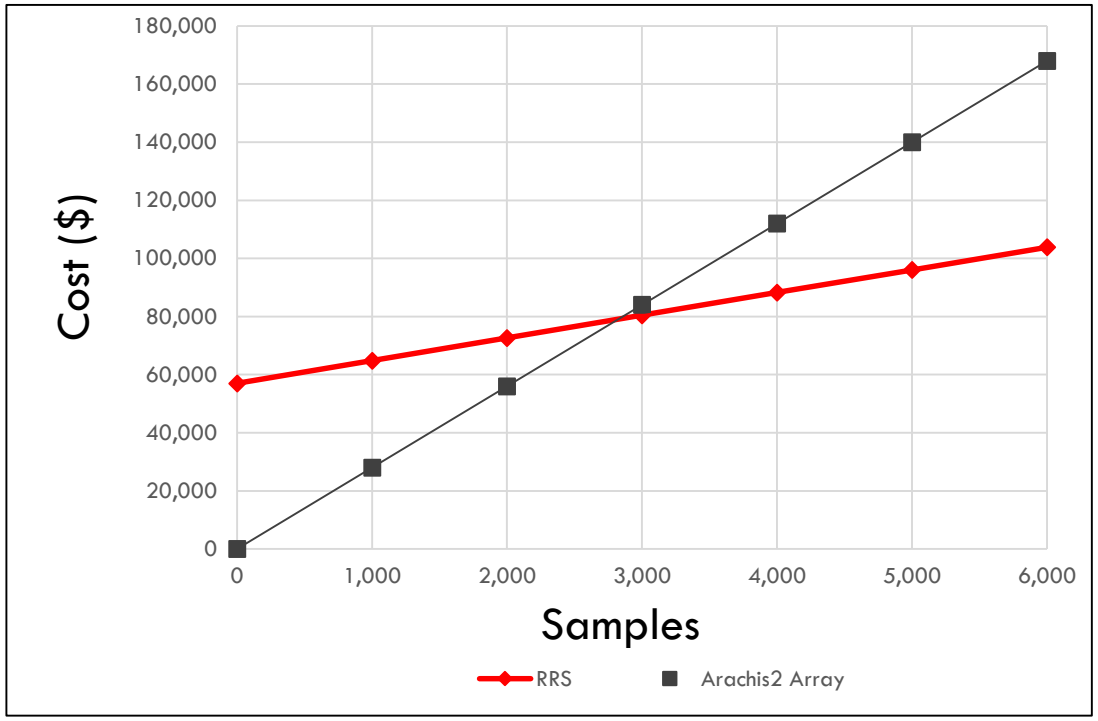


The Genotype-By-Sequencing (GBS) or Reduced Representation Sequencing (RRS) pipeline will reduce the cost per informative marker from $0.03 to $0.001



The Genotype-By-Sequencing (GBS) or Reduced Representation Sequencing (RRS) pipeline will break even in 4 years or after ~2,800 samples

Leaf Spot Characterization

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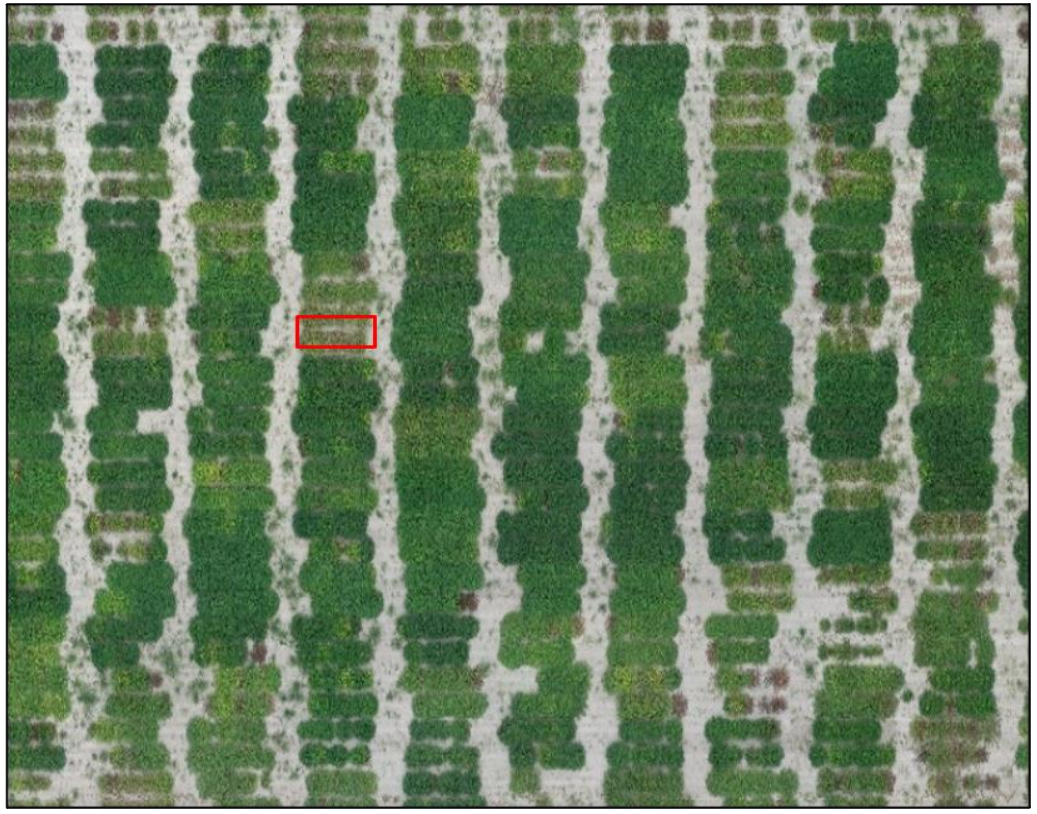
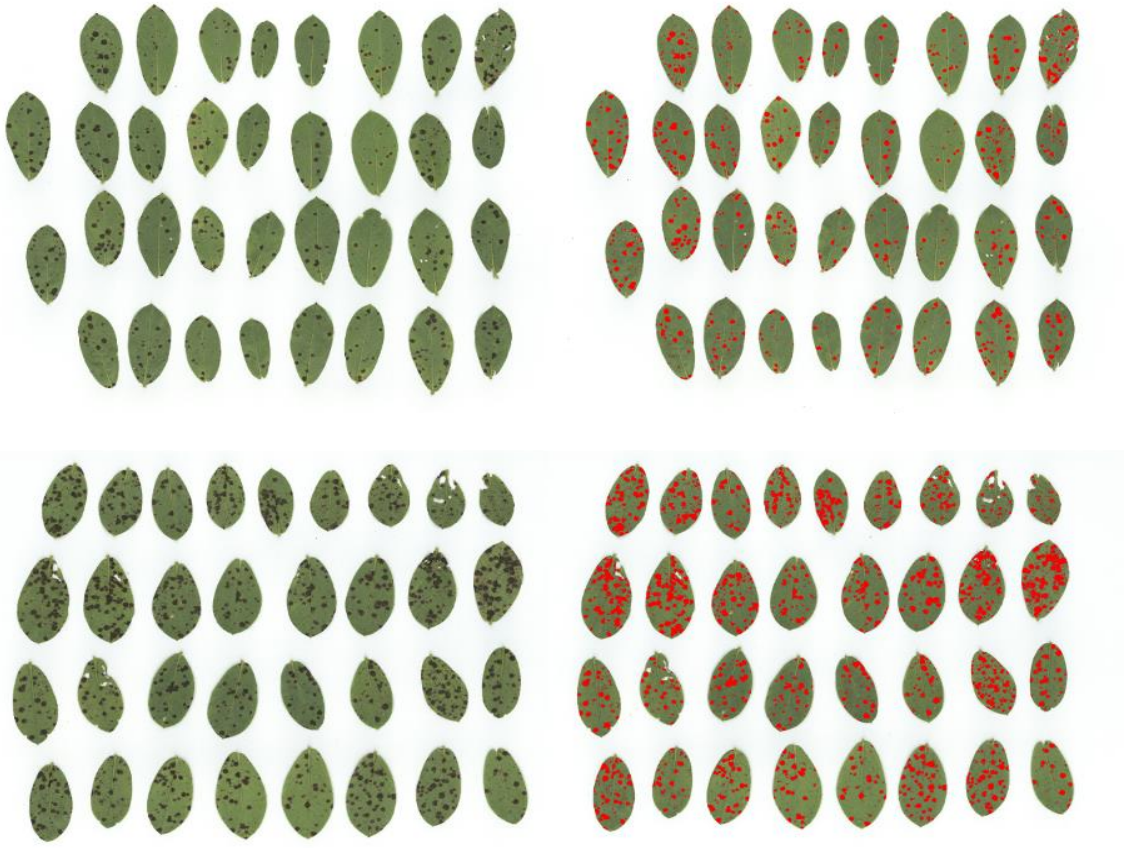
Aerial Image of Peanut Defoliation



