Molecular Research To Evaluate Glyphosate Resistance Mechanisms in Palmer Amaranth

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USDA-ARS
Fort Collins, CO

Collaborative Research Group

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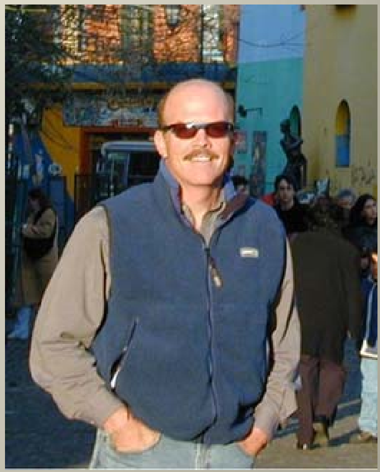

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Univ. of Adelaide

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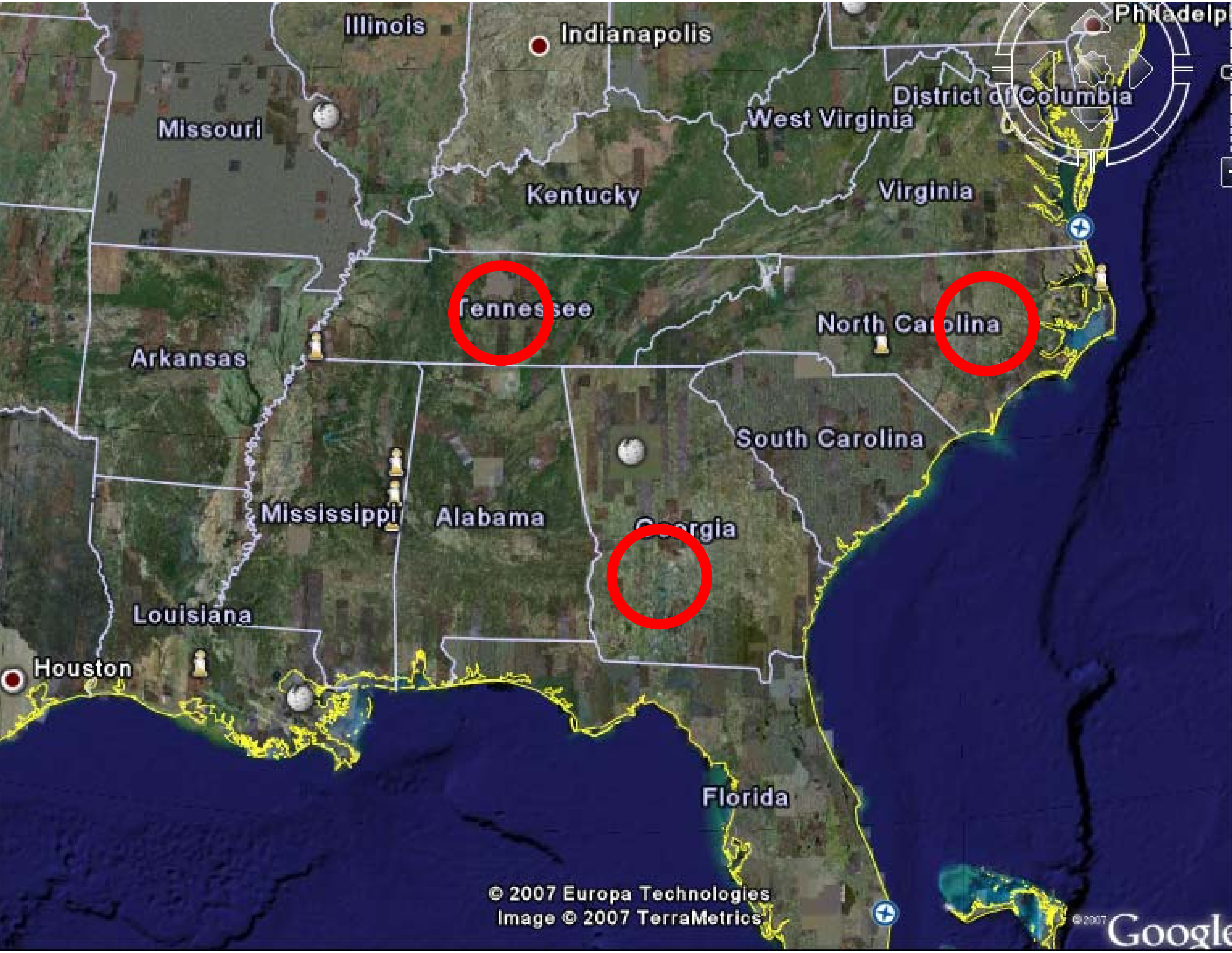
WE have 4,000 square feet of high quality greenhouse space


Illinois
Indianapolis
Philadelph
Missouri
District of Columbia
West Virginia
Kentucky
Virginia
Tennessee
North Carolina
Arkansas
South Carolina
Mississippi
Alabama
Georgia
Louisiana
Houston
Florida
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PA female flowers

PA male flowers

Assumptions

- Metabolism of glyphosate is not occurring
- No difference in uptake, absorption or translocation out of leaf (Culpepper, 2006)
- Focus of our research: target-site basis

Research Approaches

- Molecular research on possible resistance mechanisms
  - Molecular tools: RT-PCR, cloning, sequencing
  - Shikimate assay
- Genetic basis
  - Gene action and copy number
- Pollen-mediated transfer of resistance trait to other *Amaranthus* species

Shikimate Assay

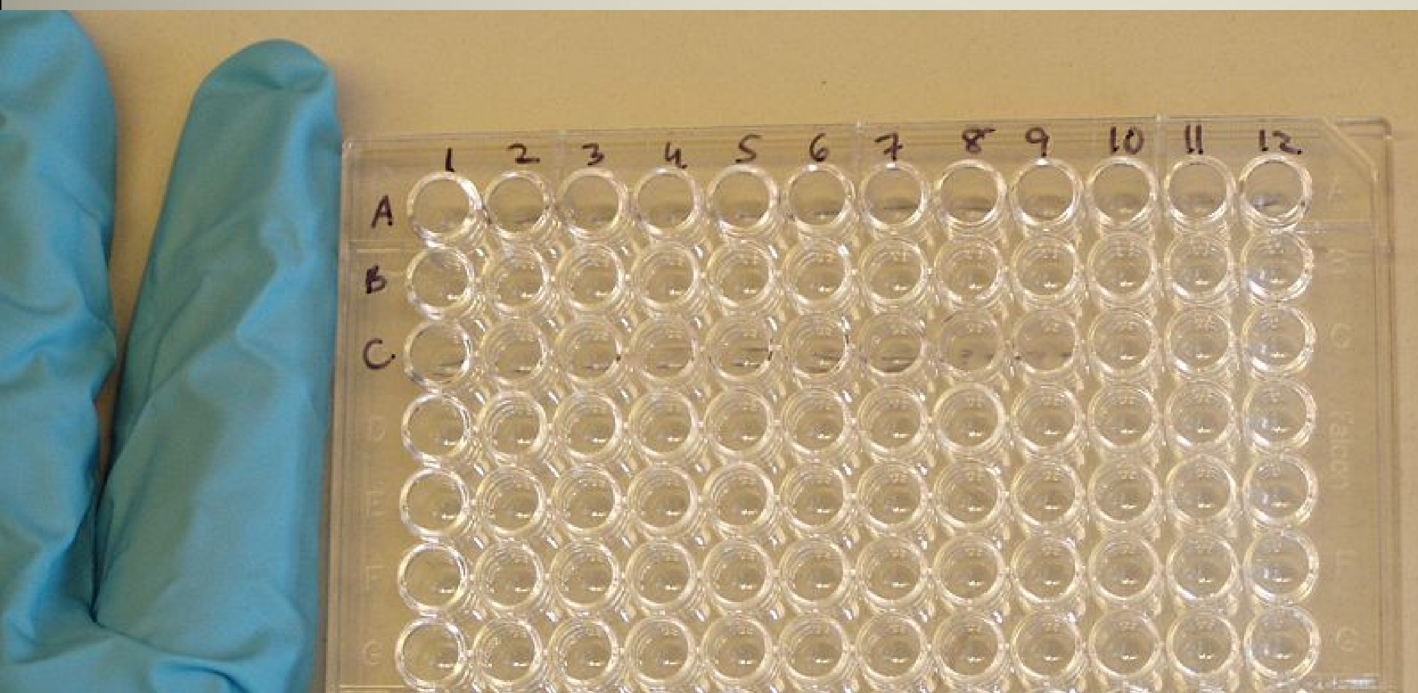
- Glyphosate inhibits EPSPS and is competitive with PEP for binding
- When susceptible plant leaf discs are treated with glyphosate
  - Shikimate accumulates
- When resistant Palmer amaranth leaf discs are treated with glyphosate
  - Shikimate does not accumulate
  - Does not necessarily mean that EPSPS in resistant plants is not sensitive to inhibition

