The Path to Seedless Cannabis: How Triploid Sterile Varieties Can Help Farmers Stop Worrying About Unwanted Pollen

Richard Philbrook
Scientist - Molecular Biology

DARK HEART NURSERY

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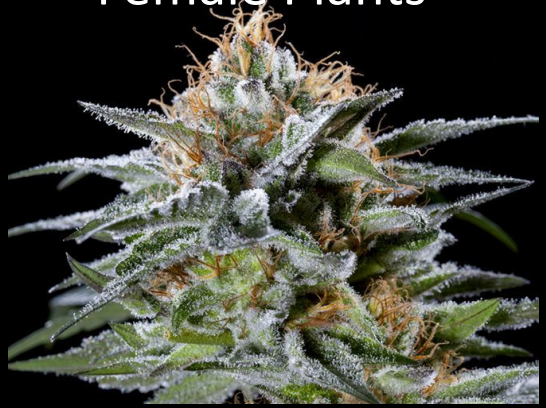
Female Plants



- Trichomes
- High in cannabinoids
- XX Sex Chromosomes
- Develops seeds

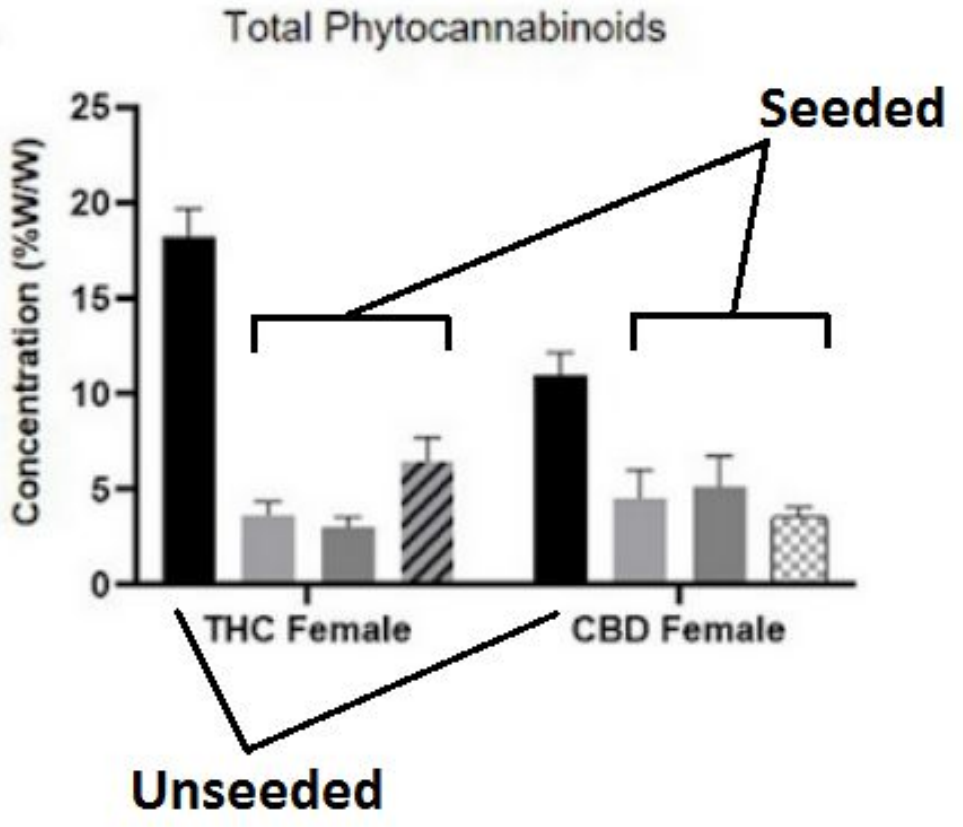
Male Plants

- Few trichomes
- Low cannabinoids
- XY Sex Chromosomes
- Develops pollen

Intersex Plants

- Male and female flowers
- Can be XX or XY
- Both pollen and seeds

Why Seedless Cannabis?



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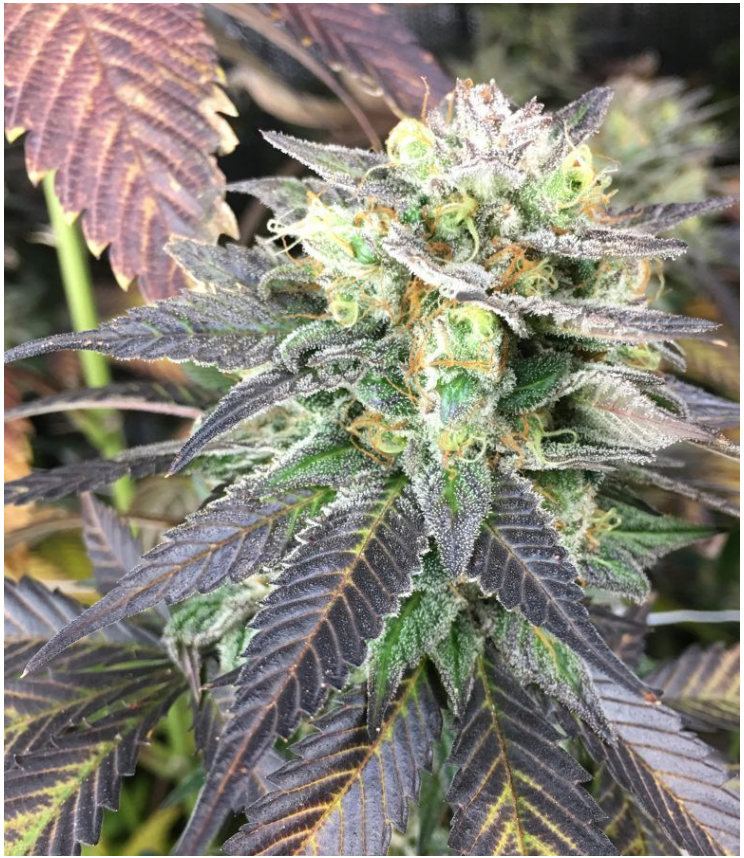
Why Seedless Cannabis?



VS

History of Sinsemilla

- Traditionally, Cannabis grown in mixed sex fields and allowed to pollinate/set seed

History of Sinsemilla

- Beginning in 1970's, growers in Mexico began culling males before plants matured
  - Consumers immediately noticed improved quality of unseeded flower

History of Sinsemilla

- Sinsemilla - “without seeds” - has since become standard method of cultivation for high-quality flower

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Current Cultivation Methods to Avoid Pollen

Female Only Clones

Feminized Seeds

Scouting for Anthers/Removing Males

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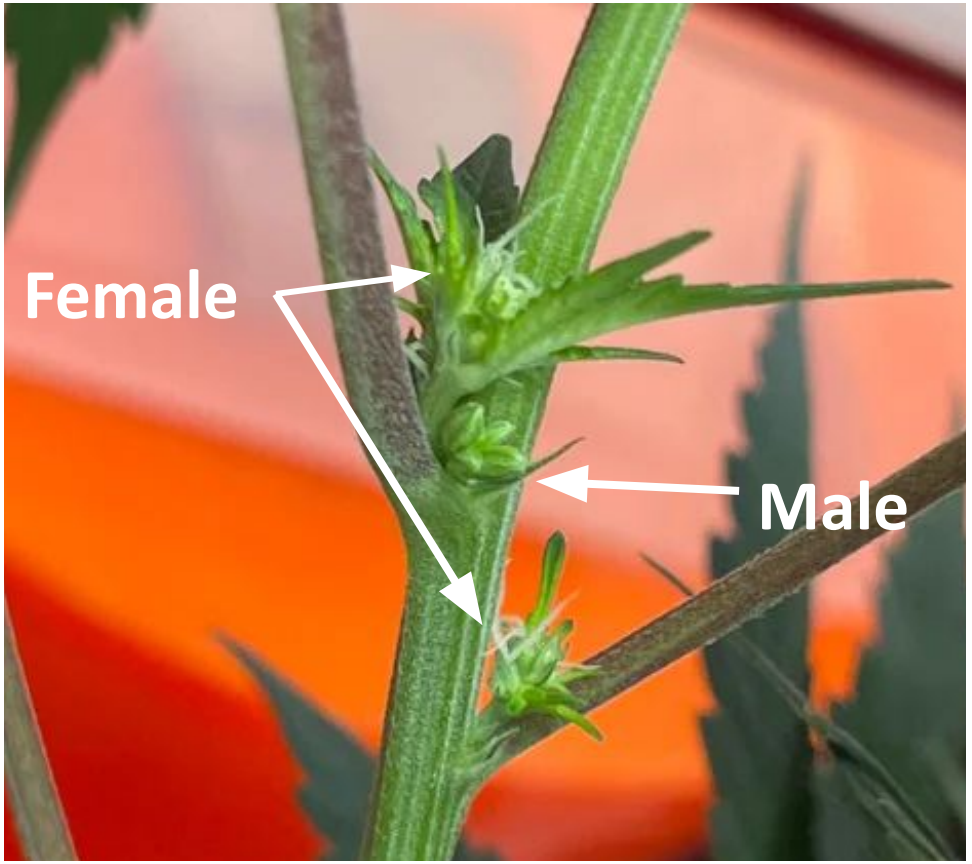
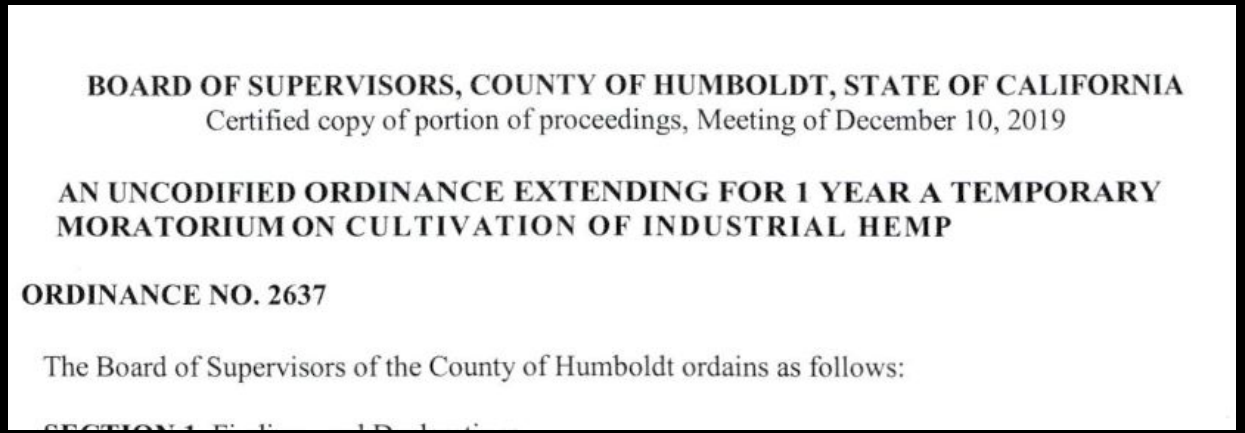
“Herming”: A Grower’s Worst Nightmare



No Current Prevention Method Stops Cross-Pollination by Hemp

- Hemp fields often contain males or monoecious varieties
  - Stokes et al 2000: 36% of all pollen sampled in Midwest was C. sativa. Before legalization!