Leaf Spot Tissue Scans for Coverage



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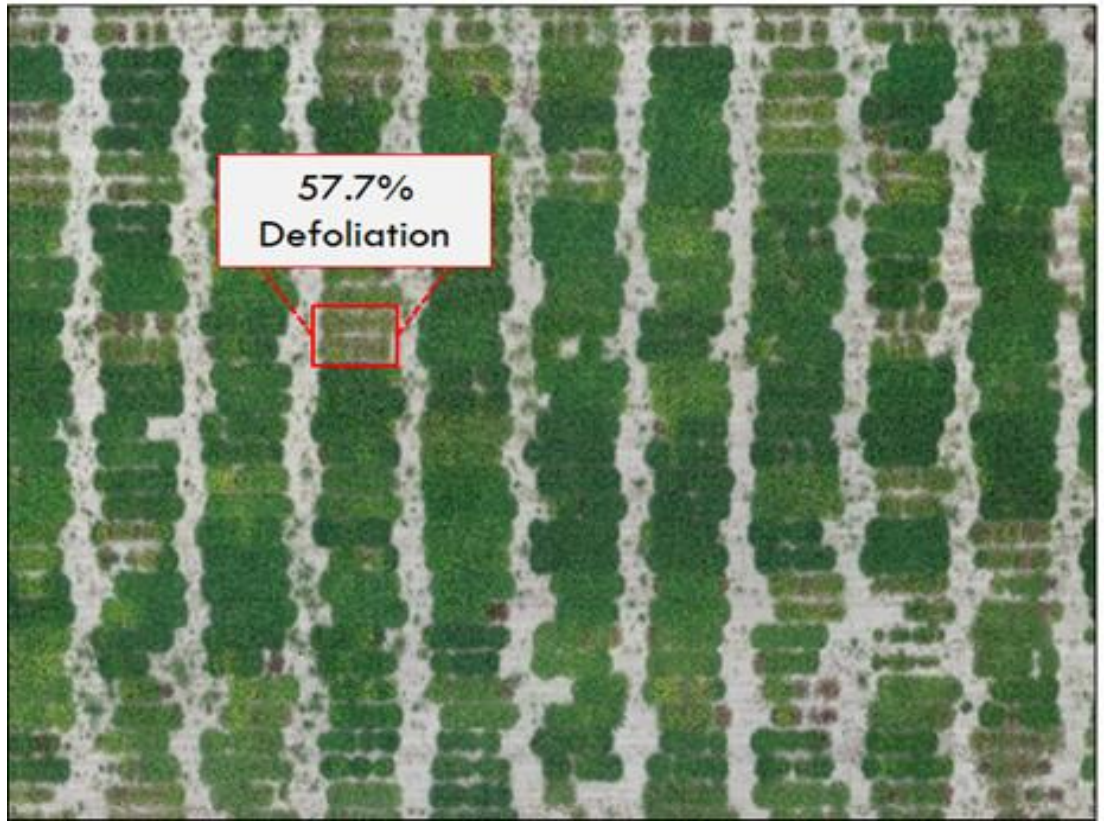