Shikimate Assay Methods

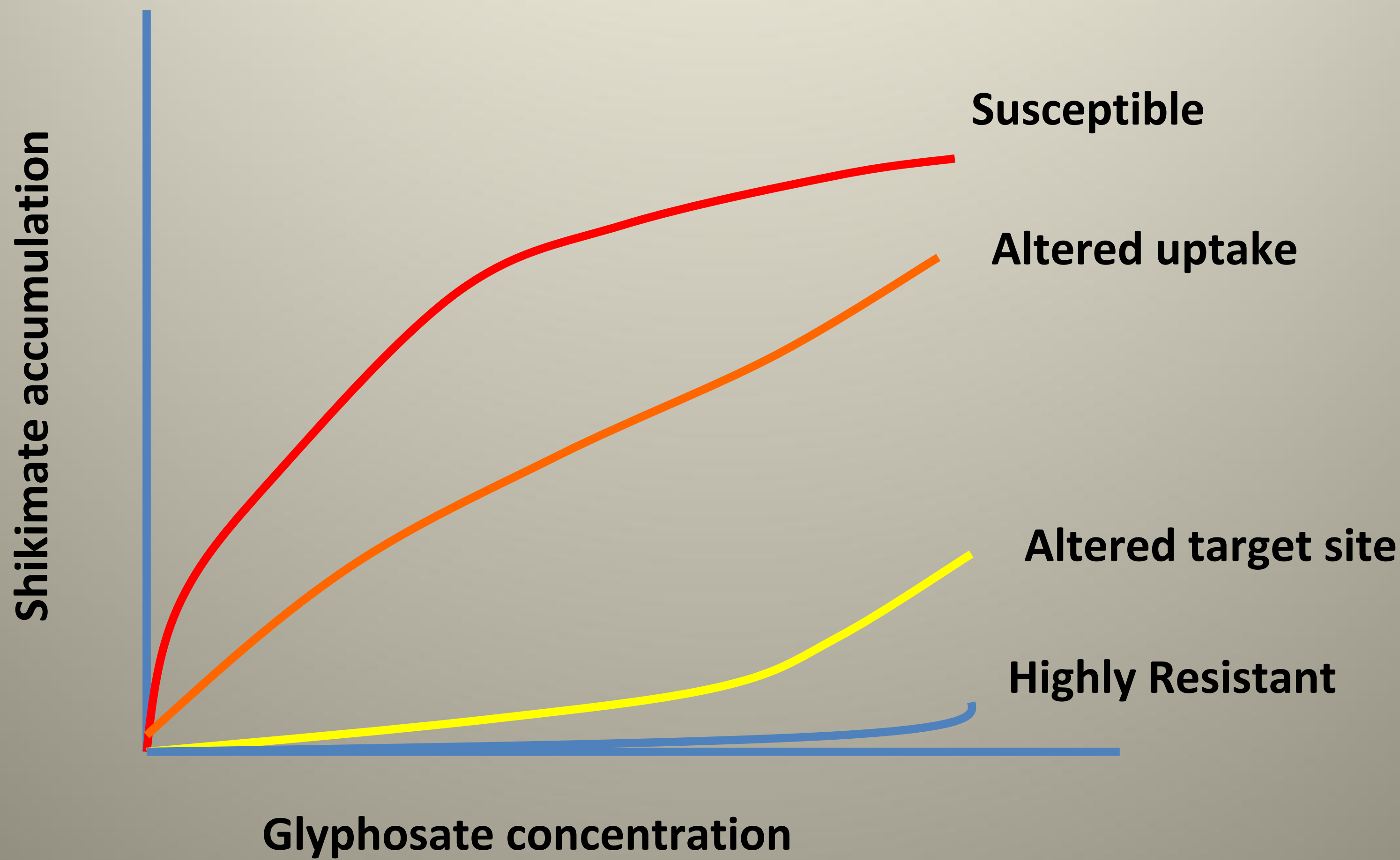
Shaner et al. 2005

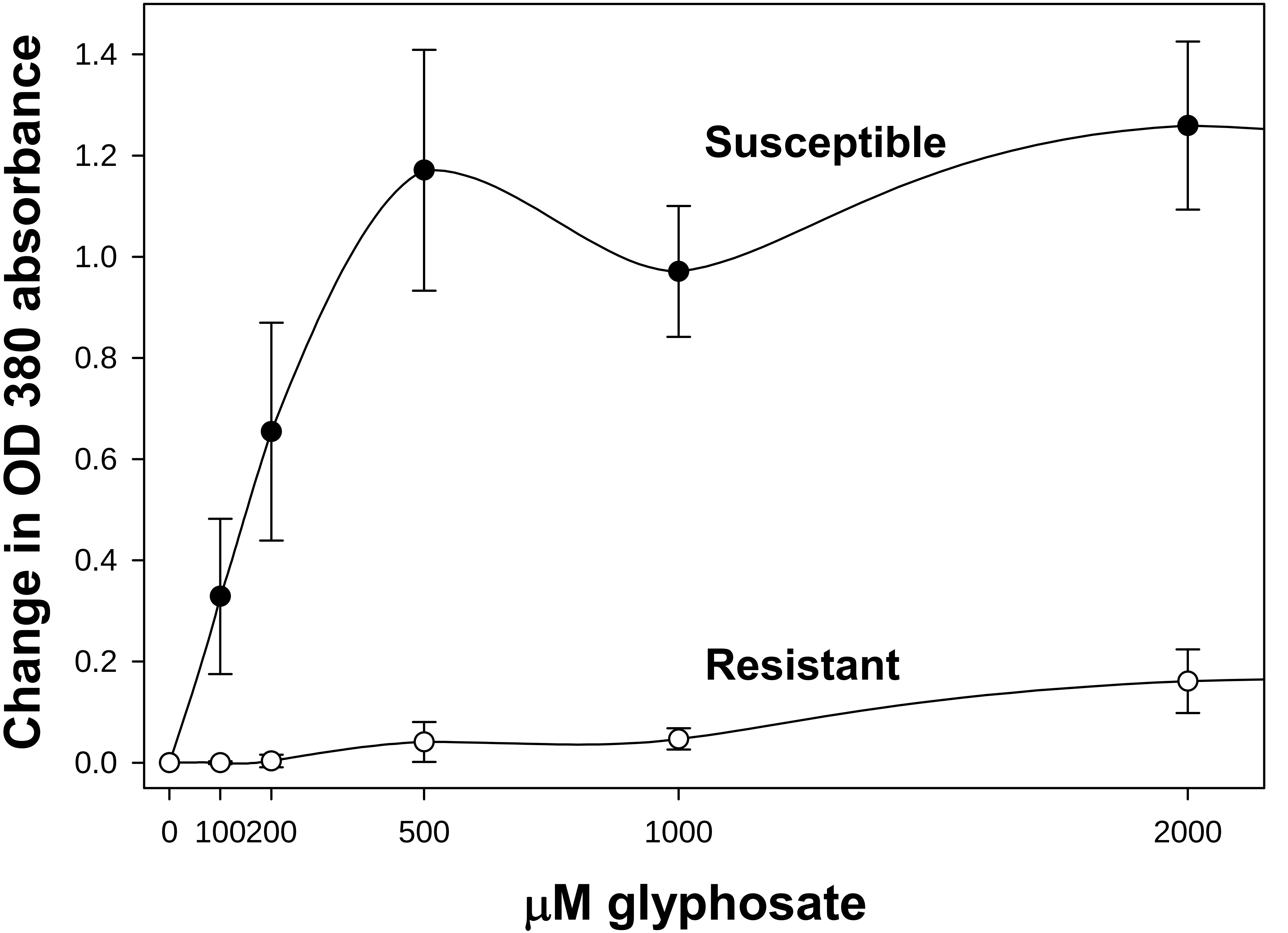
- Micro-titer plate assay
- Wells have range of glyphosate concentrations, typically 0 to 500 µM
- We use formulated glyphosate
- Buffer can be water only, ammonium phosphate, or MES
- Leaf discs are incubated under light for 16 hours
  - Can use sucrose with no light
- Use two or three reps per plant
- Sample young, expanding leaves
- Use 0 µM as background
- Establish shikimate standard curve in the same buffer
- Measure absorbance at OD 380
- Calculate ng shikimate per µL that have accumulated above the background level