- Lawsuits arising over cross-pollination
  - *Jack Hempicine LLC v. Leo Mulkey Inc.*, Case No. 18CV38712, Polk Cty. Sup. Ct.
- Humboldt County has temporary ban on hemp farming to prevent pollination



BOARD OF SUPERVISORS, COUNTY OF HUMBOLDT, STATE OF CALIFORNIA
Certified copy of portion of proceedings, Meeting of December 10, 2019

AN UNCODIFIED ORDINANCE EXTENDING FOR 1 YEAR A TEMPORARY MORATORIUM ON CULTIVATION OF INDUSTRIAL HEMP

ORDINANCE NO. 2637

The Board of Supervisors of the County of Humboldt ordains as follows:

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The Problem With Pollen

- Wind-borne pollen can travel miles, but is especially problematic within a few hundred yard radius
- A single male flower can pollinate dozens of plants
- When plants start making seeds, they halt cannabinoid/terpene production
- Current Methods of Prevention Are Still Lacking

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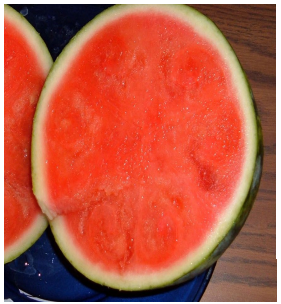
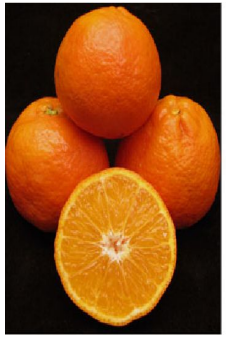
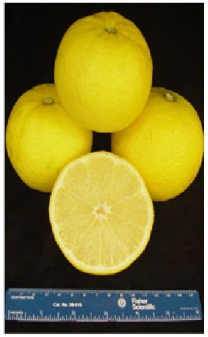
To Move Cannabis Into a Large-Scale Agricultural Crop, the Pollen Problem Must be Solved

How can We Make Cannabis Pollen Proof?

Established examples of sterile/seedless varieties in other crop species:



What do they have in Common? Triploids!

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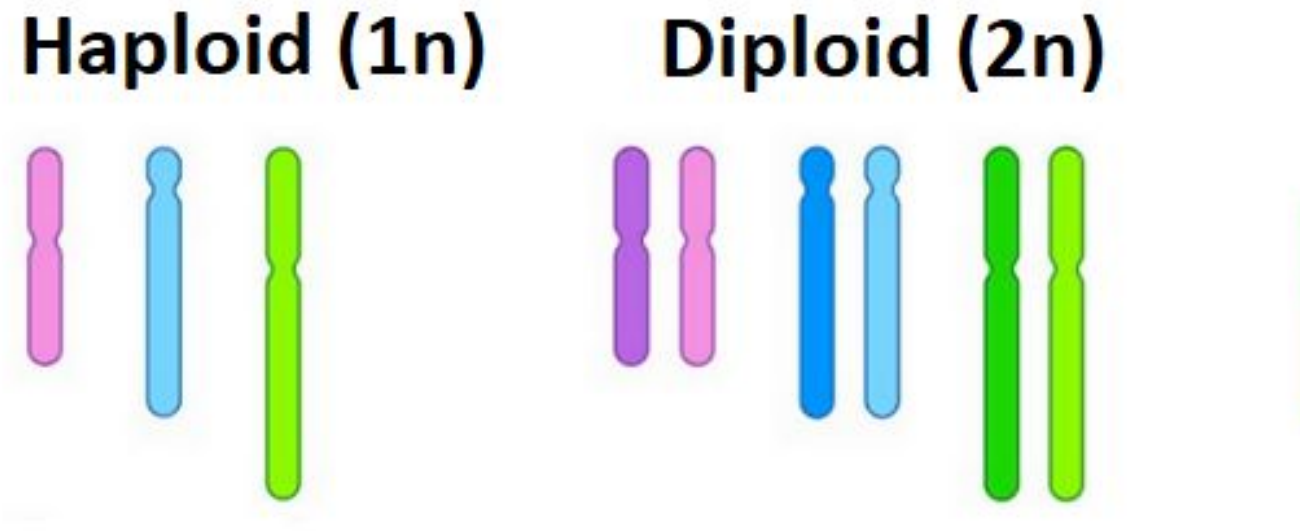
Genetics 101

- **What is Ploidy?**
  - The number of copies of each chromosome an organism contains
  - n = number of chromosomes



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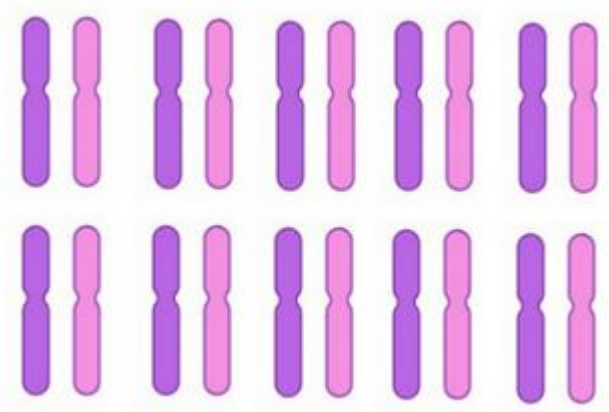
Genetics 101

- Most organisms are diploid (2n), like humans and cannabis
- Cannabis has 10 chromosomes and is diploid (2n), therefore it has 20 chromosomes total in somatic cells and 10 chromosomes total in gametes



10 pairs of chromosomes = 20 total

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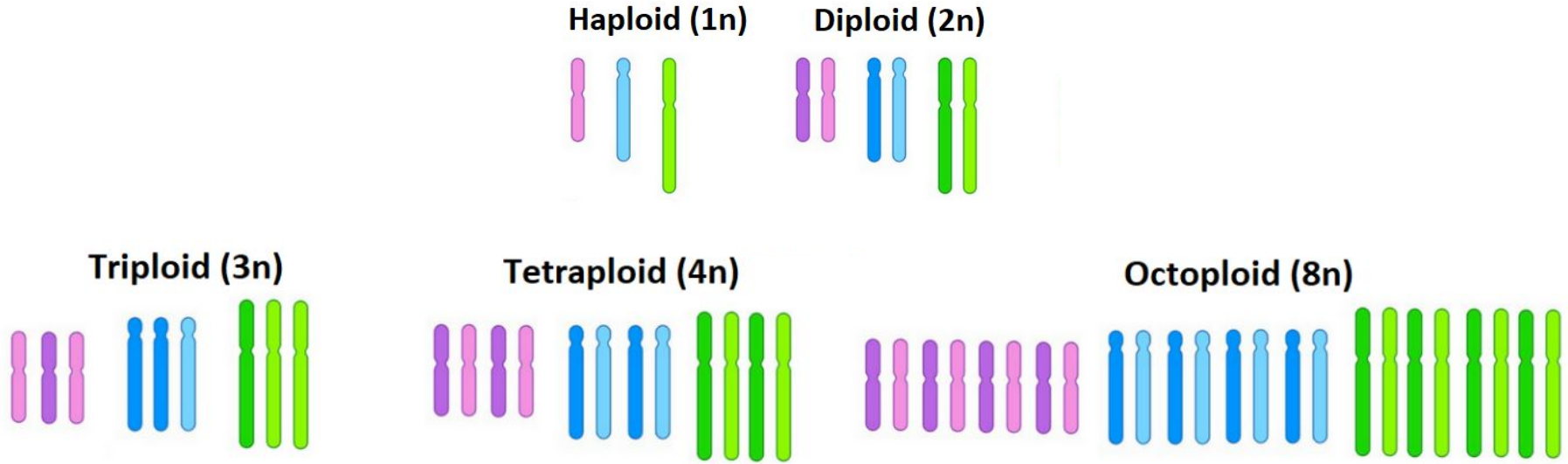
Genetics 101

- Polyploidy refers to an increased number of copies of each chromosome
  - Can occur naturally, aka endoreduplication, or be induced artificially



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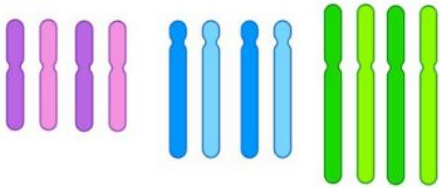
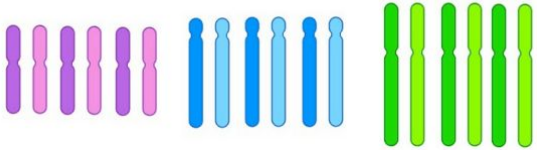
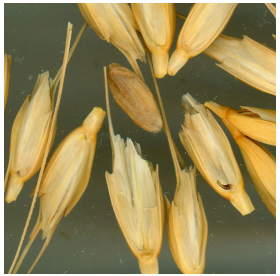
Common Polyploid Crops