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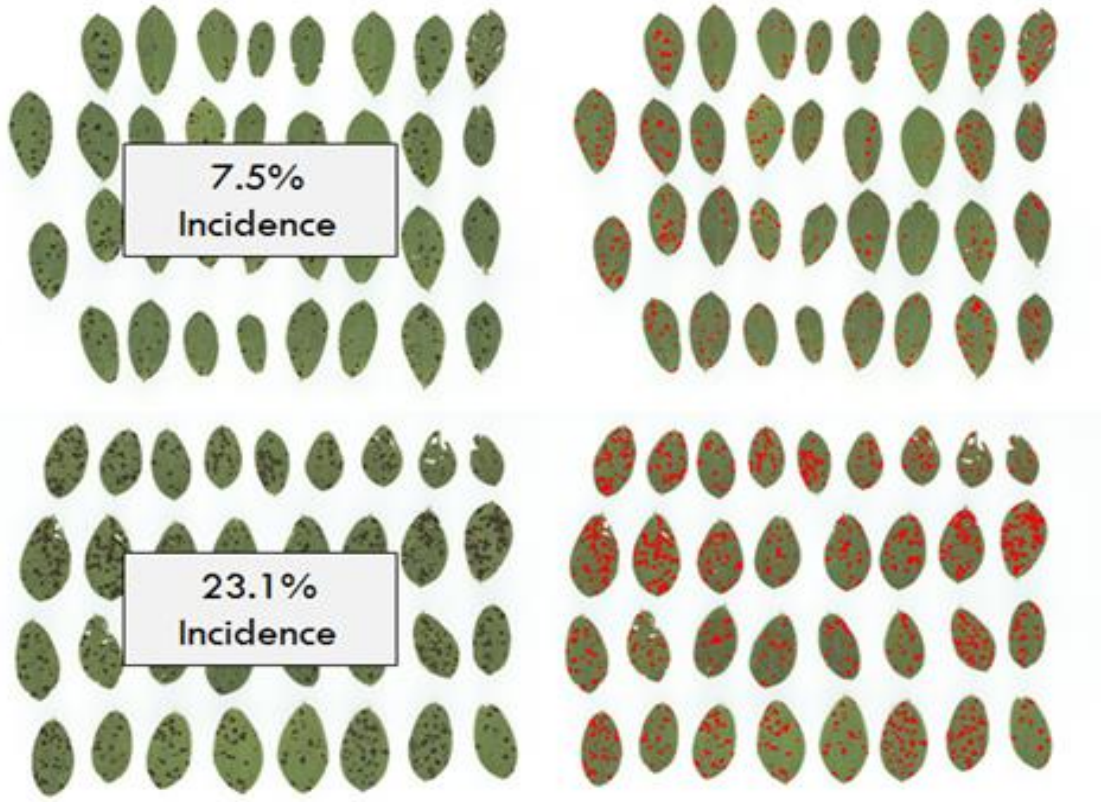

Comparison of Imaging to Visual Ratings

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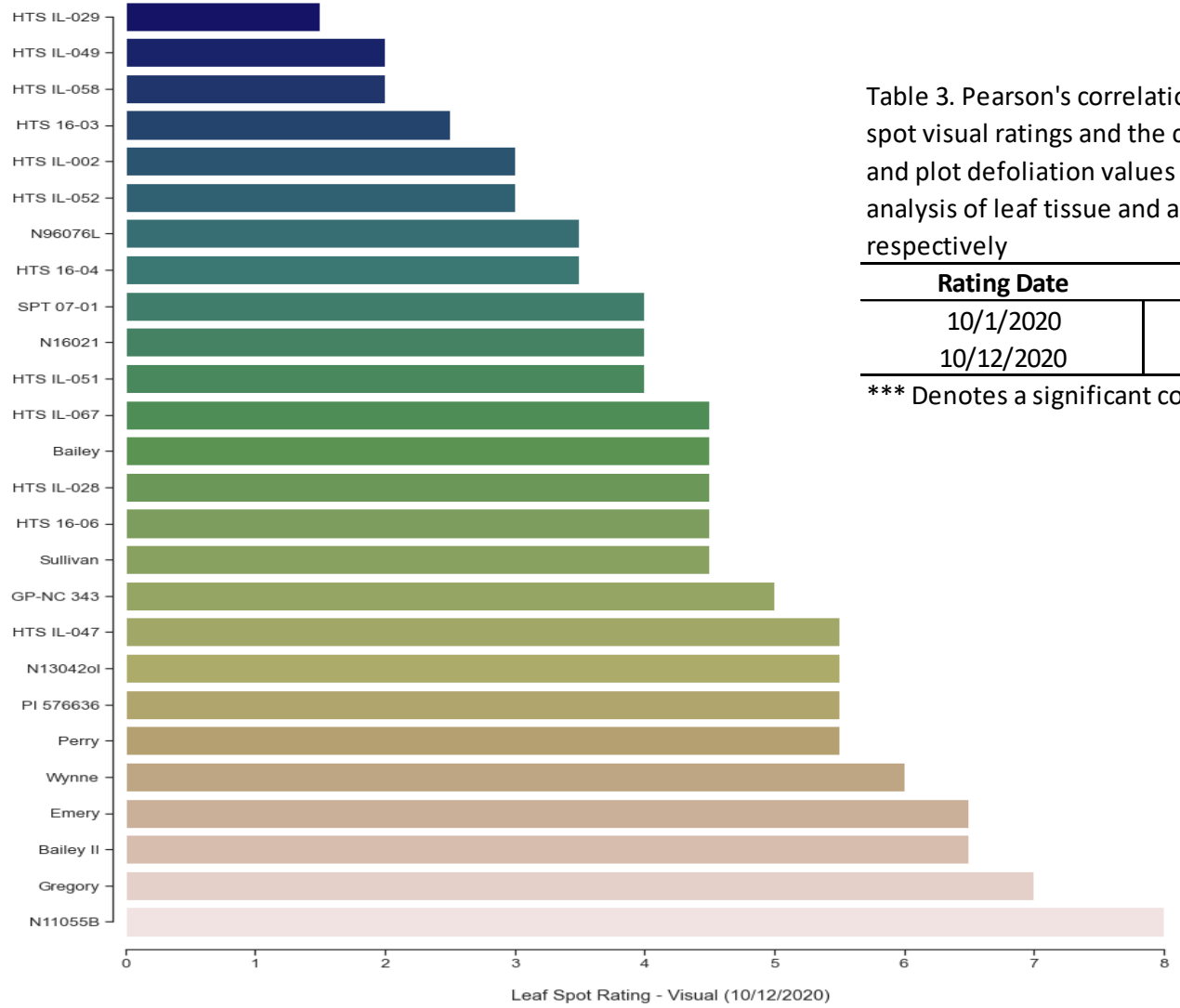

Table 3. Pearson's correlation coefficient between leaf spot visual ratings and the combined percent leaf spot and plot defoliation values determined through image analysis of leaf tissue and aerial drone imaging, respectively

Rating Date	Pearson's Correlation (r)
10/1/2020	0.59***
10/12/2020	0.64***

*** Denotes a significant correlation at the <0.0001 level

2021 Budget

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FUNDS REQUESTED:		2021
EPA Salaries and fringe benefits[1]		
Graduate Student Stipends and Support[2]		$21,000
SPA Salaries and fringe benefits[1]		
Part-time Labor	(including: 8.60% fringe benefits, and add $142/year health insurance for hourly / part-time workers who will work ≥ 30hrs/wk. for longer than 3 months)	$5,000
Supplies	(such as: lab and field consumables, fuel, etc.)	$2,500
Equipment	(greater than $500)	
Travel	(for airfare, overnight lodging and meals associated with project tasks)	
Other	(such as: motor pool rental, lab analysis, contracted services, greenhouse space, page/publication charges)	
TOTAL in Dollars		$28,500

SUPPORT FOR PEANUT WILD SPECIES BREEDING AND GERMPLASM MAINTENANCE AT NCSU

NORTH CAROLINA PEANUT GROWERS RESEARCH MEETING
NOVEMBER 30, 2020

Ryan Andres| Room 209, Unit 3, Method Road | 919.215.7338 | rjandres@ncsu.edu

Objective 1: Maintain Wild Species Greenhouse Collection

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BEFORE

- Started with 325 very weedy and overgrown pots.
- Discarded 171 either because they were duplicates or we already had sufficient seed.
- Did not harvest any seed off these pots.