Leaf Disc Assay


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C

Shikimate Assay - Theory

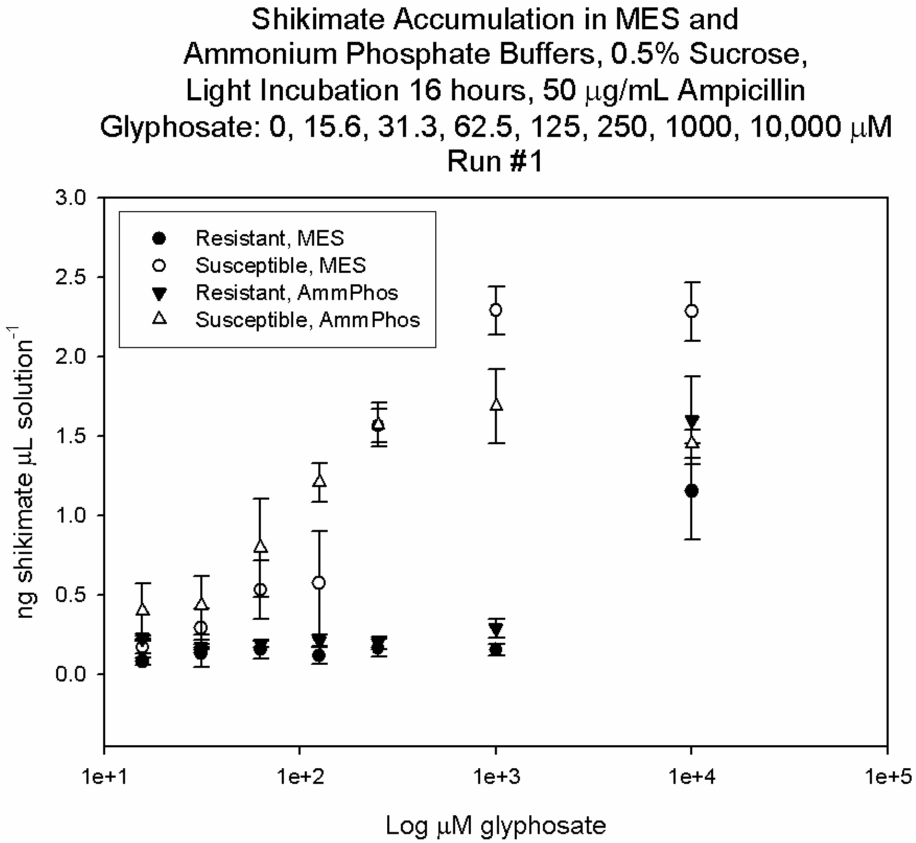


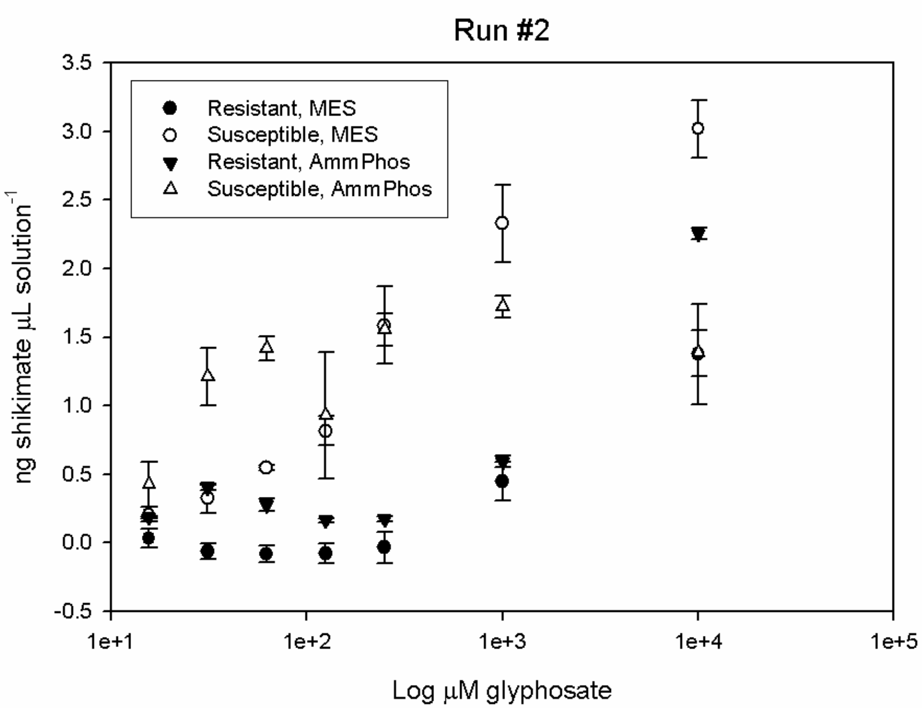


Susceptible
Resistant
Change in OD 380 absorbance
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
100
200
500
1000
2000
μM glyphosate

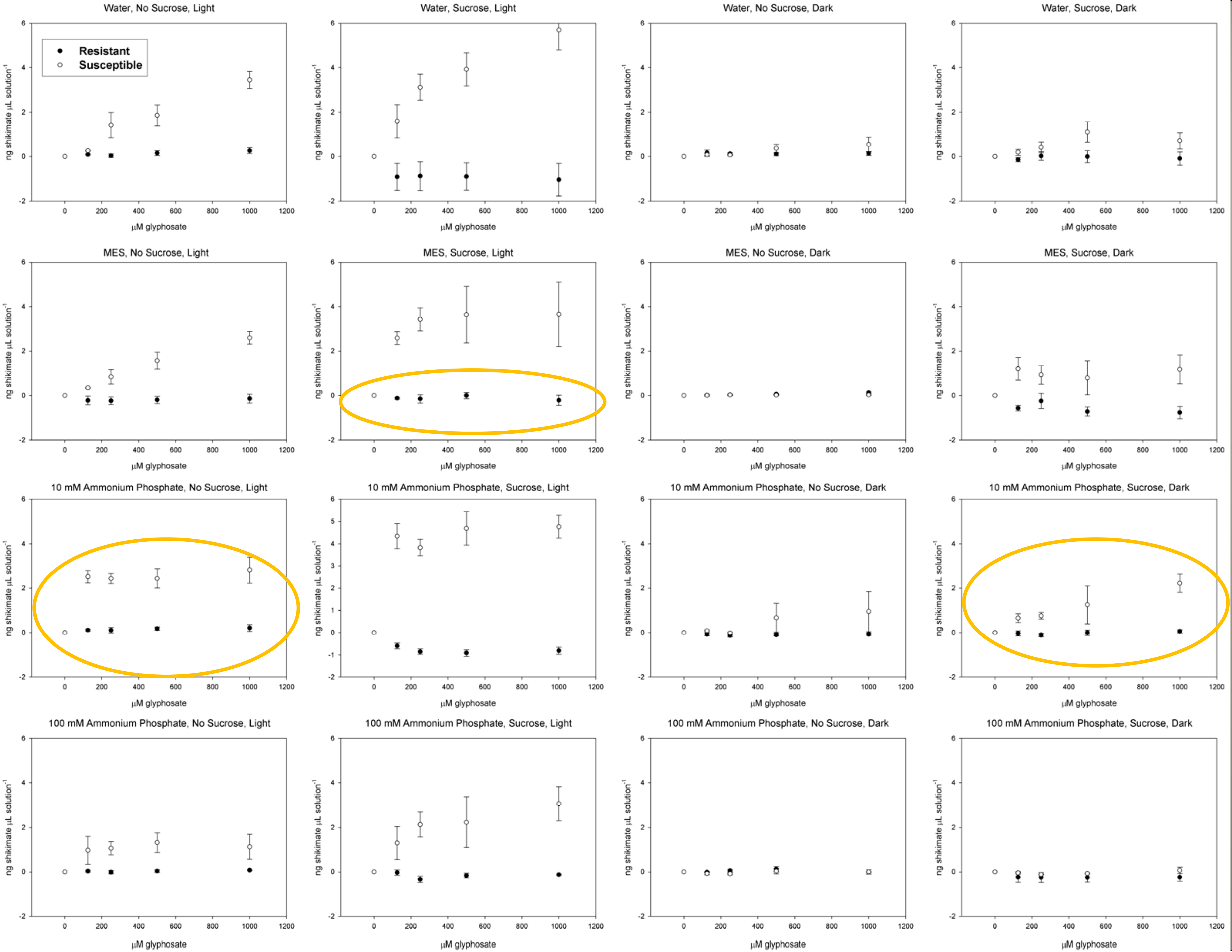
Assay Questions

- MES, ammonium phosphate, or water
  - Does buffer influence shikimate accumulation
- Sucrose and light
  - Can you run the assay without light incubation





- More shikimate accumulates in susceptible and resistant plants with ammonium phosphate
- Overall results are similar with both buffers – resistant can be distinguished from susceptible



Water, No Sucrose, Light
Water, Sucrose, Light
Water, No Sucrose, Dark
Water, Sucrose, Dark
Resistant
Susceptible
MES, No Sucrose, Light
MES, Sucrose, Light
MES, No Sucrose, Dark
MES, Sucrose, Dark
10 mM Ammonium Phosphate, No Sucrose, Light
10 mM Ammonium Phosphate, Sucrose, Light
10 mM Ammonium Phosphate, No Sucrose, Dark
10 mM Ammonium Phosphate, Sucrose, Dark
100 mM Ammonium Phosphate, No Sucrose, Light
100 mM Ammonium Phosphate, Sucrose, Light
100 mM Ammonium Phosphate, No Sucrose, Dark
100 mM Ammonium Phosphate, Sucrose, Dark
ng shikimate µL solution^{-1}
µM glyphosate

Pros and Cons

- Non-destructive
- Has utility for screening plants from the field
- Fast, easy, reliable
- Can use sucrose and avoid light requirement
- Dose-dependent response
- We have used it to phenotype plants for crossing

- Theoretically it should work across species
- In reality, you have to try it to see how a species responds
- Difficult to compare a dose from assay to a field application rate

Resistance Mechanism

- Initial hypothesis: Single amino acid change in EPSPS in resistant plants
- Methods
  - Extract RNA from resistant and susceptible plants
  - Make cDNA using Reverse Transcriptase
  - Design primers based on known *Amaranthus* and *Arabidopsis* sequences
  - Amplify gene, clone, and sequence

Approach:

Align sequences…

```
Arabidopsis      GGTGCTGATGTTGAATGTACTCTTGGCACTAACTGCCCTCCTGTTCGTGTCAACGCTAAT 720
Amaranthus       GGTTCAGATGTTGACTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAA 357
                 *** * ******** ***  ********* ** ************** ***** *****

Arabidopsis      GGTGGCCTTCCTGGTGGAAAGGTGAAGCTTTCTGGATCTATTAGTAGTCAGTACTTGACC 780
Amaranthus       GGAGGCCTTCCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACT 417
                 ** ******** ** ** ***** ***** ********  ******* ** ** ** **
```

…design primers flanking conserved region

•amplified fragment contains Pro of interest

A. palmeri

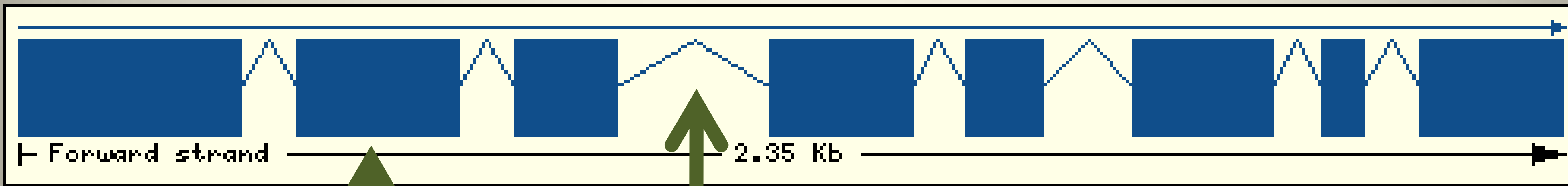
R and S

Extract RNA → Make cDNA w/ reverse transcriptase

Extract DNA → Test primers by PCR (or RT-PCR)