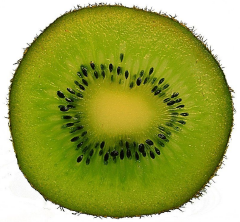
Tetraploid 4n



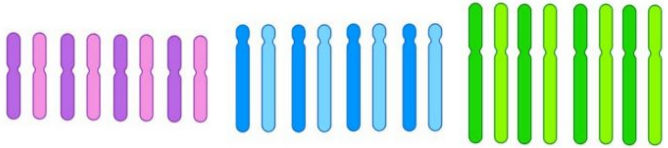
Hexaploid 6n







Octoploid 8n



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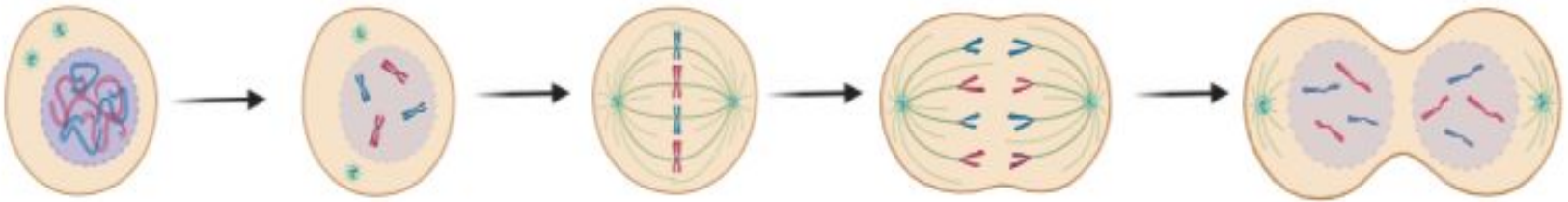
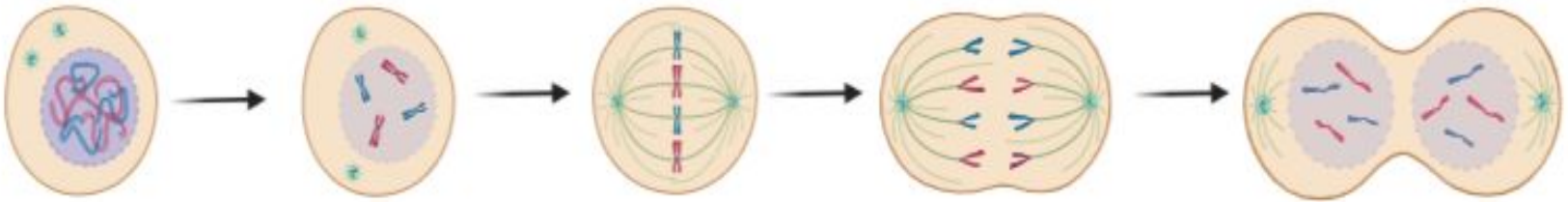
Genetics 101 - Mitosis



Interphase Prophase Metaphase Anaphase Telophase

Genetics 101 - Mitosis

Interphase



Prophase

Metaphase

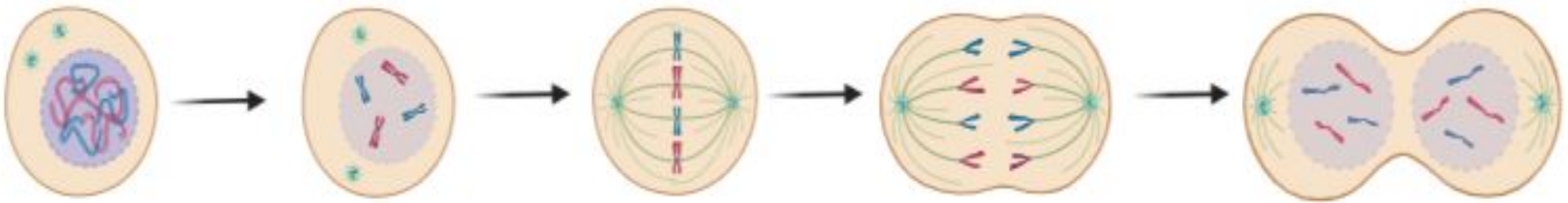
Anaphase

Telophase

Normal function, DNA replicates

Genetics 101 - Mitosis

Prophase



Interphase

Chromosomes condense, spindle starts to form

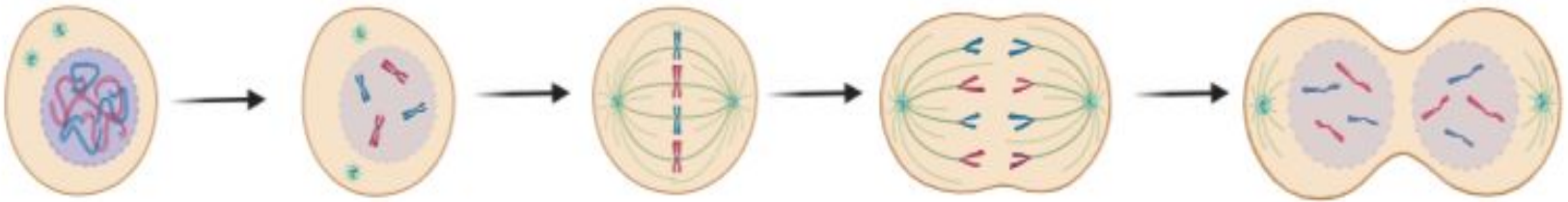
Metaphase

Anaphase

Telophase

Genetics 101 - Mitosis

Metaphase



Interphase

Prophase

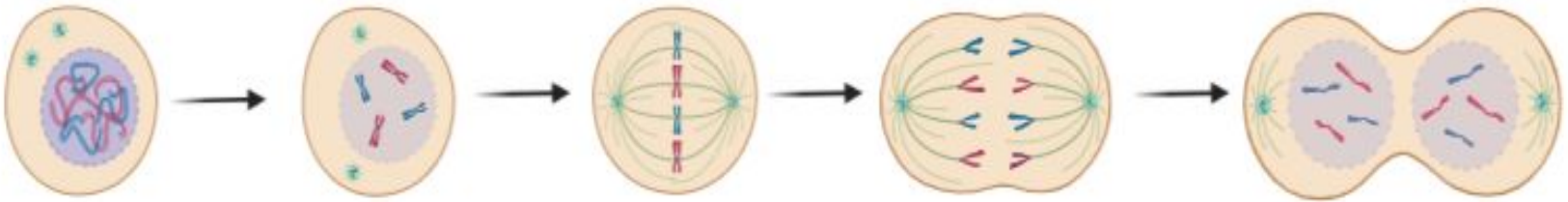
Spindle aligns chromosomes along center of the nucleus