Objective 1: Maintain Wild Species Greenhouse Collection

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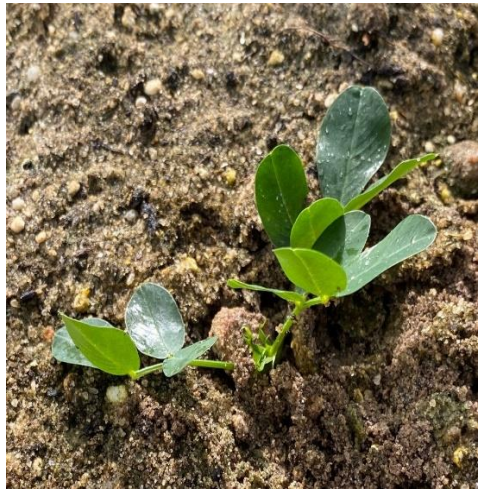
- Performed vegetative propagation on the remaining 154 pots.

1) Cut diagonally along the last node of an actively growing lateral branch.

2) Dip cut end in rooting powder.



3) Plant cutting and keep soil wet.

4) In a few weeks, cutting will produce roots and leaves.

Objective 1: Maintain Wild Species Greenhouse Collection

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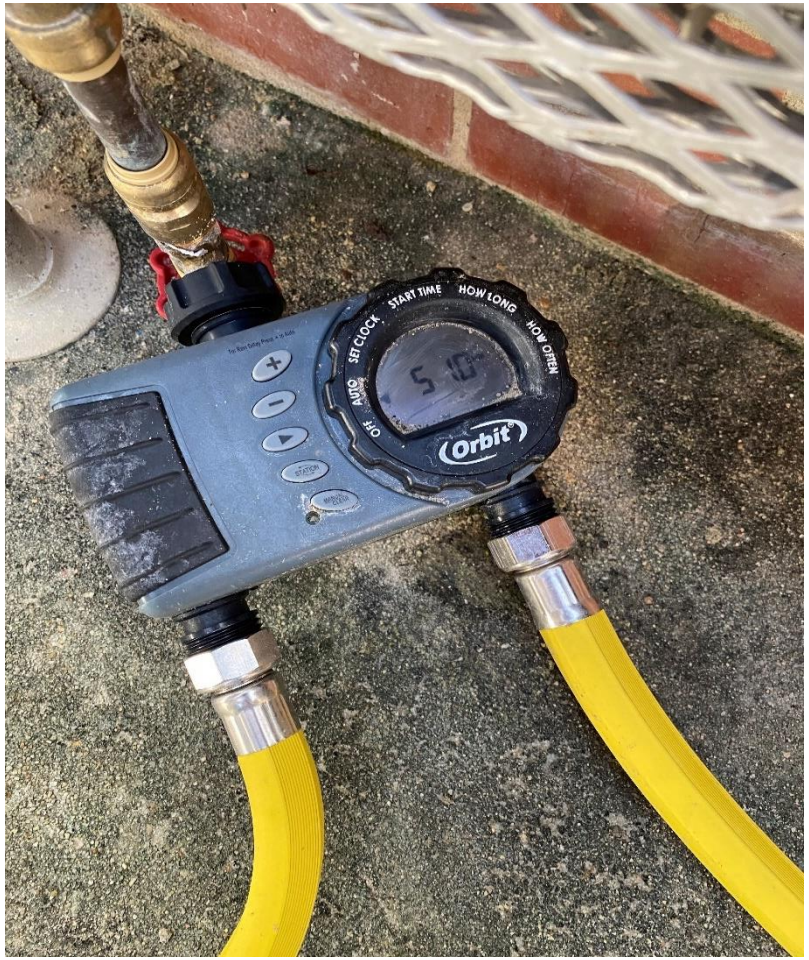

One control box for two benches.

Control box feeds a lateral tube that runs the length of the bench. Every other box has a vertical riser with a sprinkler on top.

Objective 1: Maintain Wild Species Greenhouse Collection

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- Harvest seed when its ready.
- Maintain via vegetative propagation if necessary.

AFTER

Objective 2: Inventory, Organize, and Refresh the Wild Species Seed Collection

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- Refresh: No Wild Species Nursery at Sandhills this year due to Covid-19.
  - No long-term effects.
  - Larger in 2021.

- Inventory: *475* total accessions
  - 22 known duplicates
  - 38 have questionable or incomplete information
  - 415 “high-quality” accessions