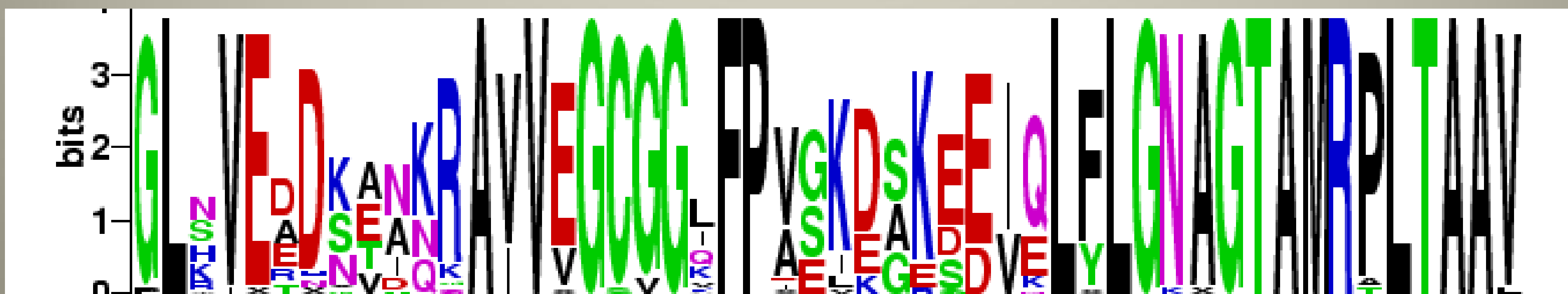
EPSPS Gene Structure

- 8 exons, 7 introns



Exon: Transcribed and

Intron: Transcribed but not translated



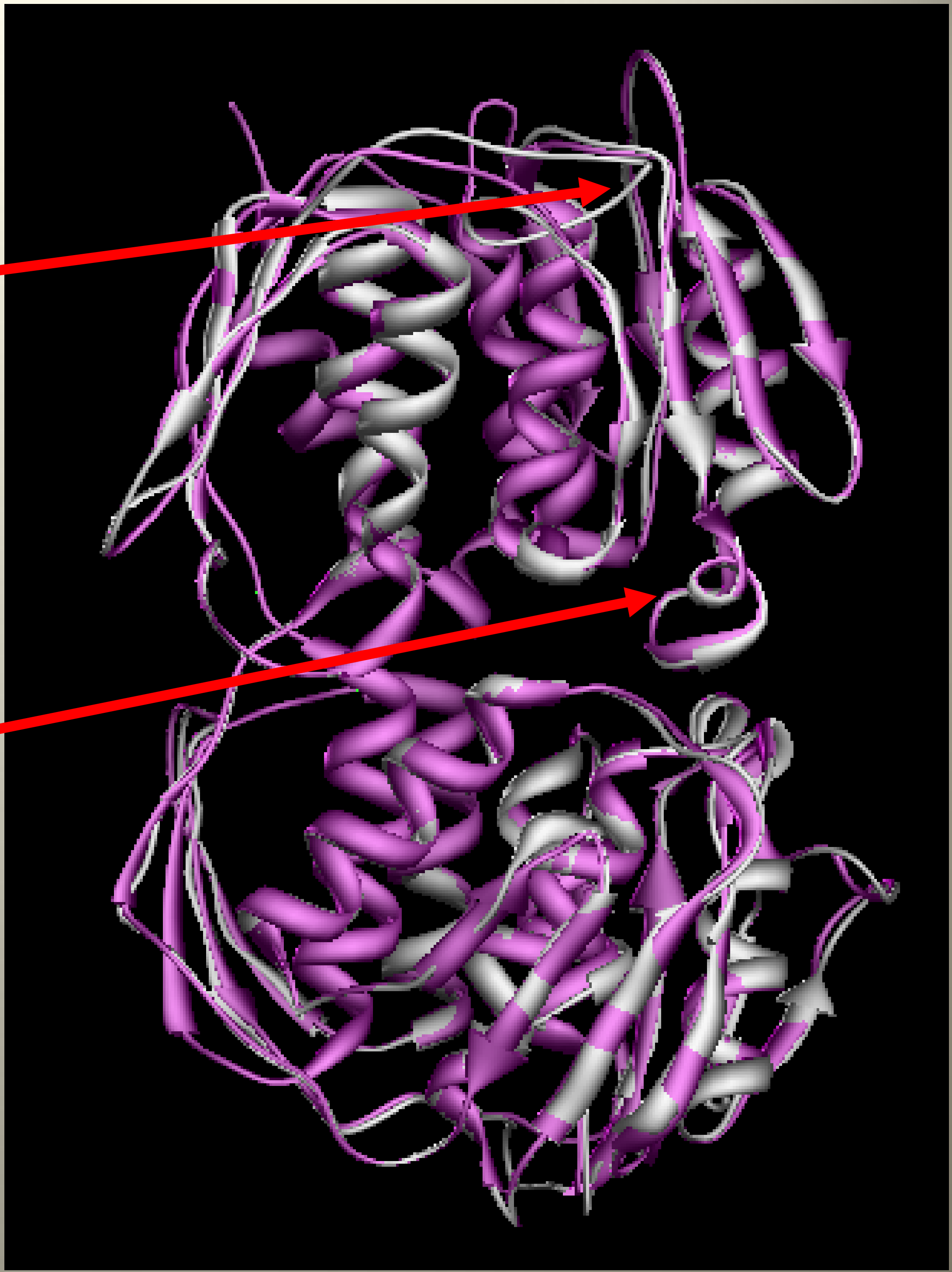
Candidate mutation in plant EPSPS – Proline 106

Species with P106 change: goosegrass in Malaysia, rigid ryegrass in Australia, and Italian ryegrass in Chile

3-D threading of Palmer sequence on crystallized *E. coli* structure 2AA9

Arg/Lys

PEP & Glyphosate binding



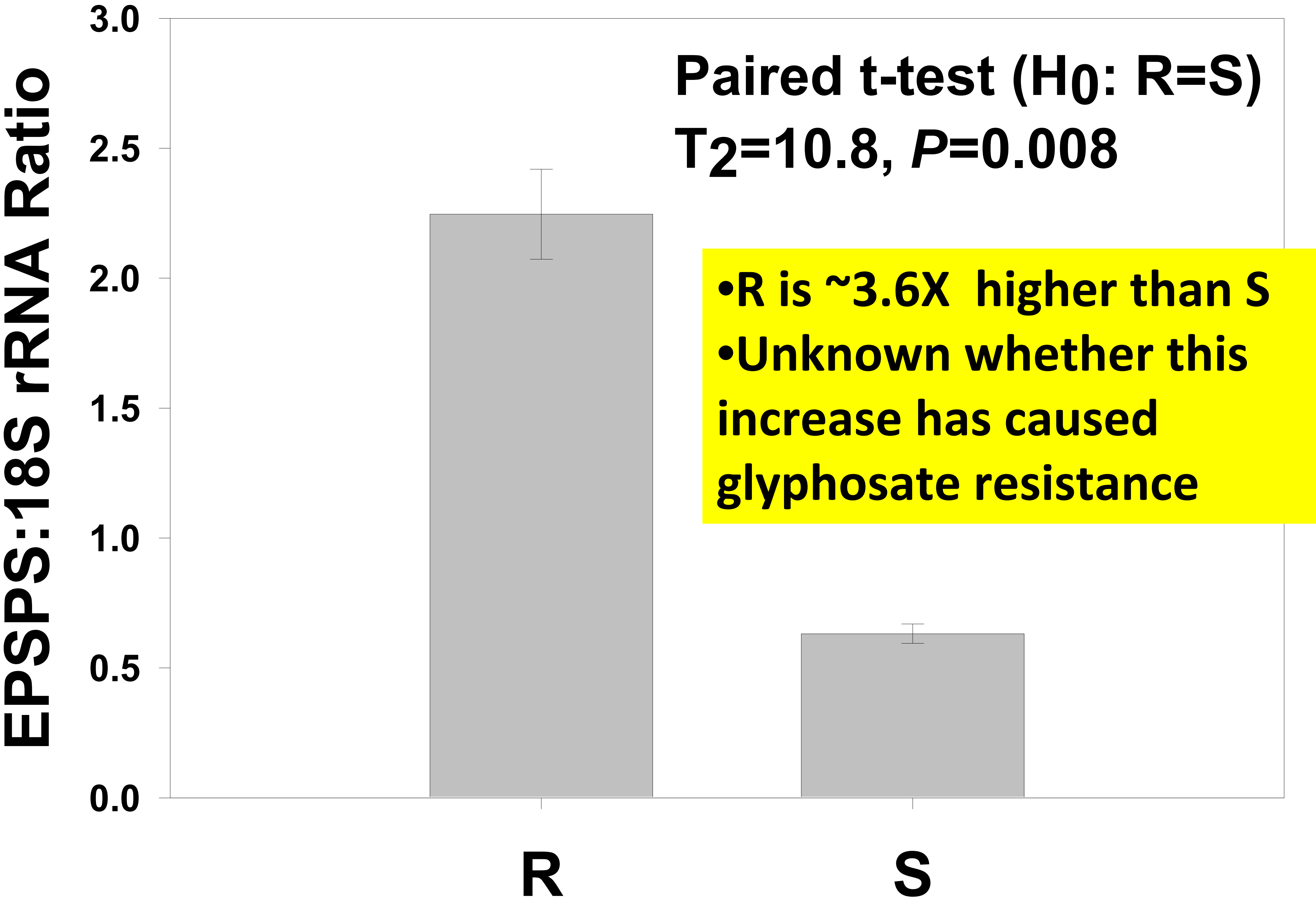
UCSF Chimera

Sequencing Conclusion

- One mutation found in Palmer amaranth resistant EPSPS
  - From arginine to lysine
- This mutation is not likely responsible
  - Occurs on outside of enzyme
  - Located in a highly variable region within a highly conserved enzyme
  - Some susceptible species have lysine at the same position