Anaphase

Telophase

Genetics 101 - Mitosis

Anaphase



Interphase

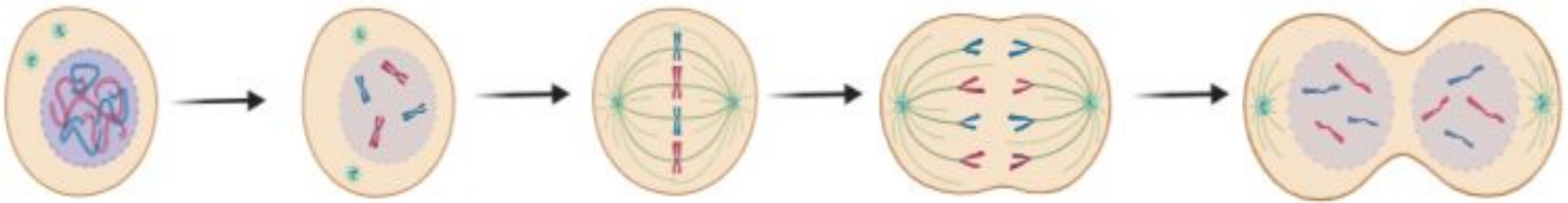
Prophase

Metaphase

Spindle pulls chromosomes apart, cell begins to divide

Telophase

Genetics 101 - Mitosis

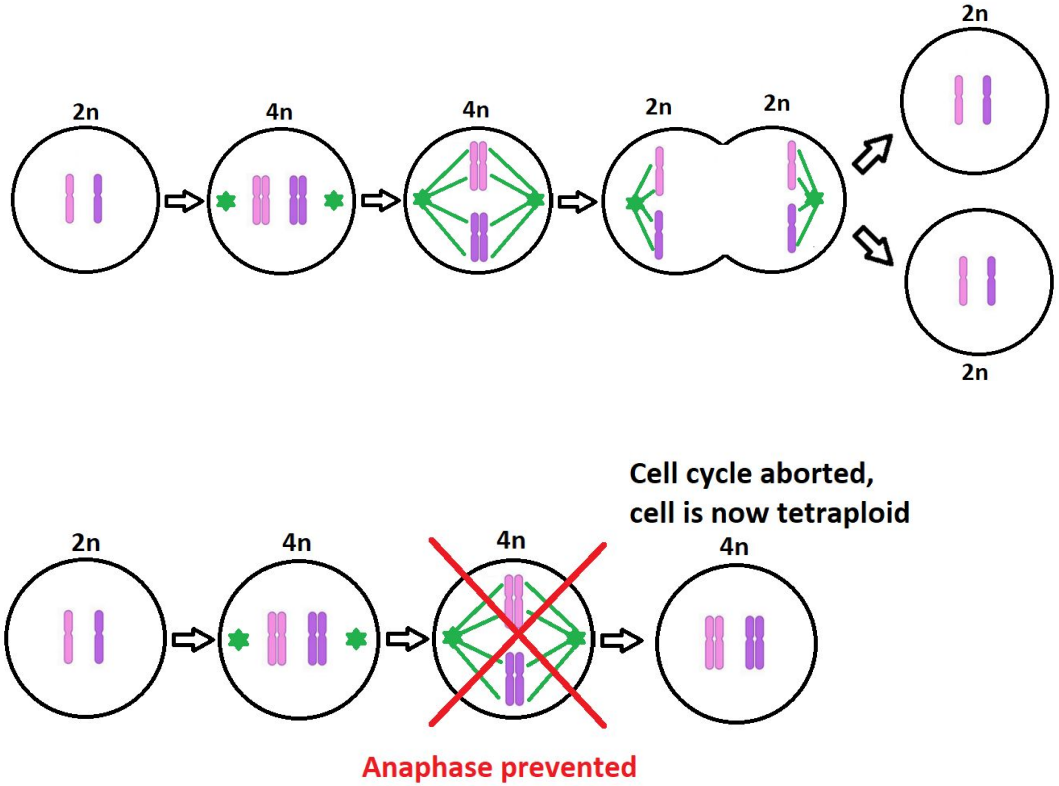


Interphase | Prophase | Metaphase | Anaphase | Telophase

Cells finish dividing, 2 identical daughter cells

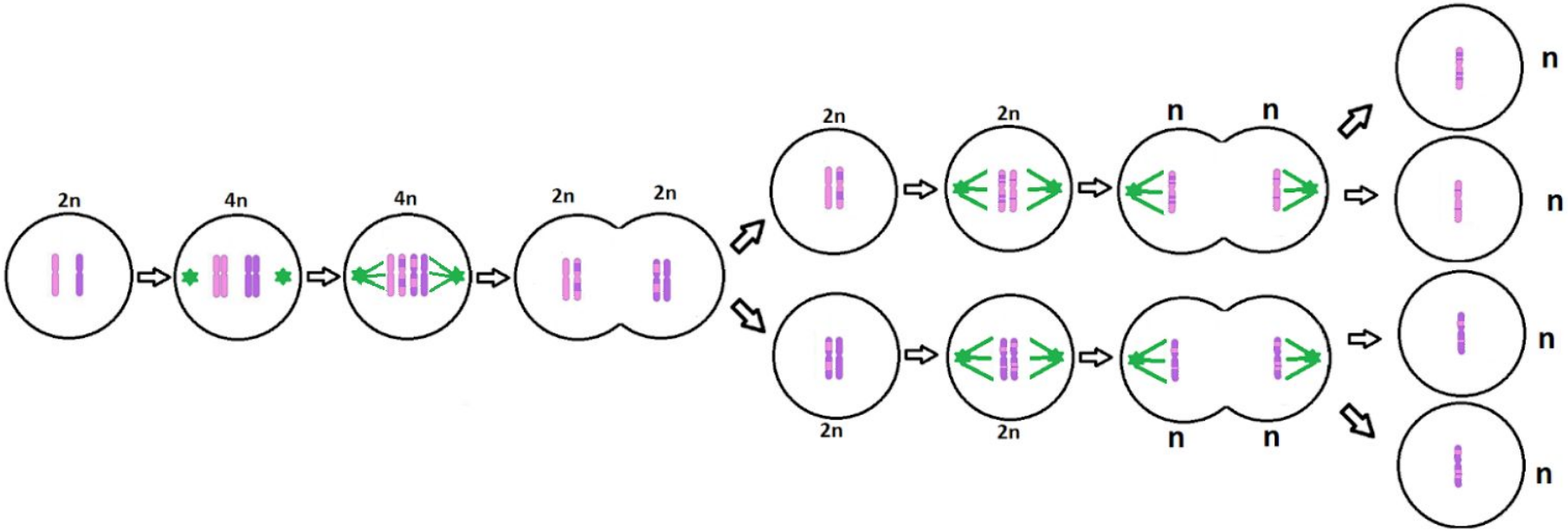
Creating Tetraploids

- Mitotic microtubule inhibitors cause genome duplication due to inability to pull apart cells.
- Oryzalin and colchicine disrupt microtubule function
- **Treating plants with these compounds will produce tetraploids**



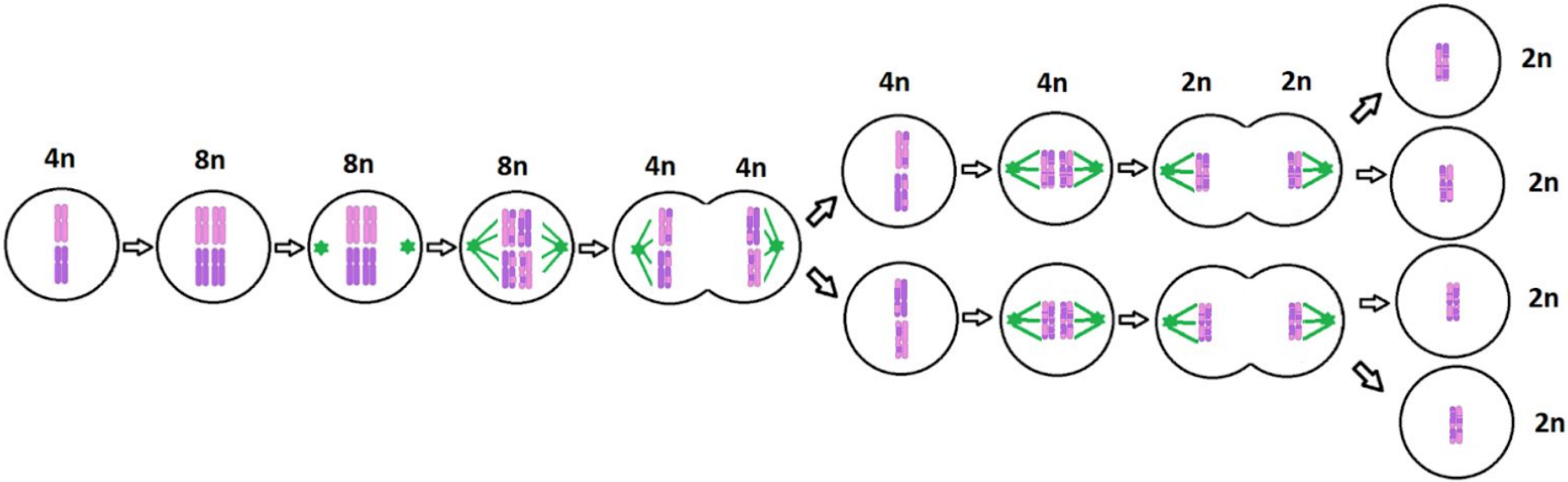
Genetics 101- Meiosis

Meiosis in a diploid results in 4 haploid gametes

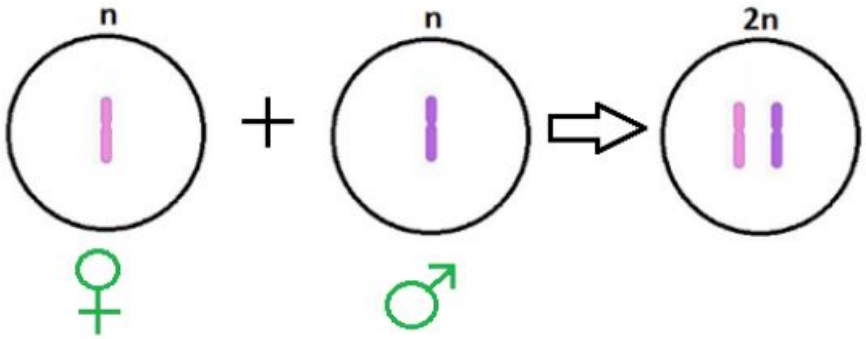


Genetics 101- Meiosis

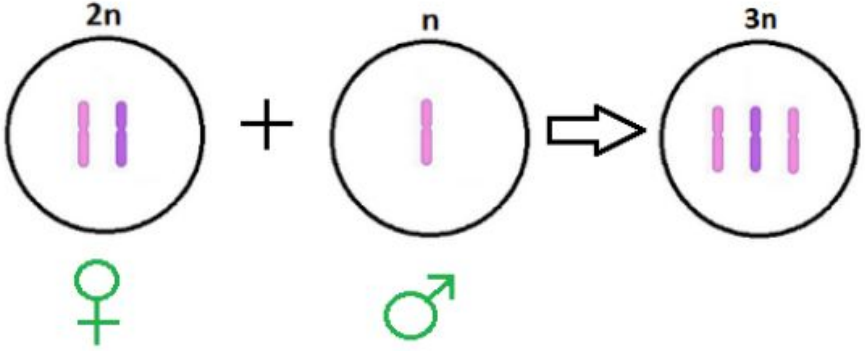
Meiosis in a tetraploid results in 4 diploid gametes



Creating Triploids



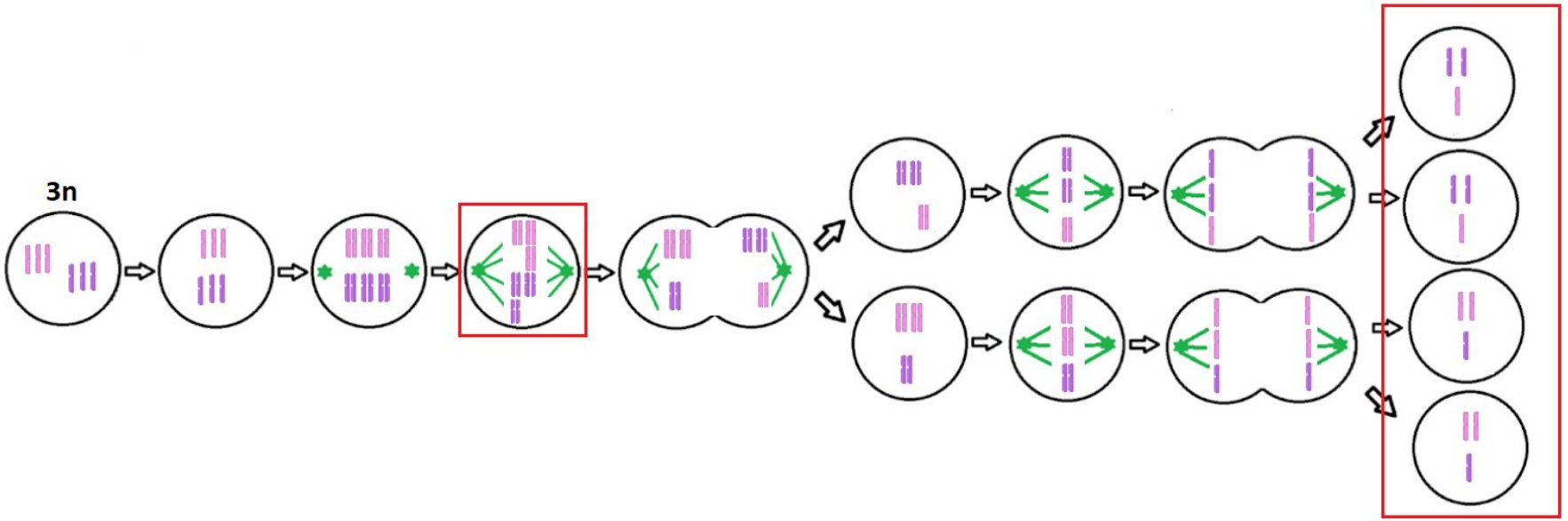
Haploid gametes combine to form diploid embryo



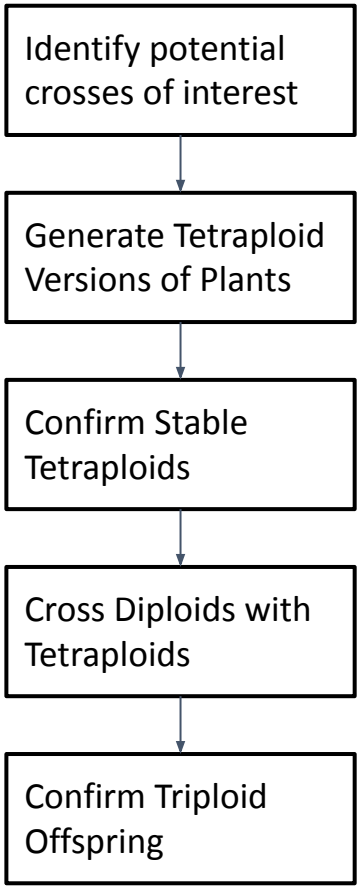
Diploid gamete and haploid gamete combine to form triploid embryo

Why Are Triploids Sterile?