Objective 2: Inventory, Organize, and Refresh the Wild Species Seed Collection

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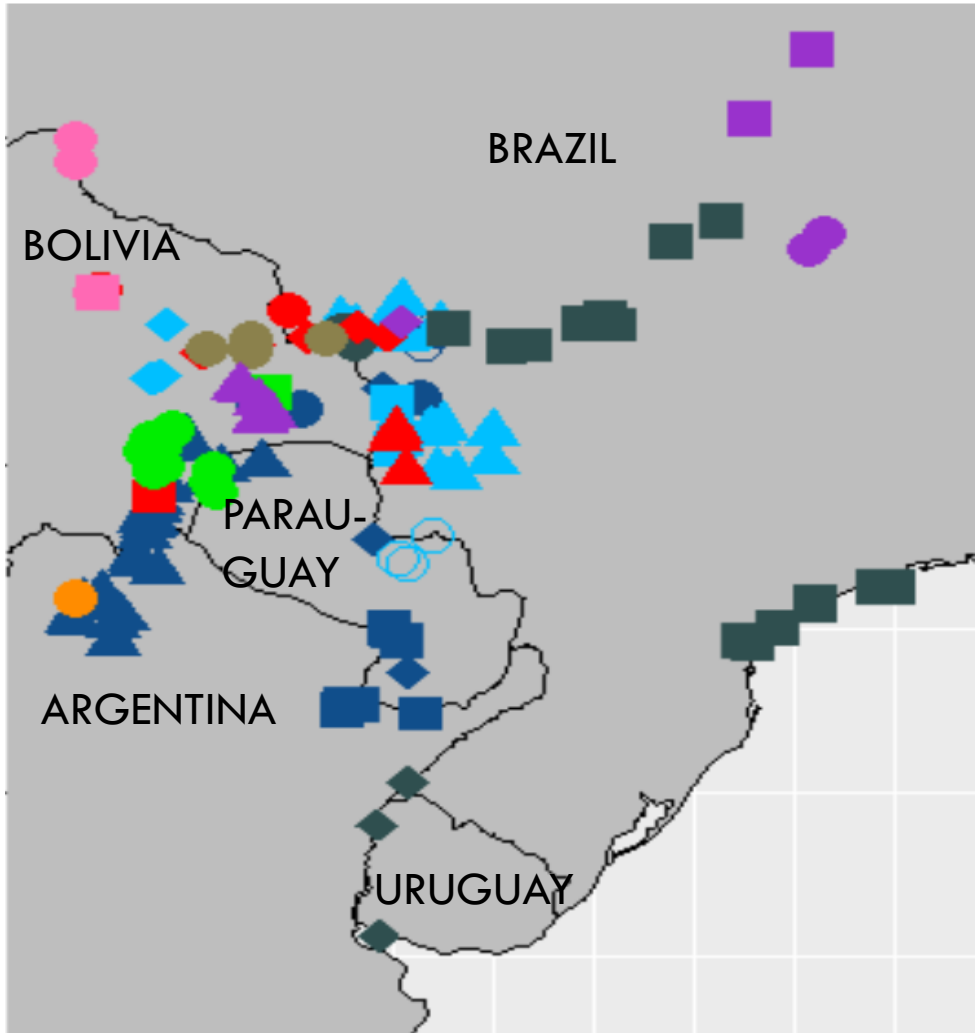
Section Arachis – 217 Accessions



A Genome (155)
- *A. cardenasii* (15)
- *A. correntina* (10)
- *A. diogoi* (6)
- *A. duranensis* (46)
- *A. helodes* (7)
- *A. herzogii* (1)
- *A. hoehnei* (4)
- *A. kempff-mercadoi* (6)
- *A. kuhlmannii* (19)
- *A. microsperma* (4)
- *A. simpsonii* (5)
- *A. stenosperma* (23)
- *A. villosa* (9)

AB Genome (7)
- *A. monticola* (7)
  - *A. hypogaea*

B Genome (14)
- *A. gregoryi* (1)
- *A. ipaensis* (1)
- *A. magna* (7)
- *A. valida* (4)
- *A. williamsii* (1)

D Genome (5)
- *A. glandulifera* (5)

F Genome (5)
- *A. benensis* (4)
- *A. trinitensis* (1)

K Genome (17)
- *A. batizocoi* (15)
- *A. cruziana* (2)

Unknown Genome (6)
- *A. decora* (3)
- *A. palustris* (2)
- *A. praecox* (1)

Species Unknown (8)
- Unknown

Accessions from these 3 species have been successfully used by our program to broaden the genetic diversity of the cultivated breeding program.

Objective 2: Inventory, Organize, and Refresh the Wild Species Seed Collection

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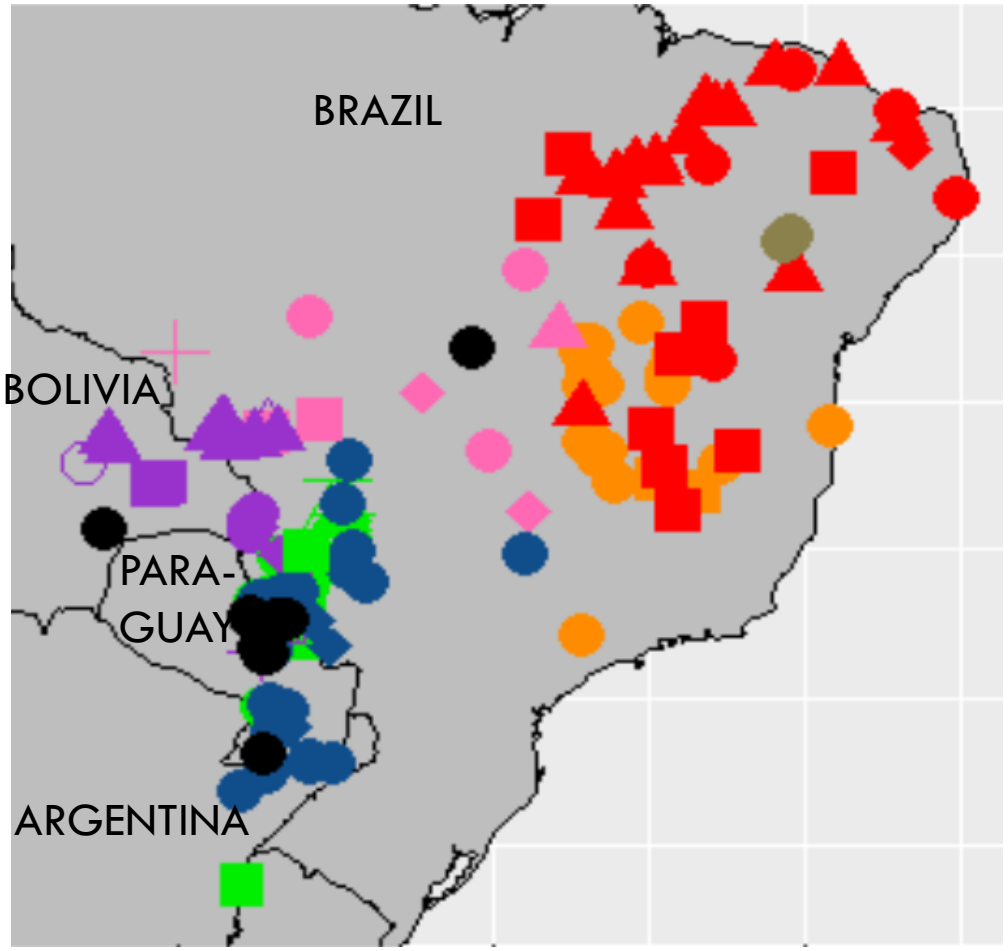
Other Sections – 196 Accessions