Expression Level

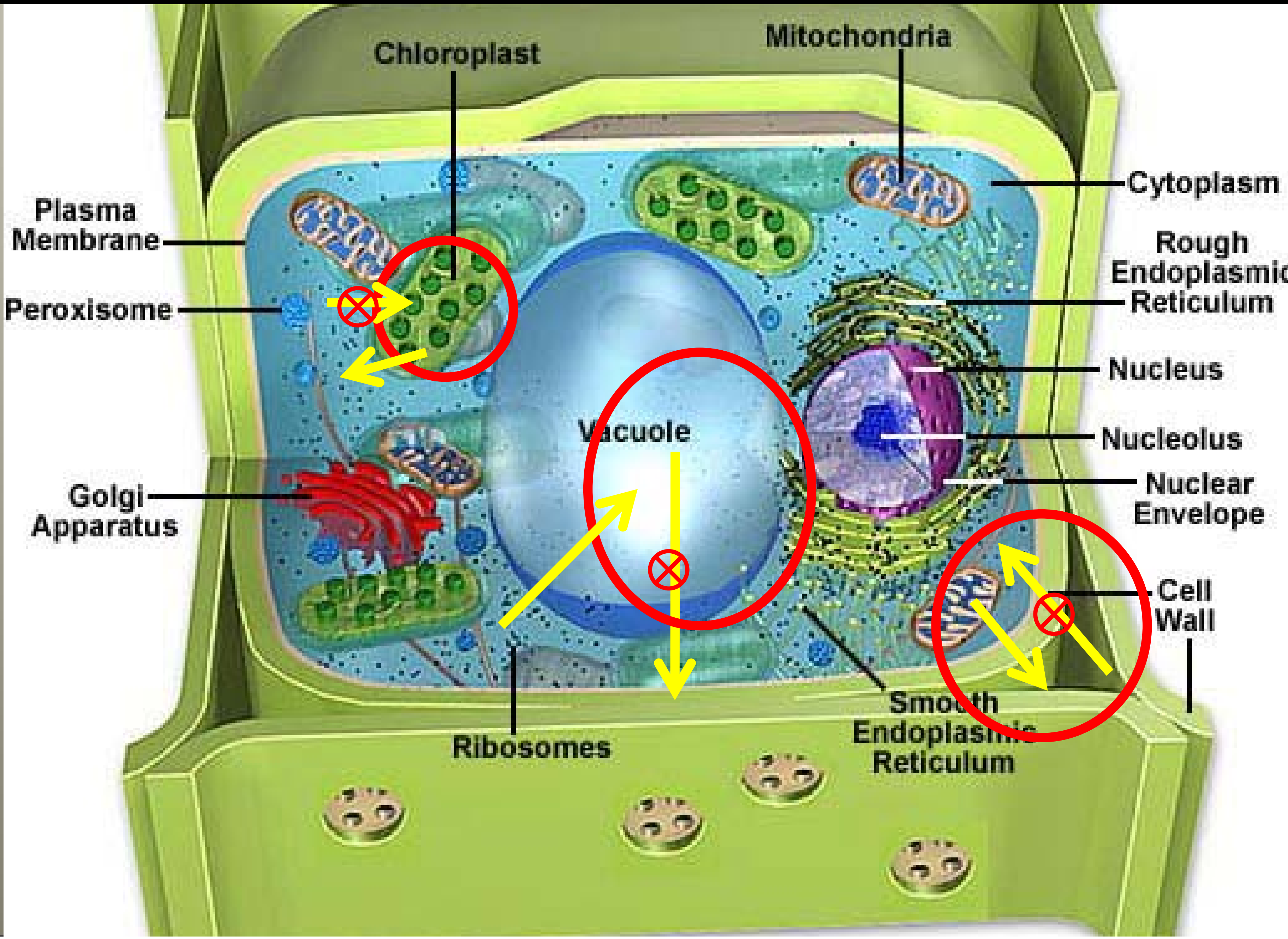
- Over-expressing EPSPS conferred glyphosate tolerance in transgenic plants (Klee 1987)
- EPSPS is under transcriptional regulation in plants
  - Existing mechanism to up-regulate transcription
  - Resistant plants could either have increased transcription, or reduced negative regulation of EPSPS
- Hypothesis: Resistant plants exhibit higher levels of EPSPS transcript than susceptible
- Method: Semi-quantitative RT-PCR from cDNA
  - Not as good as quantitive real time PCR
  - Good way to start

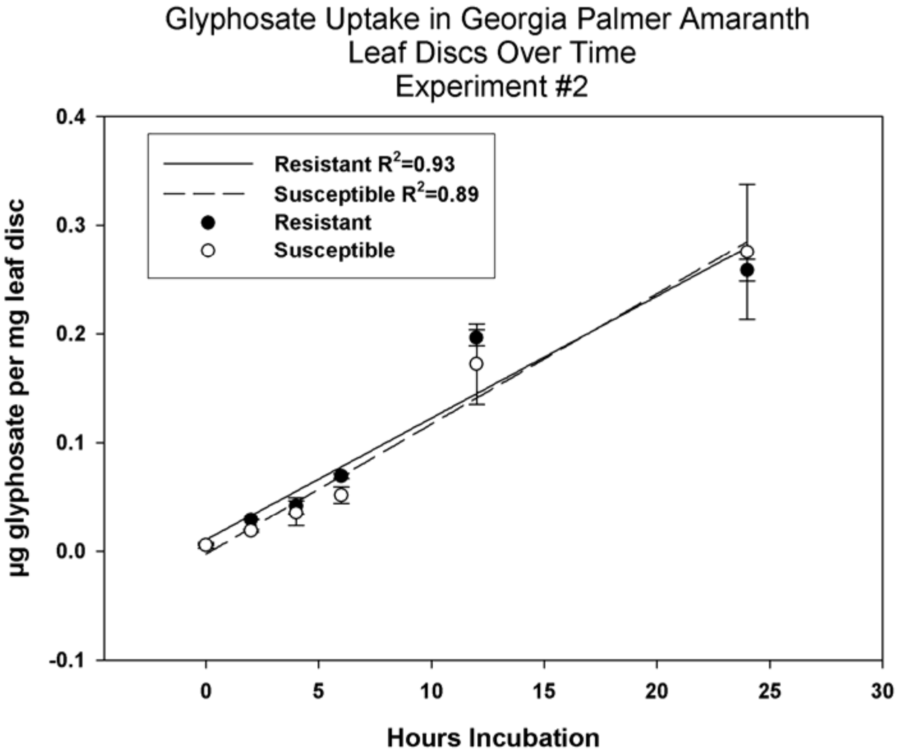

EPSPS:18S rRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Paired t-test (H_0: R=S)
T_2=10.8, P=0.008
•R is ~3.6X higher than S
•Unknown whether this increase has caused glyphosate resistance
R
S

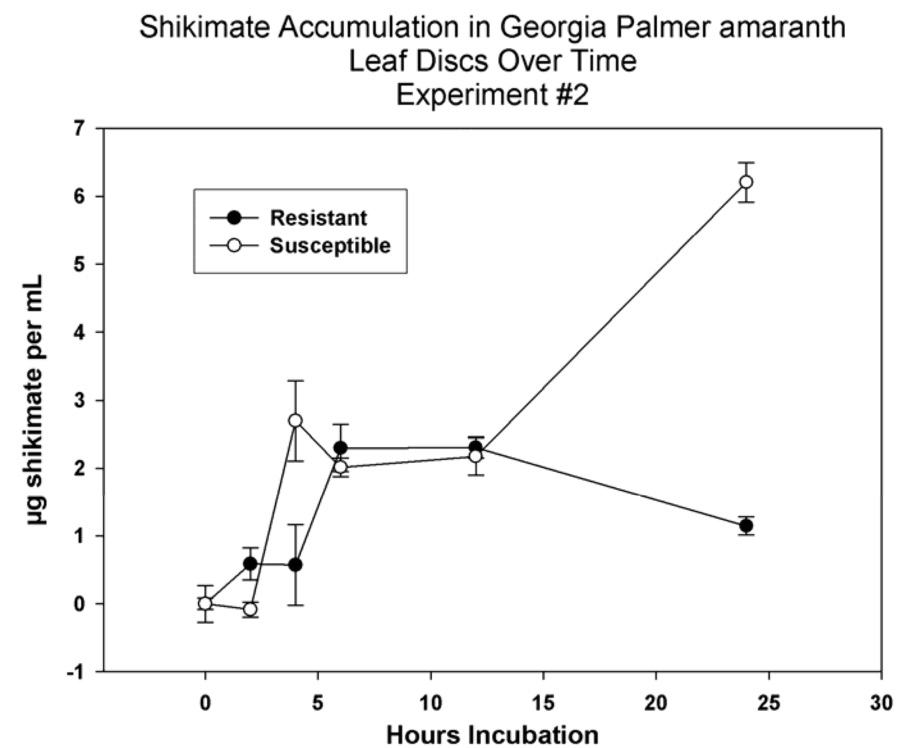
Glyphosate Uptake and Desorption

- Glyphosate must translocate to its site of action in the chloroplast
- Hypothesis: Resistant plants may have reduced glyphosate concentration in chloroplasts by
  - Sequestering glyphosate in vacuole
  - Pumping out of chloroplast
  - Pumping out of cytoplasm into apoplast
- Do resistant and susceptible plants uptake ^{14}C glyphosate equally in leaf disc assay?