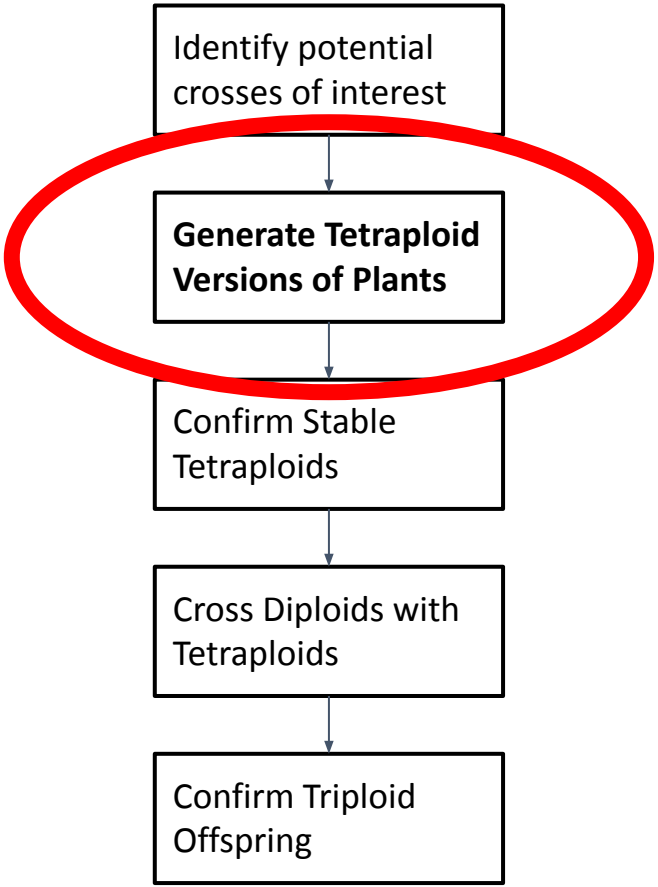
Odd numbers of chromosomes are unable to pair properly
Abnormal Gene dosages abort development



Putative Triploid Breeding Program:

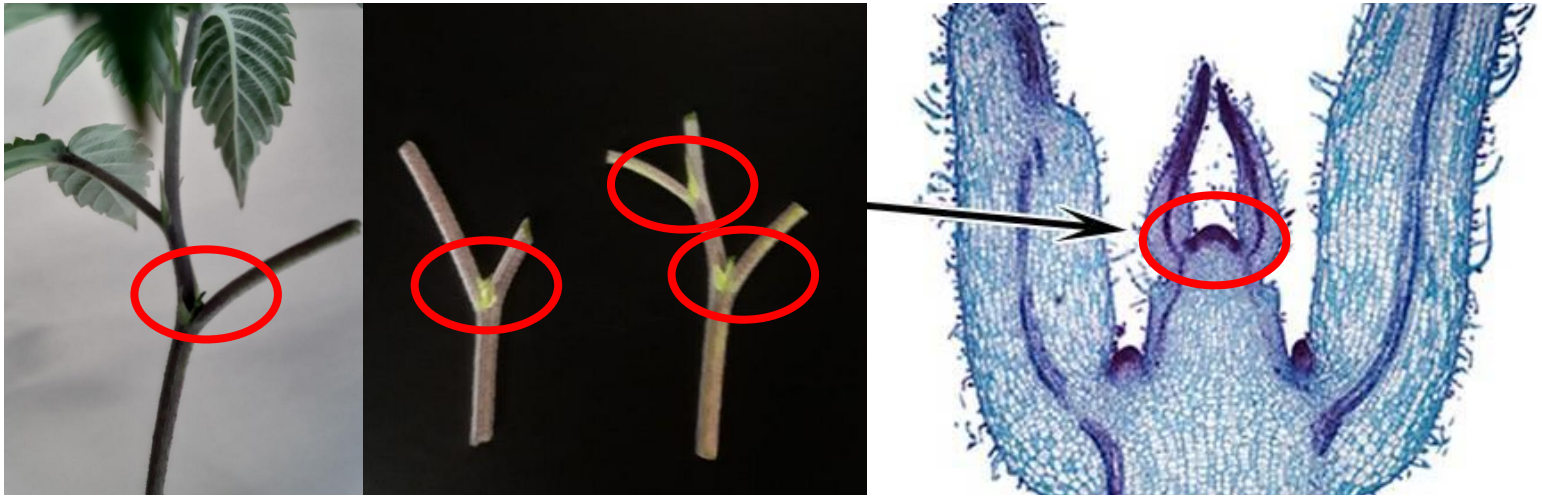


Triploid Breeding Program:



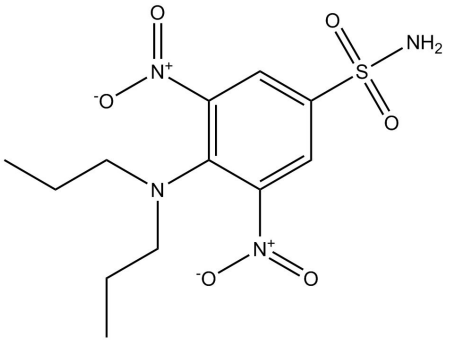
Creating Tetraploids

- Target Meristems
  - Areas of cell division ∴ undergoing mitosis

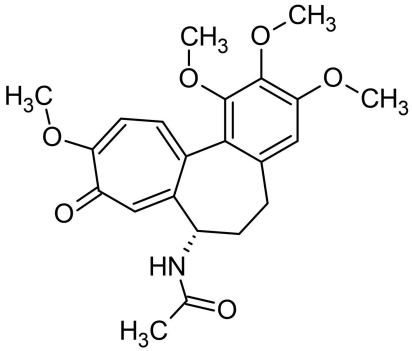


Creating Tetraploids

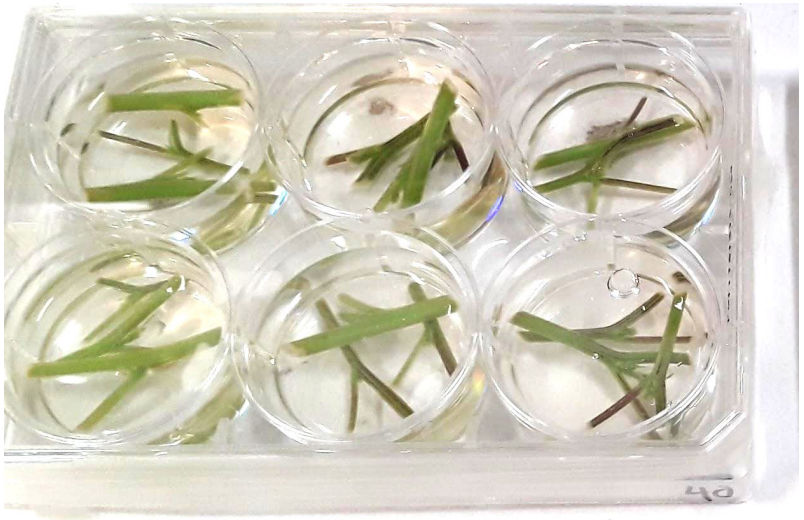
- Apply Oryzalin or Colchicine to meristems
- Keep in darkness for 24-36 hours
  - Approximate cell cycle length



Oryzalin

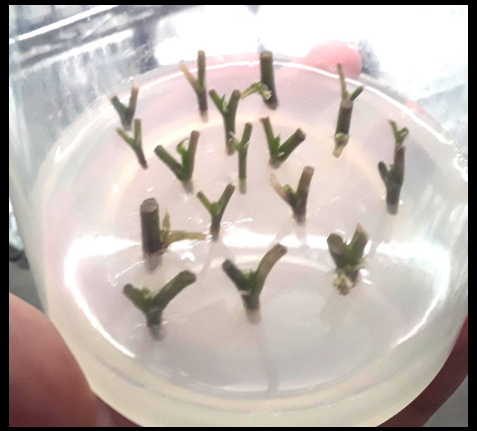


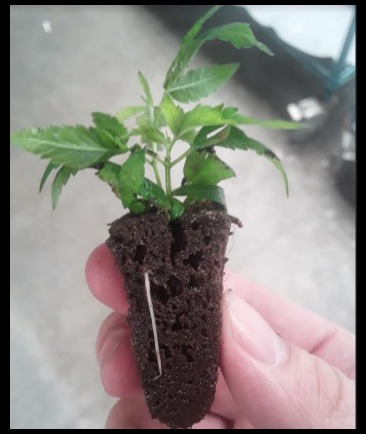
Colchicine



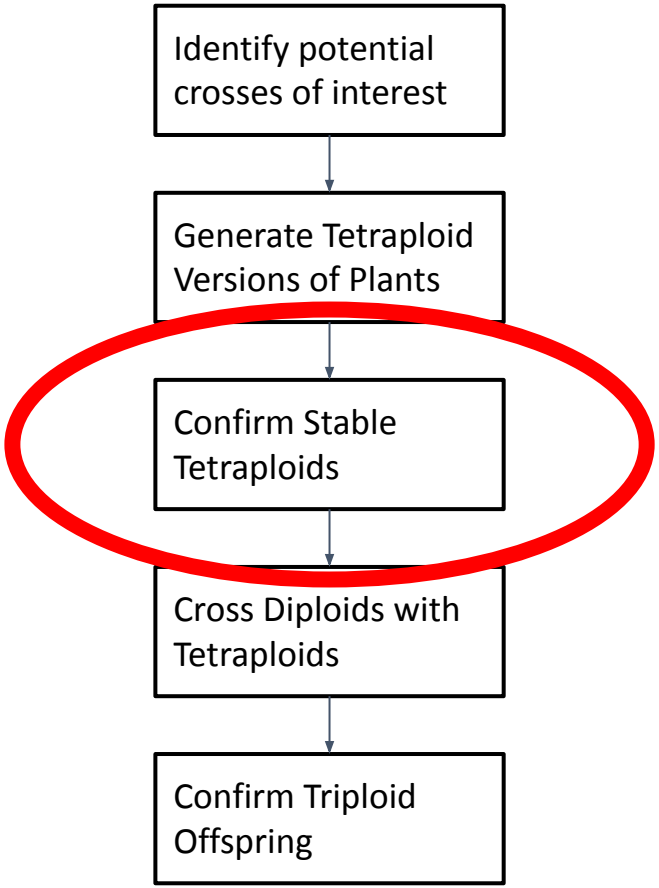
Creating Tetraploids

- Regenerate plants in tissue culture for 3-6 months
- Root and acclimatize plantlets when ready