Section Caulorrhizae (27)
- *A. pintoi* (25)
- *A. repens* (2)

Section Erectoides (42)
- *A. archeri* (1)
- *A. benthamii* (2)
- *A. cryptopotamica* (2)
- *A. hermannii* (3)
- *A. major* (10)
- *A. paraguariensis* (21)
- *A. porphyrocalyx* (1)
- *A. stenophylla* (2)

Section Extranervosae (10)
- *A. burchelli* (3)
- *A. lutescens* (2)
- A. macedoi (2)
- *A. retusa* (1)
- *A. villosulicarpa* (2)

Section Heteranthae (40)
- *A. dardanoi* (7)
- *A. pusilla* (13)
- *A. seridoensis* (1)
- *A. sylvestris* (19)

Section Procumbentes (28)
- *A. appressipila* (6)
- *A. chiquitana* (3)
- *A. kretschmeri* (4)
- *A. lignosa* (1)
- *A. matiensis* (10)
- *A. pflugeae* (2)
- *A. rigonii* (1)
- *A. subcoriacea* (1)

Section Rhizomatosae (38)
- *A. burkartii* (1)
- *A. glabrata* (33)
- *A. pseudovillosa* (4)

Section Triseminata (2)
- *A. triseminata* (2)

Section Unknown (11)
- Unknown

Used as forage cultivars.

Exploratory research on use of wild peanut as a cover crop because:
1) Low-Growing
2) Form dense mats
3) Nitrogren-fixing

Objective 2: Inventory, Organize, and Refresh the Wild Species Seed Collection

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- Organize: Currently have 5 freezers that look like this.
- Discard anything older than 15 years.
  - Includes 2 additional freezers at Reedy Creek
- Keep only 3 most recent bottles of each accession.
- Eventually genotype to eliminate duplicates and misclassifications in Section Arachis.

Objective 3: Develop a genotyping strategy to characterize the wild species collection.

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- With 218 wild species in Section Arachis, which ones should we cross with?
- Traditional Route: 1st step would involve full sequencing of 10 wild species.
  - $50,000 & ~2 years to analyze the data
- Alternate Route: Wild species primary utility is their collection of novel R (Resistance) genes.
  - Not found in cultivated material.
- R genes are very similar to one another and that makes them easy to find in the peanut genome.

Objective 3: Develop a genotyping strategy to characterize the wild species collection.

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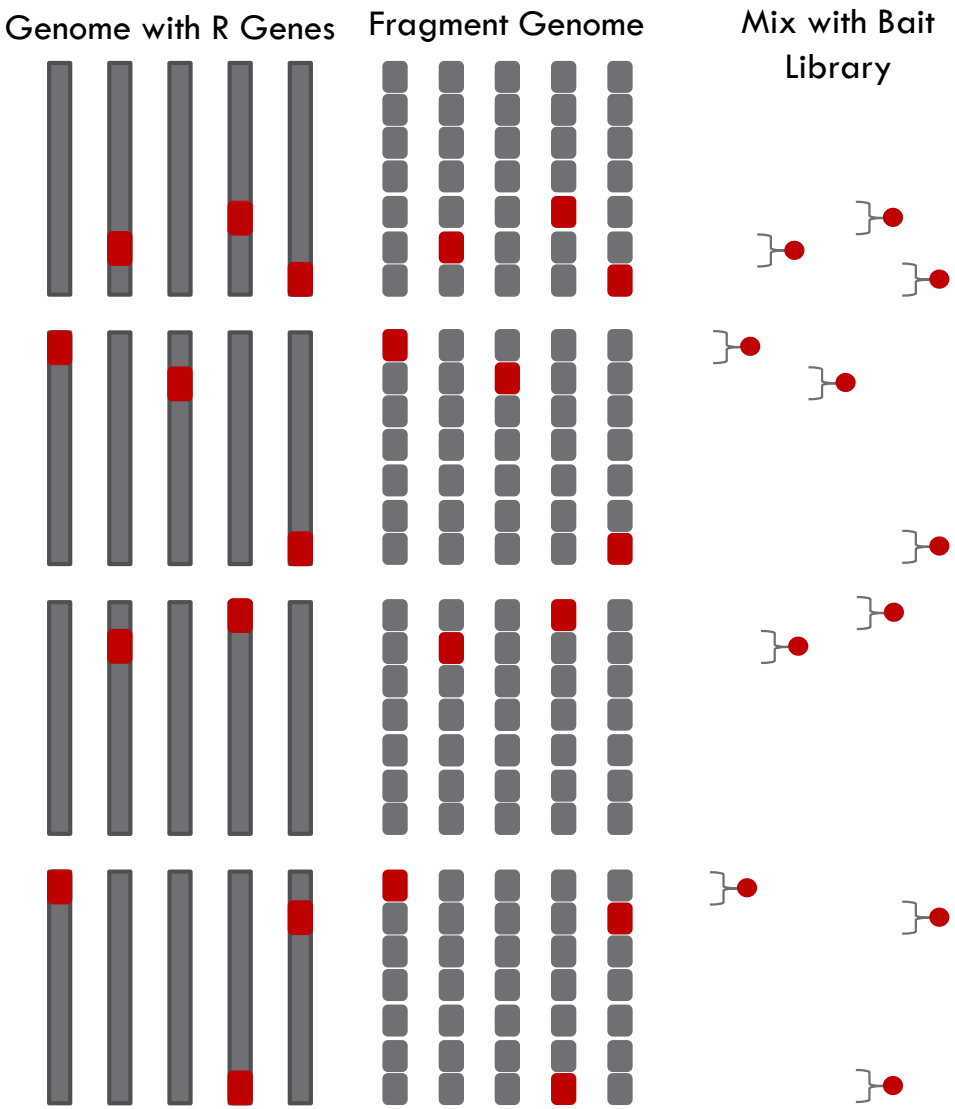

- Sequence just the R genes.
- Get 95% of the relevant data.
- $8,800
- Data analysis ~2 months.

Objective 4: Continue the wild species crossing program.