Possible Cellular Mechanisms

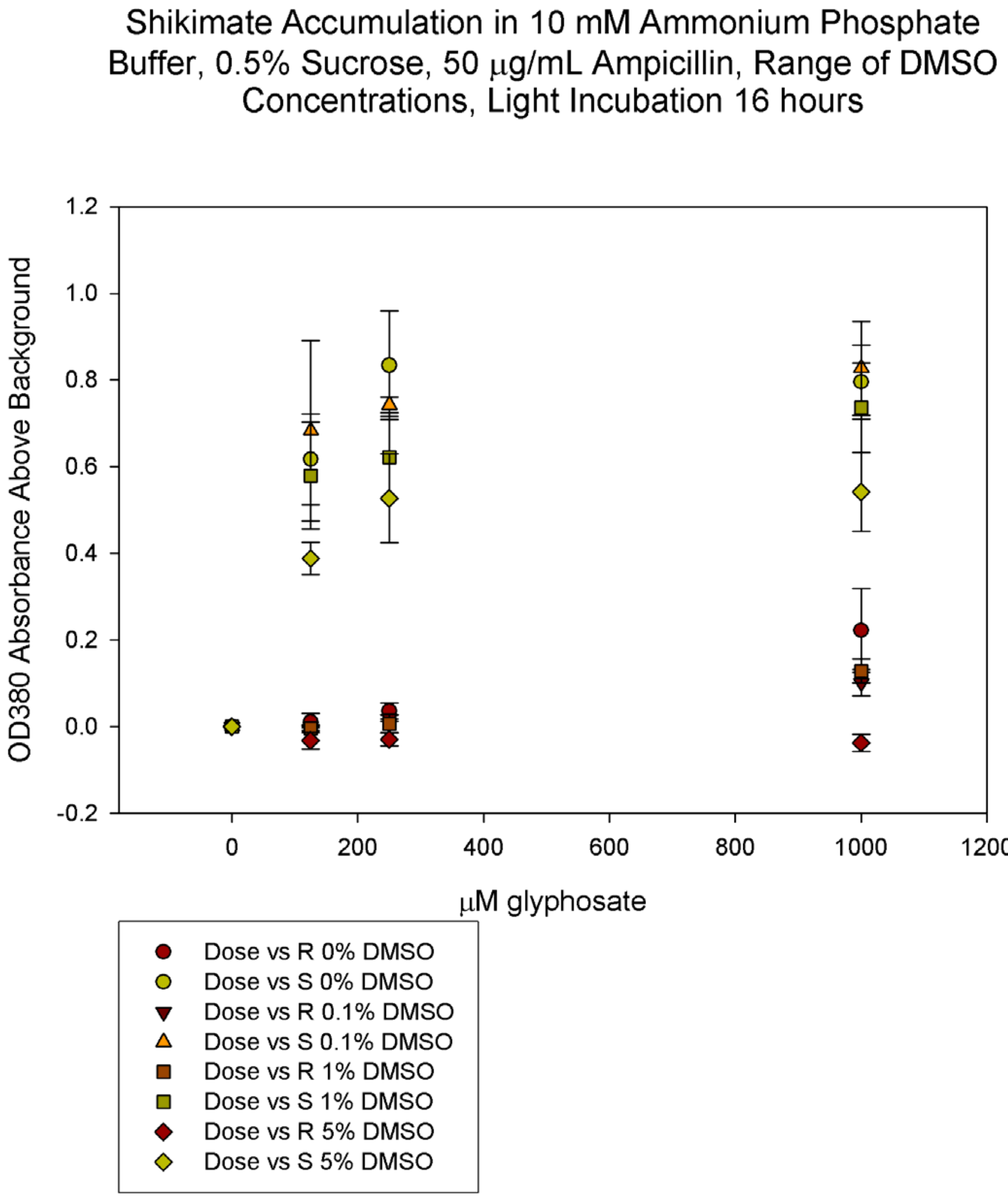






•Glyphosate taken up into R and S leaf discs
•Shikimate accumulates in S, but not in R at 250 µM

- Agrees with previous work in Georgia
- Leaf disc assay works well for measuring uptake
- Difficult to determine whether chloroplast uptake is different using this method



DMSO does not appear to release glyphosate from vacuole to make it available for EPSPS inhibition and cause shikimate accumulation in resistant plants

Resistance Mechanisms Summary

- A mutation in EPSPS is unlikely to be the mechanism
- Some data to support that increased levels of EPSPS transcript are involved in mechanism
  - More experiments planned
- Uptake of ^{14}C in leaf discs similar
  - Inconclusive as to whether cellular localization is different
  - More experiments planned

Proposed Mechanism

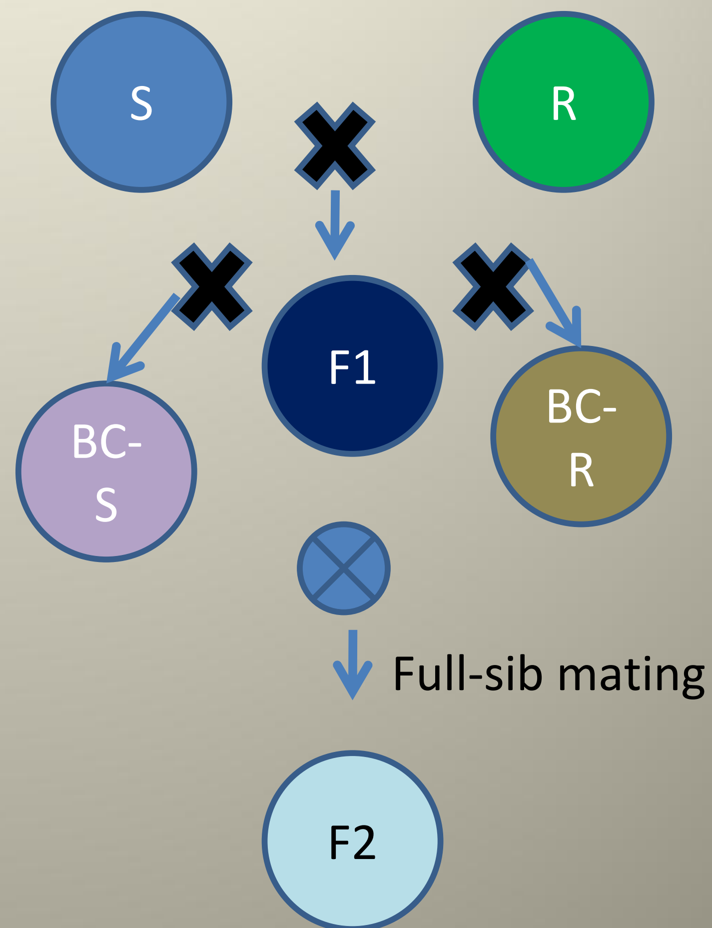
- Resistant plants produce more EPSPS
  - More pre-EPSPS and mature EPSPS is available to bind glyphosate in cytoplasm and chloroplast
- Resistant plants may have a mechanism to exclude glyphosate from the chloroplast, reducing the concentration of glyphosate at the target site
- Combination of exclusion and increased EPSPS permits shikimate pathway function

Planned Experiments

- Measure EPSPS transcription and shikimate accumulation following foliar glyphosate application
  - Resistant, Susceptible, Heterozygous F1
  - Segregating F2 population
    - Determine whether increased EPSPS level is associated with resistance
- Quantify glyphosate concentration in the chloroplasts of R and S plants
  - Methods still to be determined, will involve isolating chloroplasts