Triploid Breeding Program:



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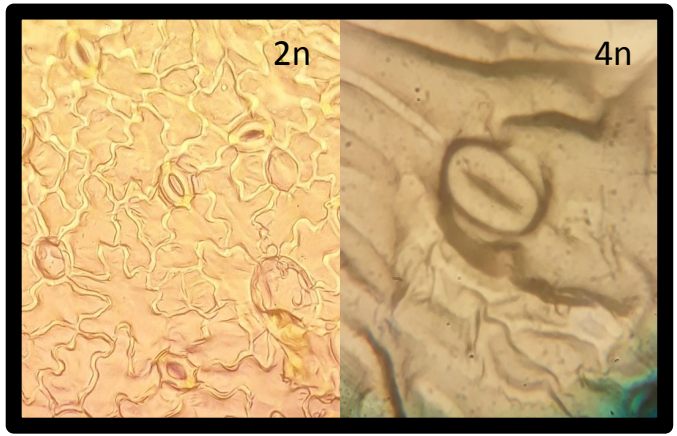
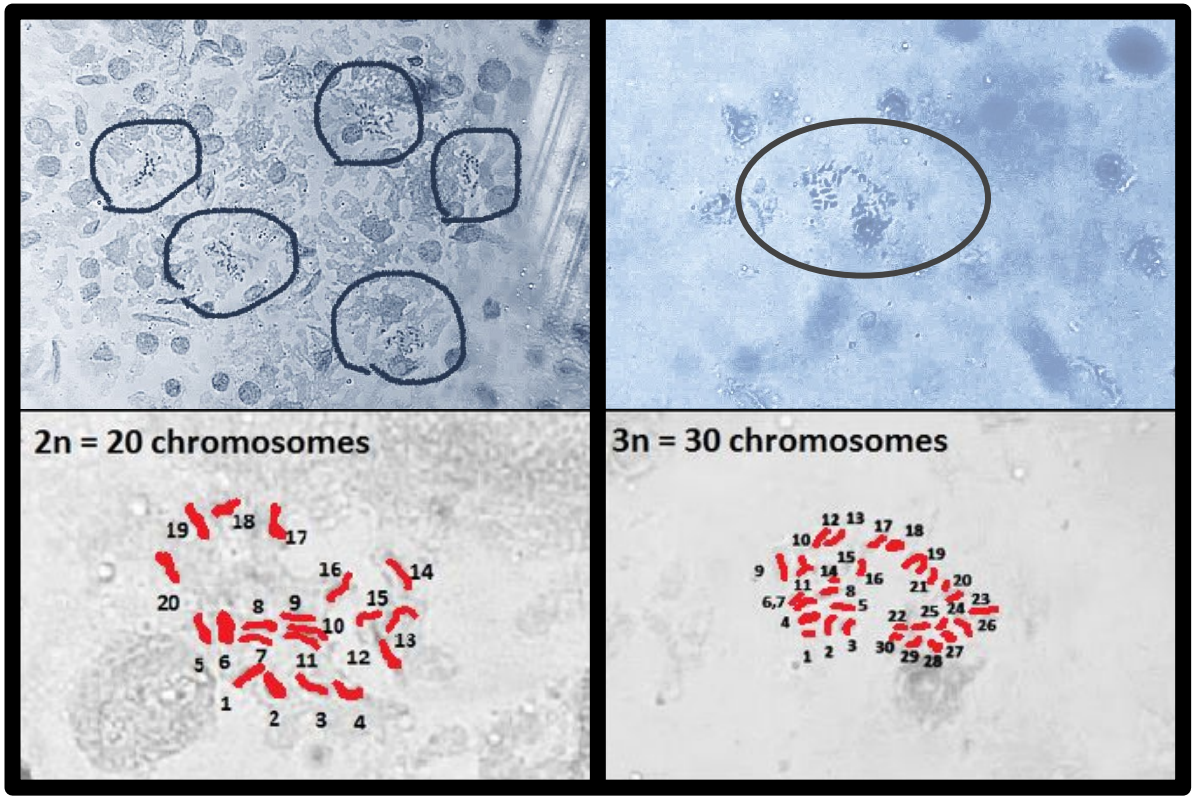
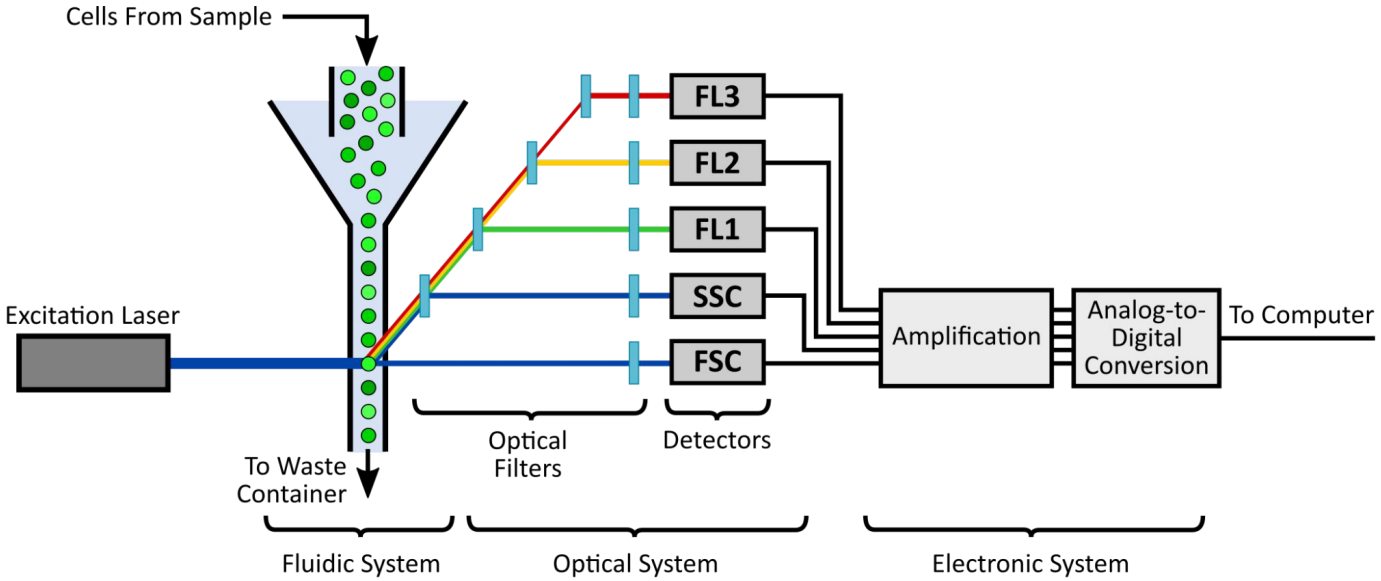
Measuring Ploidy - Stomata Size and Chromosome Squashes





Measuring Ploidy - Flow Cytometry

- Flow cytometers pass nuclei/cells individually through the path of a laser



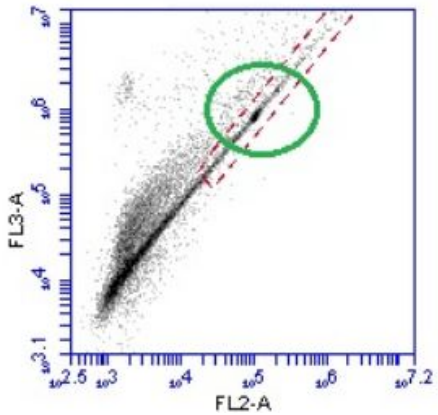
Measuring Ploidy - Flow Cytometry

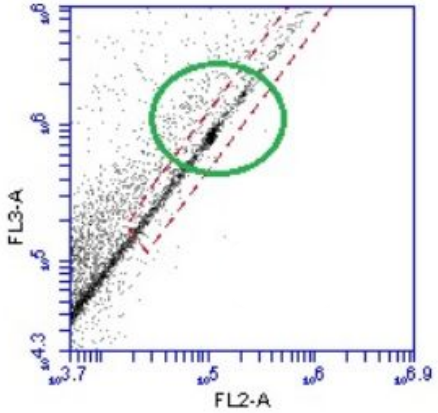
- DNA stained with a dye fluoresces when excited by the laser, and intensity of light is measured
- Nuclei with larger genomes will produce more fluorescence, therefore ploidy can be determined

Becton-Dickinson
Accuri C6 Flow Cytometer

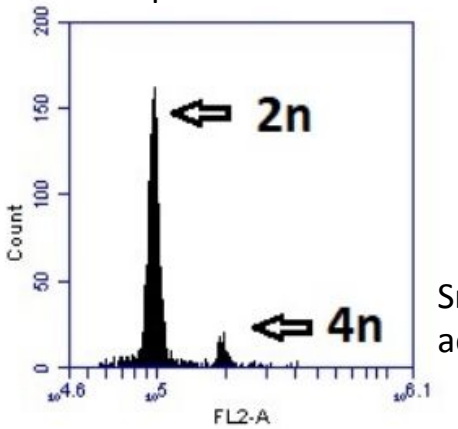
Developing Flow Cytometry Protocol

- Chop leaf tissue in nuclei extraction buffer
- Filter out particulate, treat with RNAse
- Stain DNA with propidium iodide
- Analyze via flow cytometer