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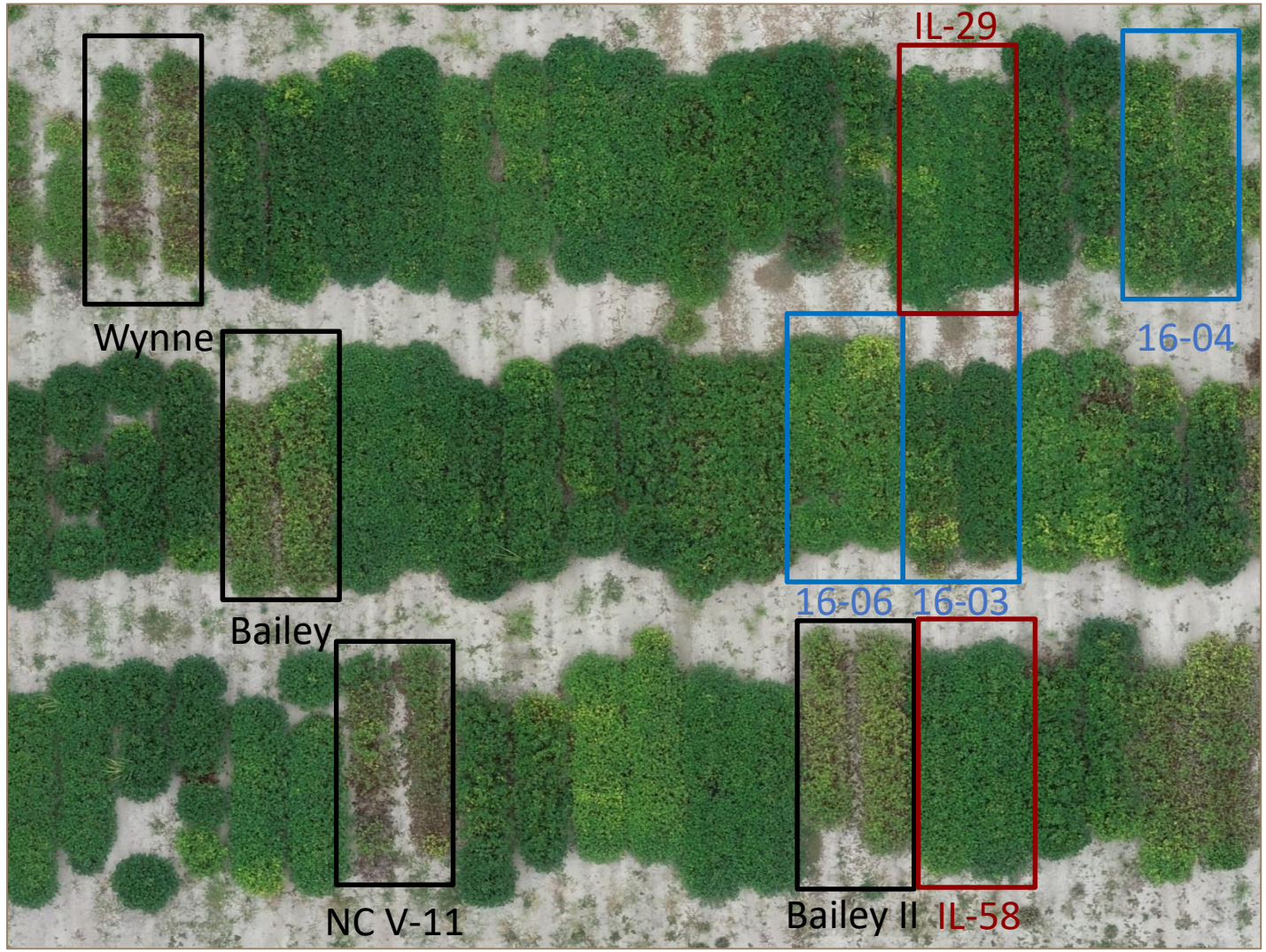
- We have breeding lines with elite leaf spot resistance from both *A. cardenasii* and *A. diogoi* backgrounds.
- The objective is to combine them in a single breeding line.
- This makes it much easier to transfer both sources of resistance to future cultivars.



Objective 4: Continue the wild species crossing program.

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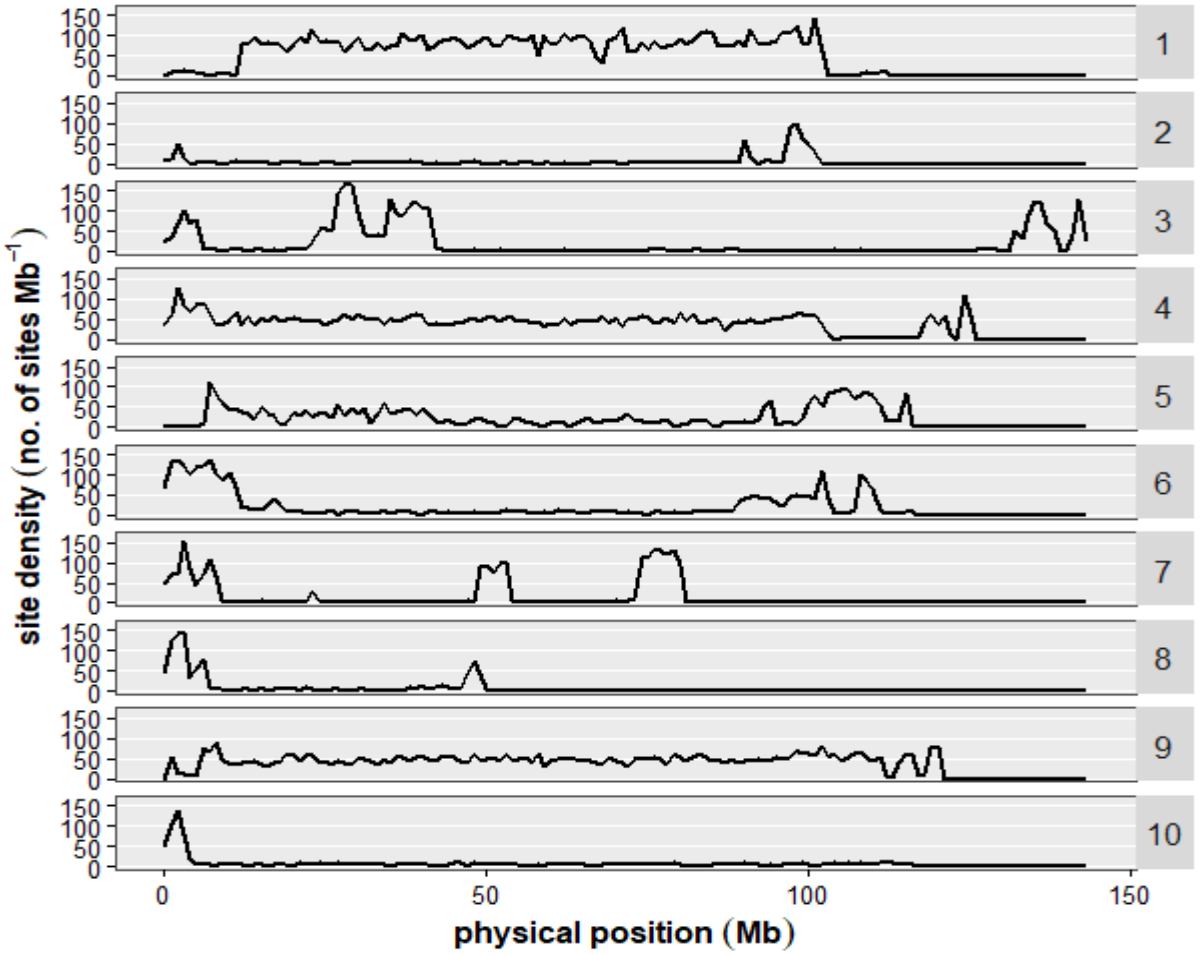
- 1st step: Sequenced 18 relevant lines.
- 2nd step: Aligned those sequences to the reference genome.
- Peaks in the graphs on the right represent regions of wild species DNA.
- Design molecular markers to determine which of these blocks lead to resistance.