Genetics Research

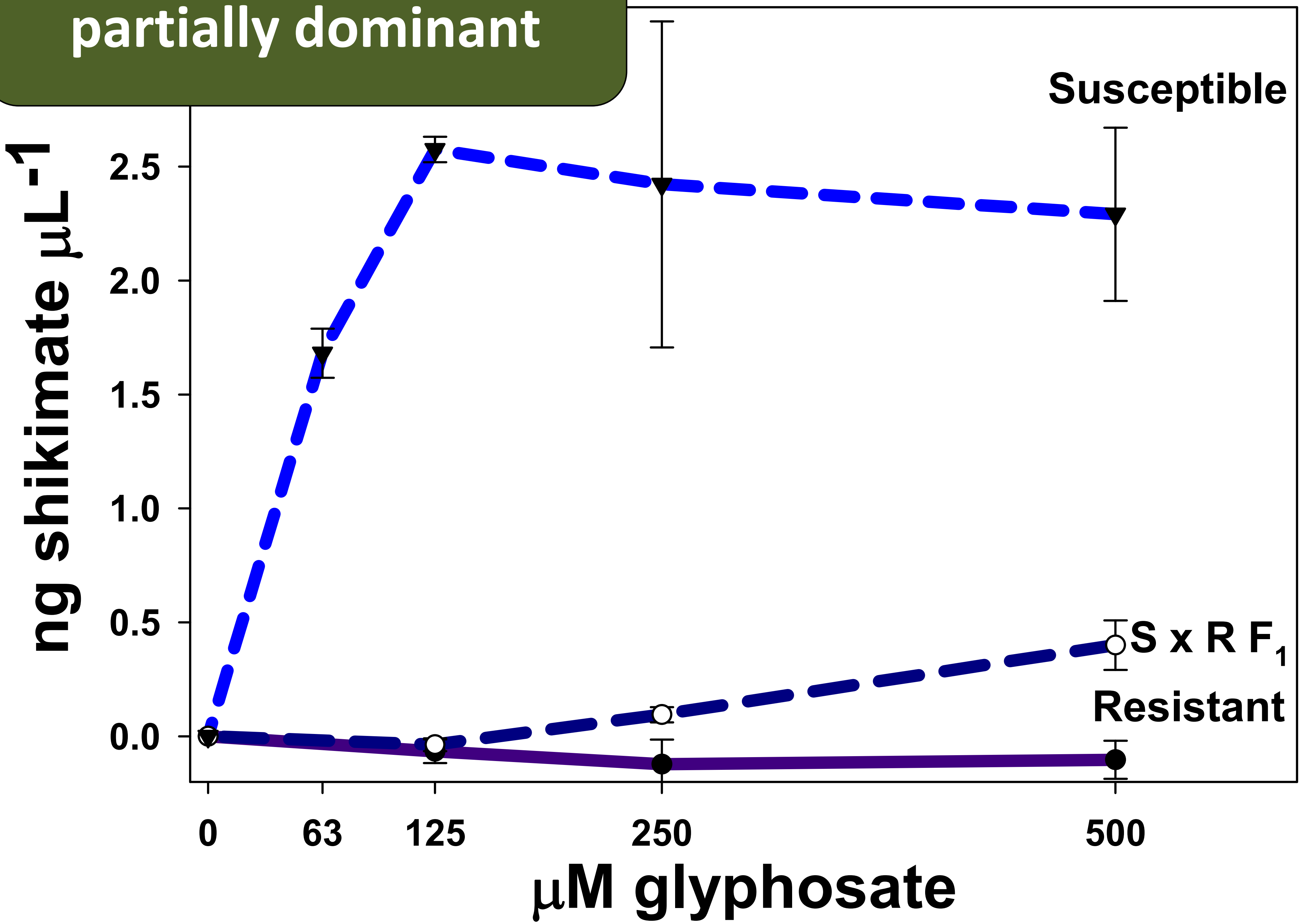
- Diallel using resistant and susceptible as male and female parents
  - F1, backcross and F2 populations
- Crosses made by bagging female flowers and hand-pollinating
- At least two different plants for each cross

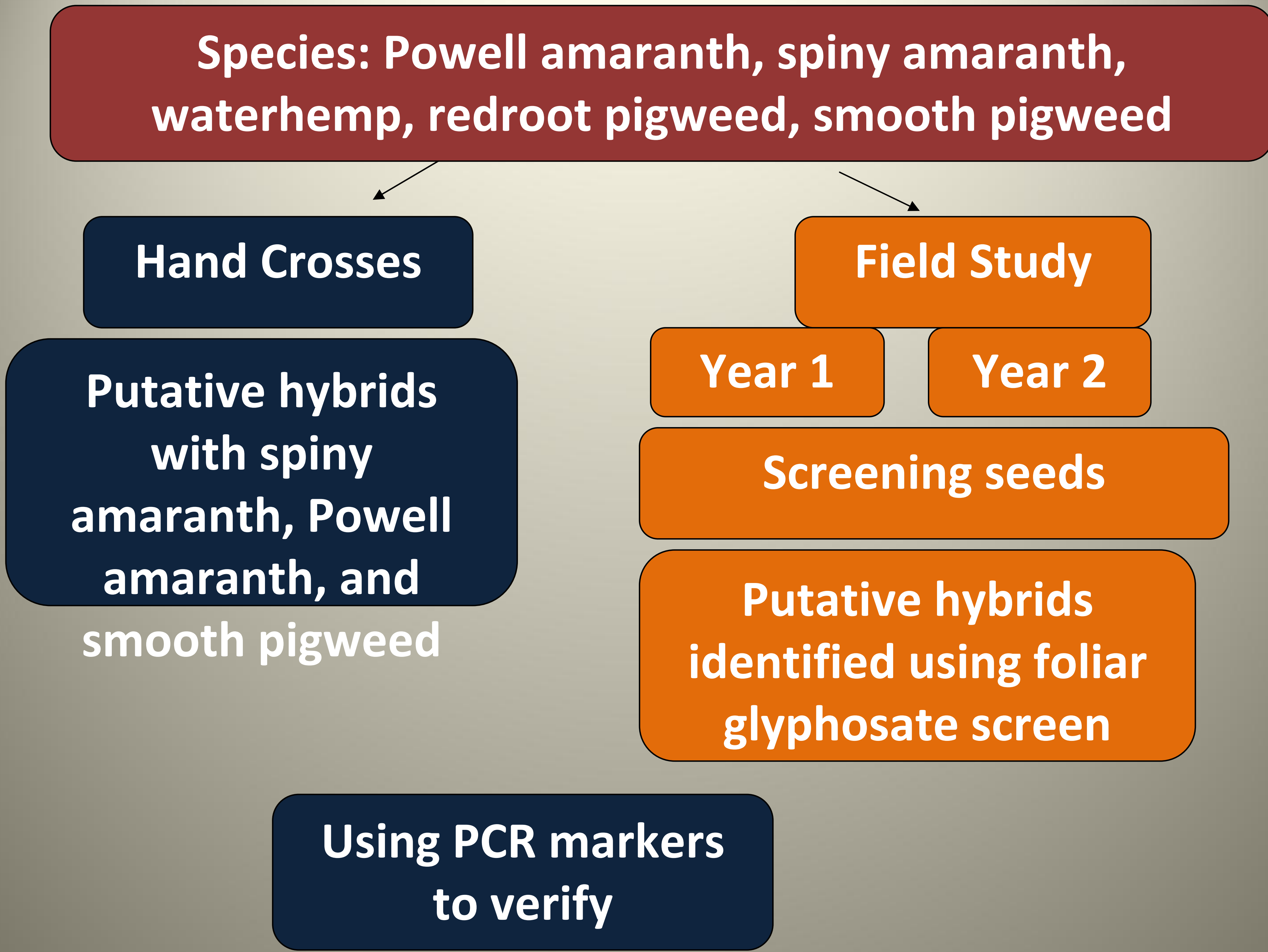


Segregating Population – Use to determine gene number and action

Crossing Technique

F_1 data indicate gene is partially dominant




Species: Powell amaranth, spiny amaranth, waterhemp, redroot pigweed, smooth pigweed
Hand Crosses
Putative hybrids with spiny amaranth, Powell amaranth, and smooth pigweed
Field Study
Year 1
Year 2
Screening seeds
Putative hybrids identified using foliar glyphosate screen
Using PCR markers to verify