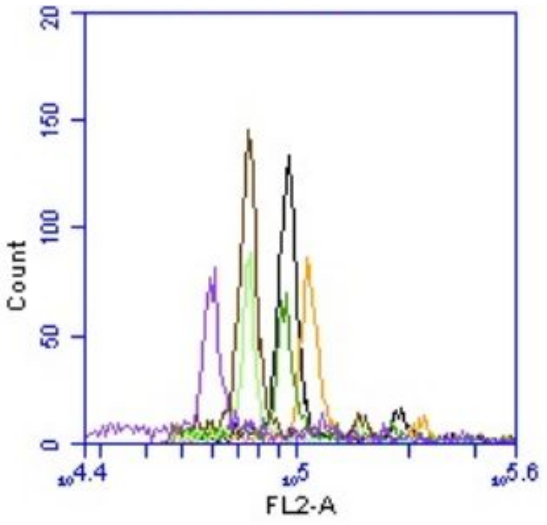


Diploid C. sativa

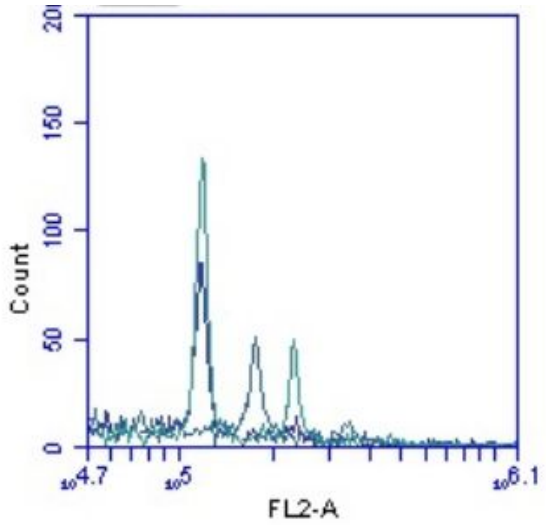


Small 4n peak = actively dividing cells

Variation Between Samples Using Flow



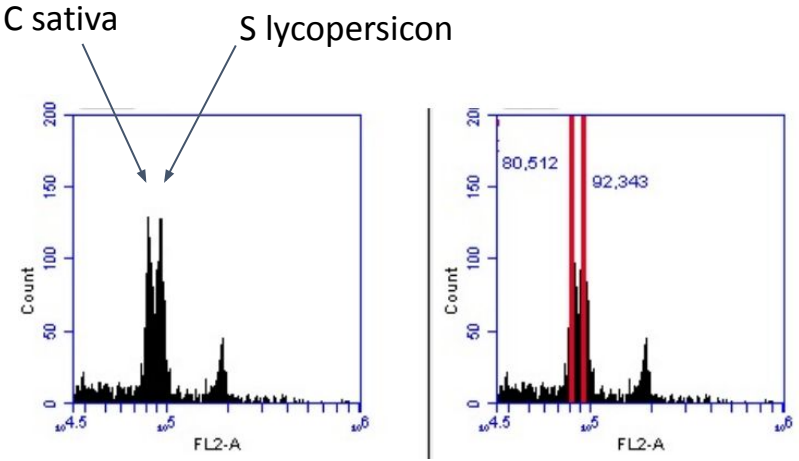
Diploid plant variation



"Faux triploid"

Results compared between samples were not consistent

Using Internal Controls as Reference



Comparing peaks within a single sample

Tomato Genome = 900MBp
Cannabis Genome = 810MBp

900/810 = 1.1 times bigger

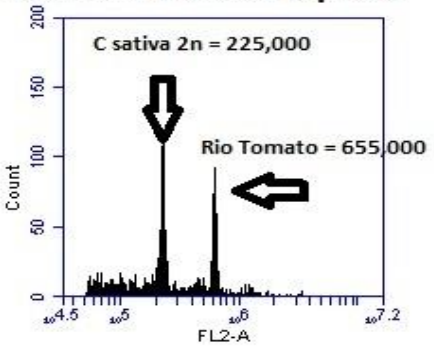
92,343/80,512 = 1.1

We are able to compare unknown genomes with known genomes by co-chopping together in a single run.

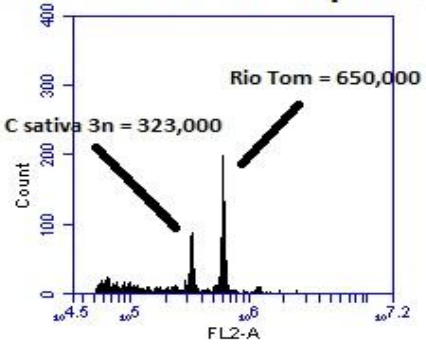
Using Internal Controls as Reference

- Use “Rio” tomatillo variety as internal control
- Rio genome size ≈ 3x that of diploid C. sativa, or about 6n

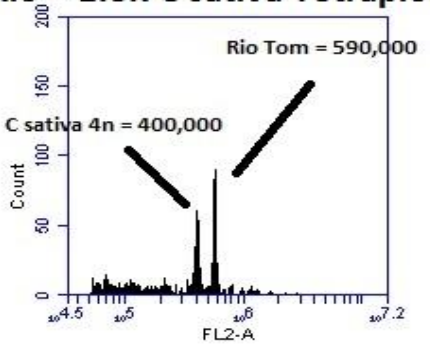
Rio = 3x C sativa Diploid



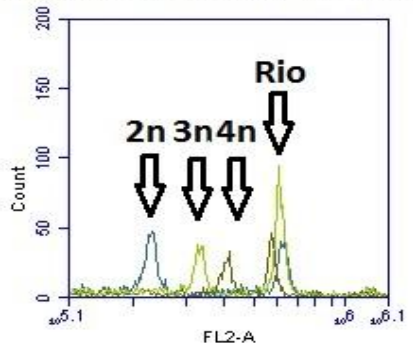
Rio = 2x C sativa Triploid



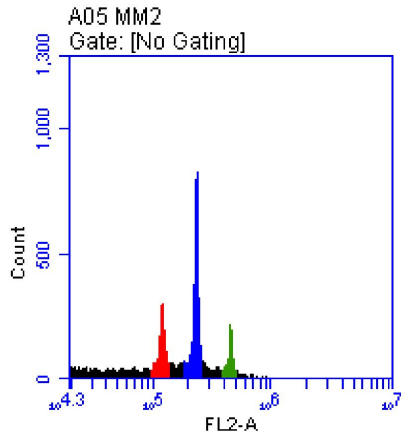
Rio = 1.5x C sativa Tetraploid

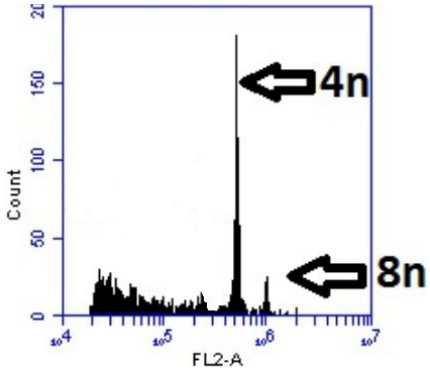


Overlay of 2n, 3n, 4n with Rio



Results of Oryzalin Treated Plants





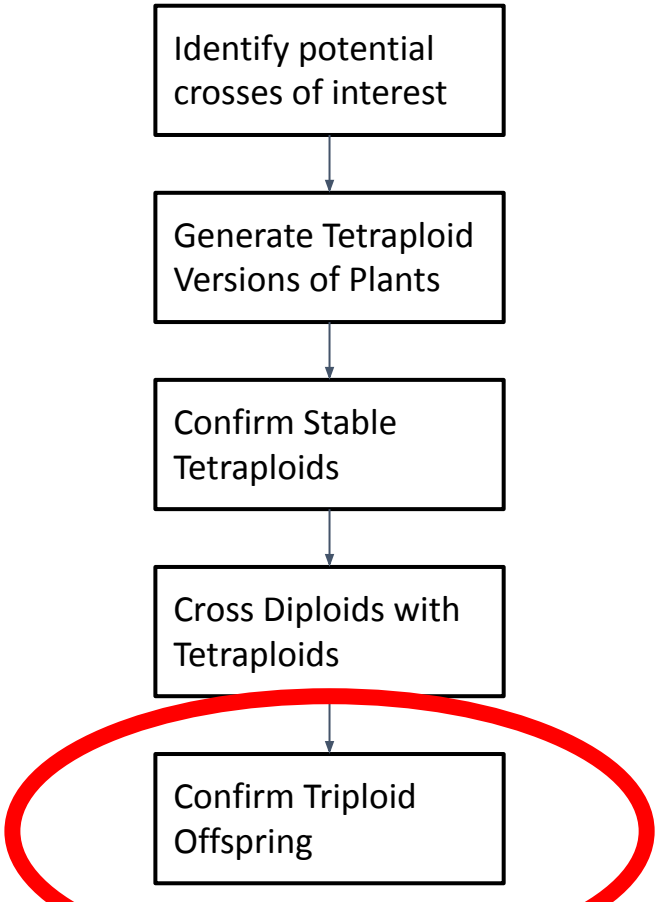
Most Samples Mixed Polyploid

~10% of treated plants fully tetraploid

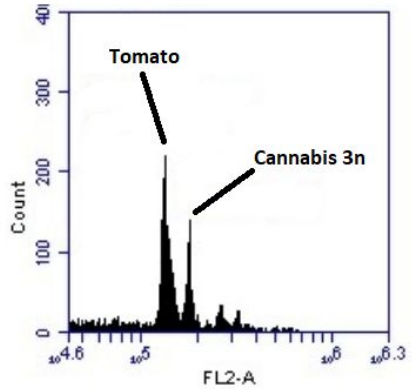
Behavior of Polyploids

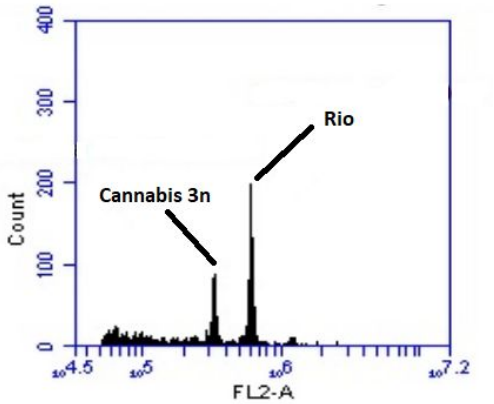
- Triploids/Tetraploids have slightly wider leaves, virtually no change in vegetative growing behavior
- Hexaploids and greater show significant change in morphology

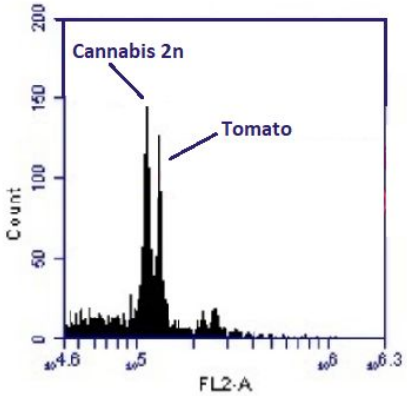
Triploid Breeding Program:

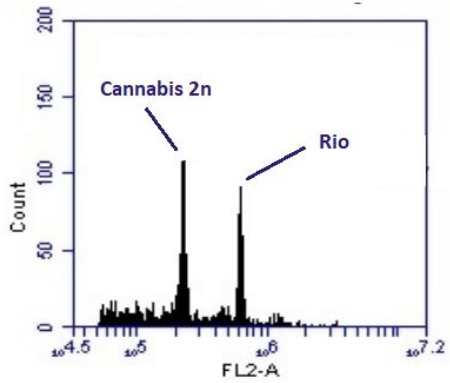


Confirming Triploid Plants



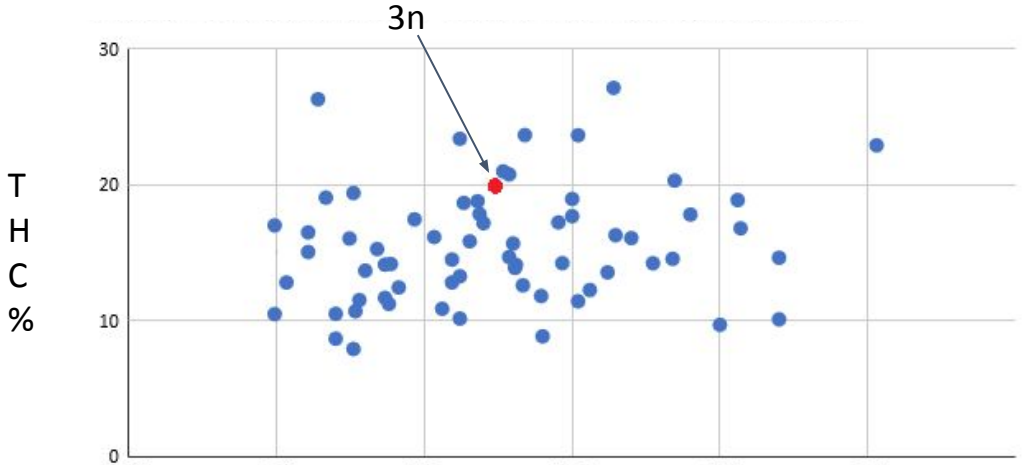






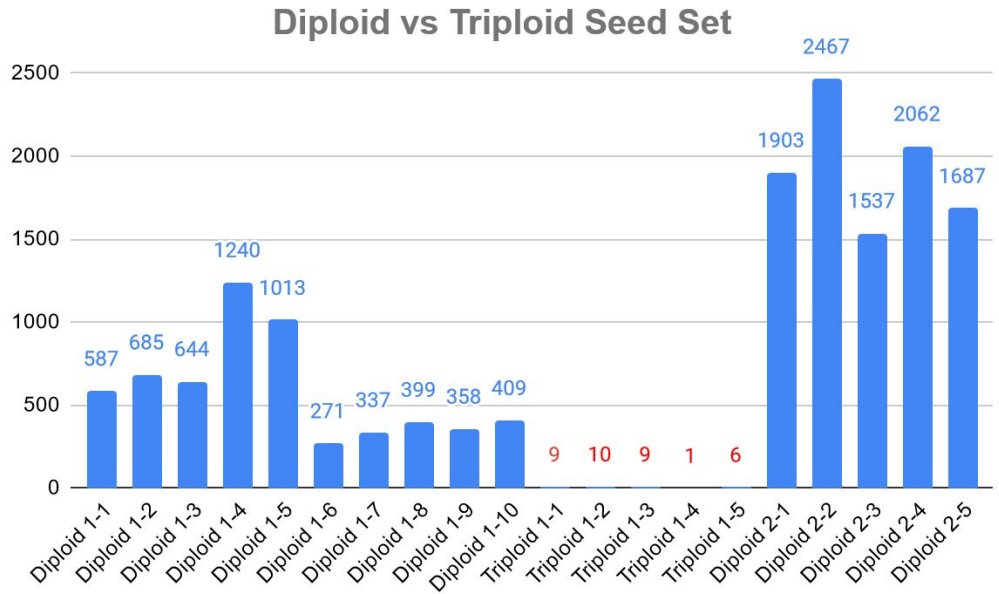
Triploid Behavior

- In the process of field testing sterility/yield/cannabinoid % compared to diploid varieties
- Current data does not control for strain differences



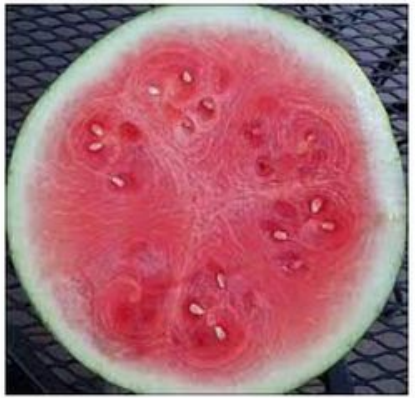
Sterility

- Triploid and diploid plants were both intentionally pollinated with the same batch of pollen



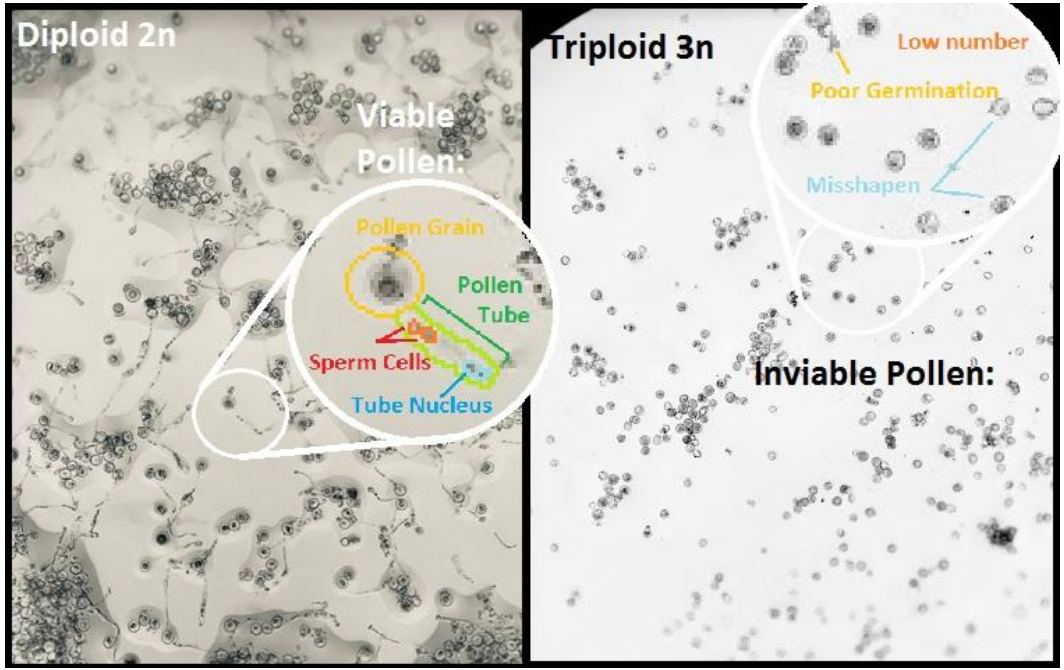
Sterility

- Triploid plants made virtually no seed
- Seed that was formed was mostly non-viable
- Flow cytometry suggests these seedlings are aneuploid



Sterility

- Triploid and diploid plants were both masculinized using same silver thiosulfate spray
- Triploid pollen did not germinate, was low in number
- Diploid pollen was numerous and germinated well



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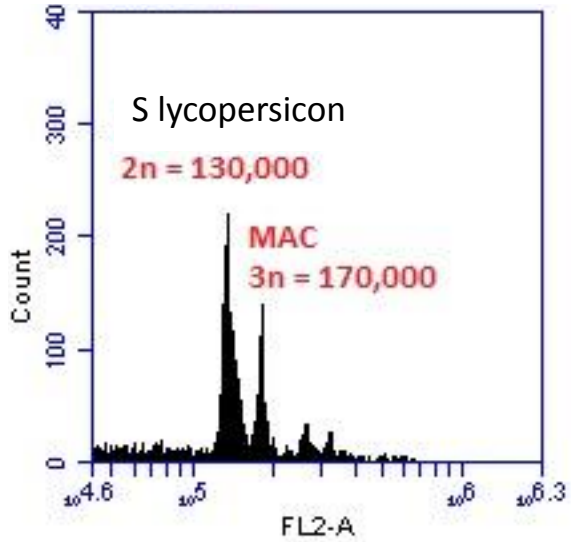
Natural Polyploidy/Is this GMO?

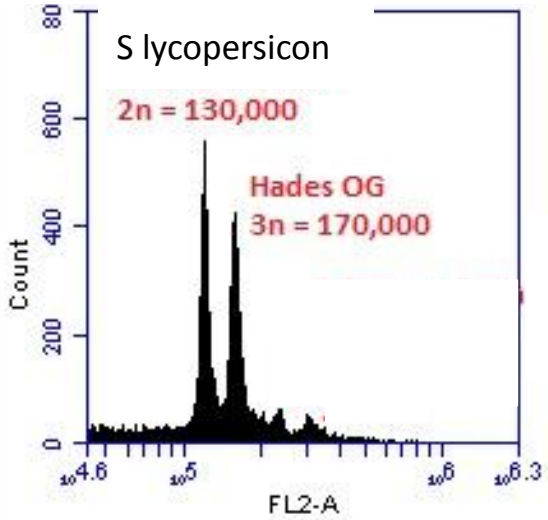
MAC1 is a very popular clone-only connoisseur strain

Hades OG picked as pheno winner out of thousands of plants in 2016

Natural Polyploidy/Is this GMO?

- Both created via conventional breeding methods
- Further breeding efforts were unsuccessful
- **Flow cytometry revealed both strains are naturally triploid**





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Thank you

Dark Heart Lab Team

- Jeremy Warren PhD - Director of Plant Science
- Kay Watt PhD - Head Breeder
- Max Vetterli - Lab Manager
- Lab Members:
  - Sydney Gerstenberg, Noah Shepherd, M McAdam, Sarah Thompson, Taylor Giamo, Daniel Belcher, Chris Atkinson
- Advisors:
  - John Yoder PhD, Bryce Faulk PhD, Ken Owens PhD, Swetha Kaul PhD

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