A. Diogoi Introgression Blocks in IL-29



Objective 4: Continue the wild species crossing program.

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- In summer 2000, VA-98R was crossed with the *A. correntina* accession GKP 9530.
- After 20 years we may finally have 3 plants out of this cross with the right number of chromosomes.
- Send seed from these plants to USDA to check the chromosome number.

Objective 4: Continue the wild species crossing program.

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- 46 synthetic allotetraploids in the greenhouse from 11 different wild species crosses.
- Keep looking for normal levels of fertility in these plants.

♀ Parent	Genome	♂ Parent	Genome	# Plants
A. batizocoi	KK	*A. cardenasii*	AA	2
A. batizocoi	KK	*A. correntina*	AA	2
A. batizocoi	KK	*A. stenosperma*	AA	6
A. gregoryi	BB	*A. stenosperma*	AA	3
A. ipaensis	BB	*A. cardenasii*	AA	3
A. ipaensis	BB	*A. correntina*	AA	15
A. ipaensis	BB	*A. diogoi*	AA	5
A. ipaensis	BB	*A. duranensis*	AA	1
A. ipaensis	BB	*A. stenosperma*	AA	4
A. magna	BB	*A. cardenasii*	AA	4
A. magna	BB	*A. stenosperma*	AA	1

Objective 5: Evaluate wild species lines for disease resistance and yield potential.

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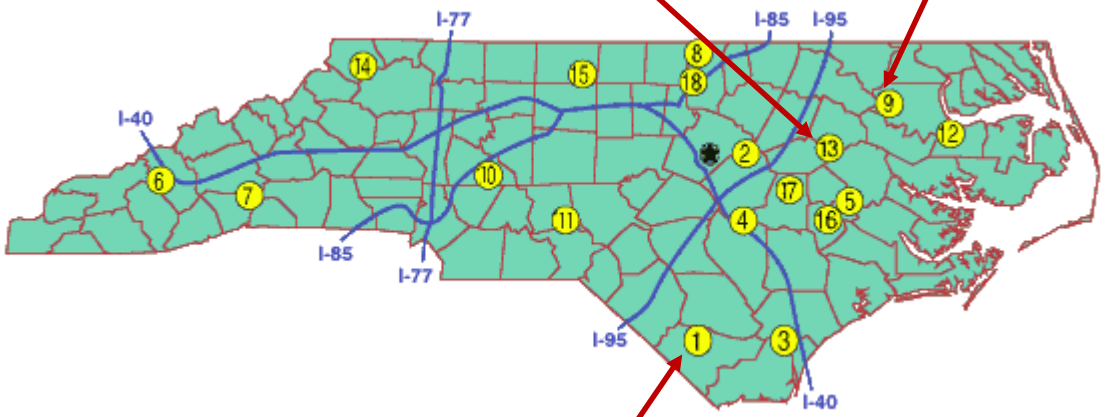
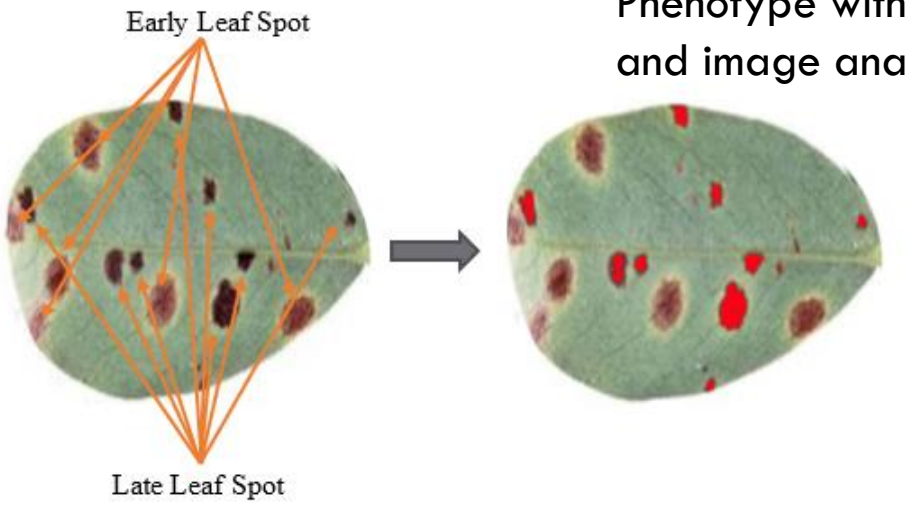
Upper Coastal Plain Research Station
Rocky Mount, NC

Peanut Belt Research Station
Lewiston, NC



Border Belt Tobacco Research Station
Whiteville, NC

Phenotype with leaf scans and image analysis.



Phenotype with drone-based whole-plot defoliation.

Budget

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BUDGET

FUNDS REQUESTED:		**2021**	**2022**	**2023**
EPA Salaries and fringe benefits[1]		$21,218		
Graduate Student Stipends and Support[2]				
SPA Salaries and fringe benefits[1]				
Part-time Labor	(including: 9.05% fringe benefits, and add $151/year health insurance for hourly / part-time workers who will work ≥ 30hrs/wk. for longer than 3 months)			
Supplies	(such as: lab and field consumables, fuel, etc.)	$7,500		
Equipment	**(greater than $500)**			
Travel	(for airfare, overnight lodging and meals associated with project tasks)			
Other	(such as: motor pool rental, lab analysis, contracted services, greenhouse space, page/publication charges)			
TOTAL in Dollars		**$28,718**	**$**	**$**