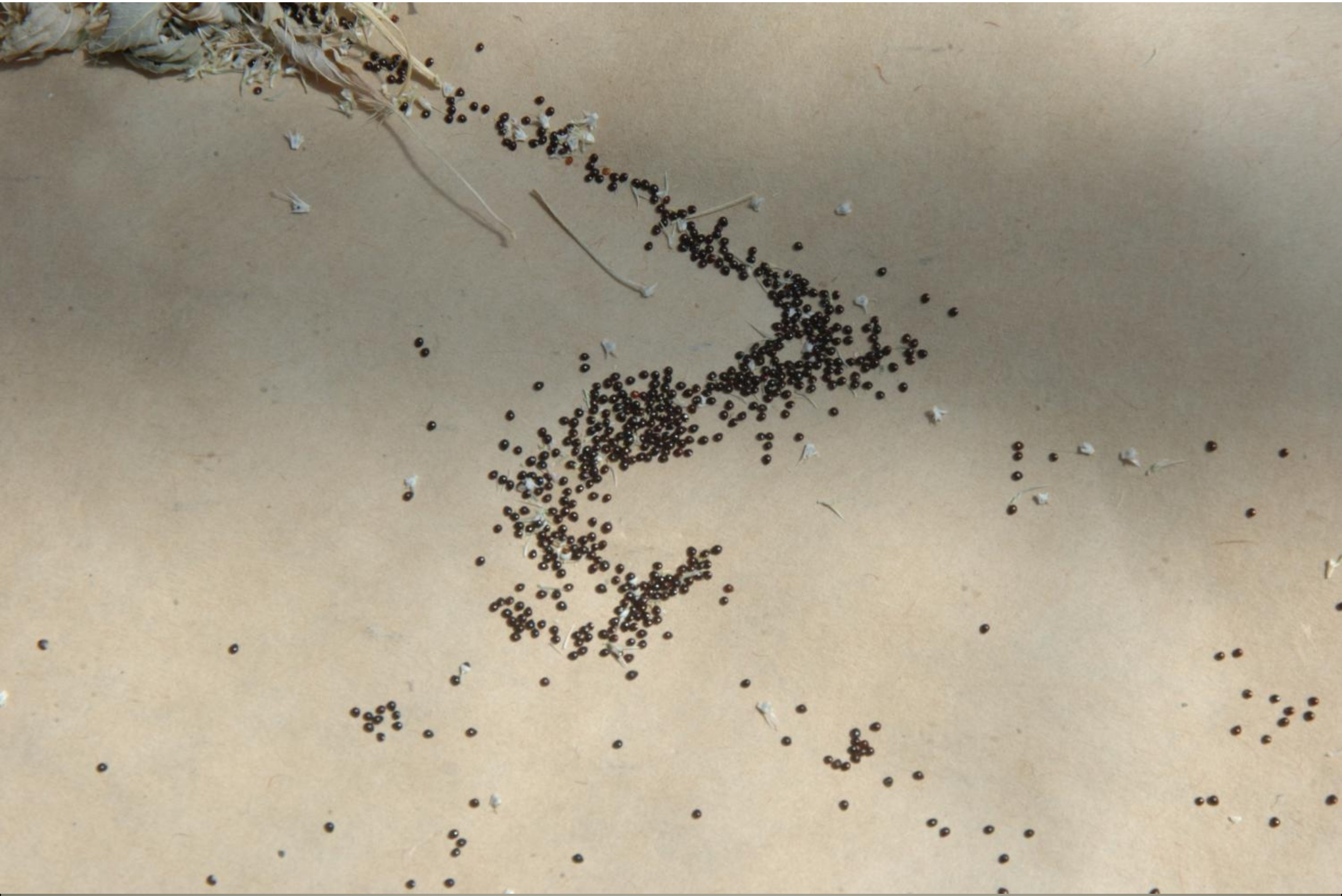
Gene Flow Study - 2006 & 2007

Methods

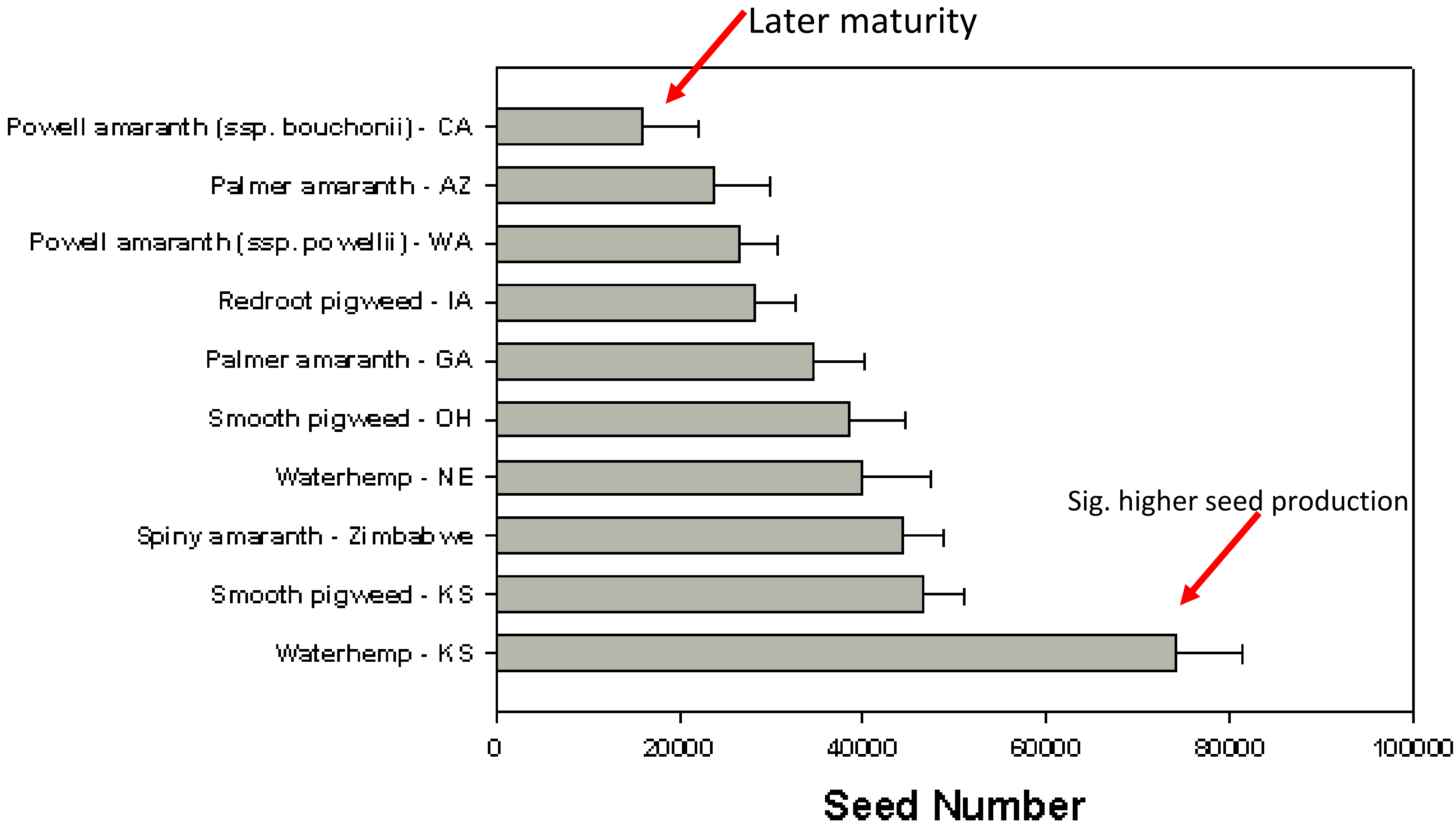
- Species used:
  - Waterhemp (2)
  - Smooth pigweed (2)
  - Spiny amaranth
  - Redroot pigweed
  - Powell amaranth (2)
  - Palmer amaranth (2)
- Seed sources: USDA-GRIN, Ames, IA; Kansas State U.; U. of Georgia

Resistant Palmer amaranth males

Harvest



Average Seeds Per Plant



High throughput screening – over 10,000 seeds screened per plant from gene flow study

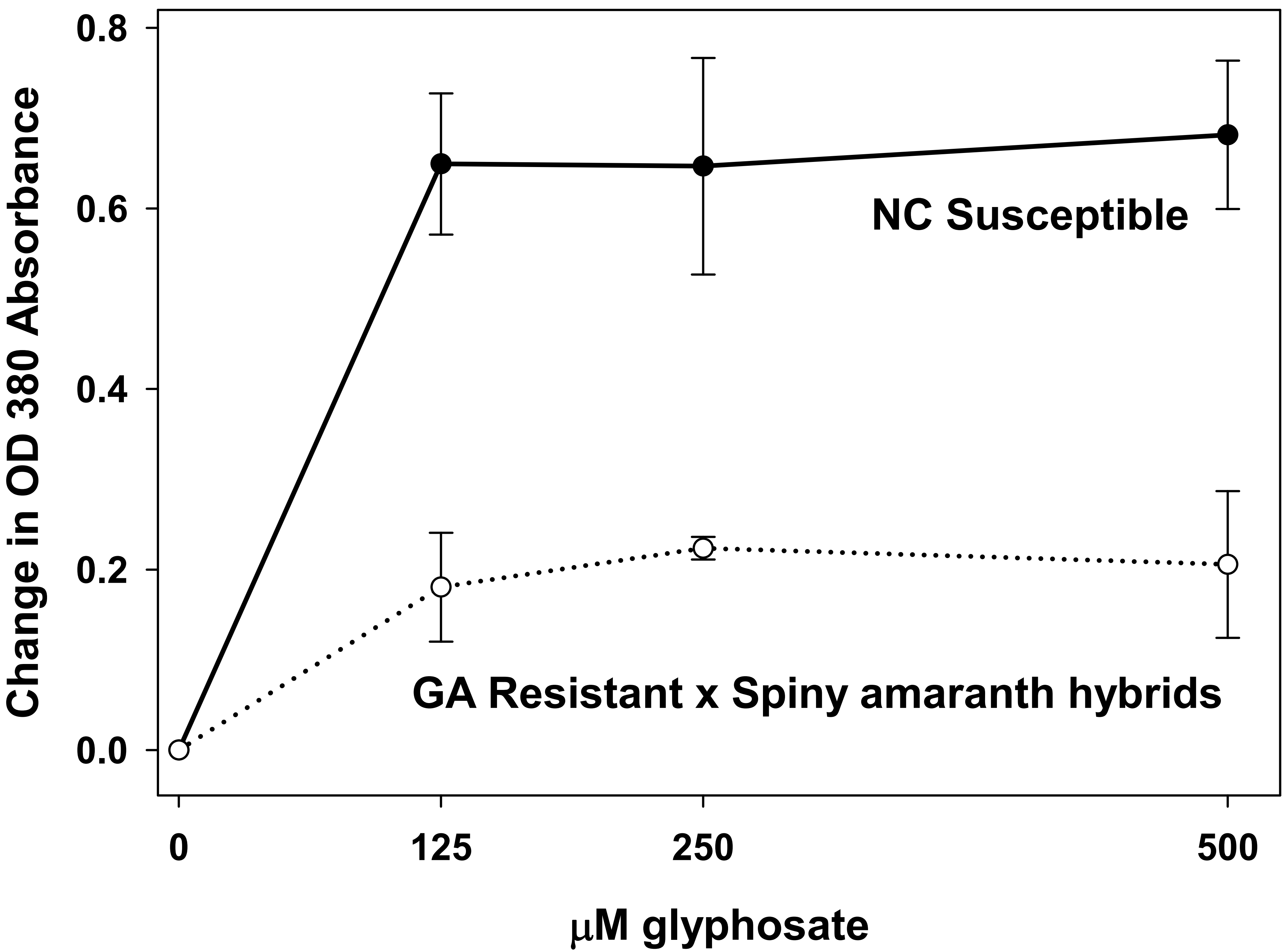
Example: Seeds from susceptible Palmer amaranth in study

Putative Hybrids

Palmer/Powell

Spiny Amaranth

Palmer/Spiny



0.8
0.6
0.4
0.2
0.0
Change in OD 380 Absorbance
NC Susceptible
GA Resistant x Spiny amaranth hybrids
0
125
250
500
μM glyphosate

Conclusions

- Glyphosate resistance in GA Palmer amaranth appears to be due to complex and not yet fully understood mechanism(s)
- This project has provided excellent graduate student training
- This research may help in evaluating future cases of new glyphosate resistant weeds
- We thank Monsanto for their support of this research project