Cannabis Glandular Trichome Relevance to Harvest

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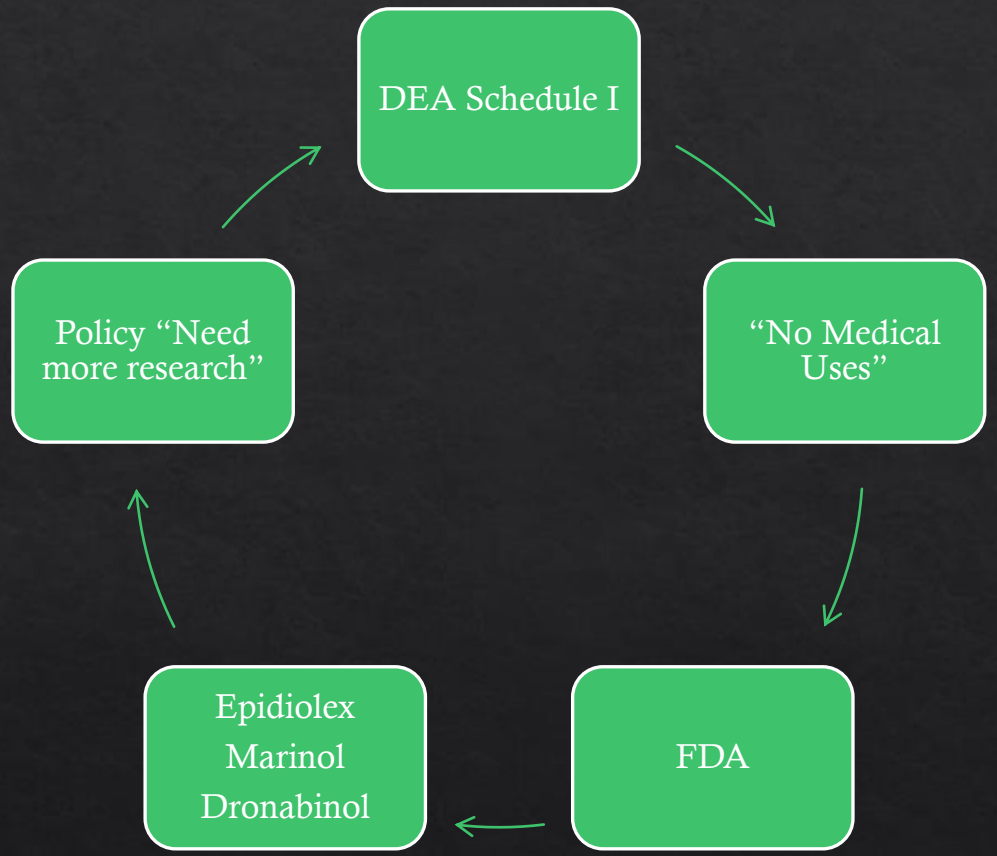
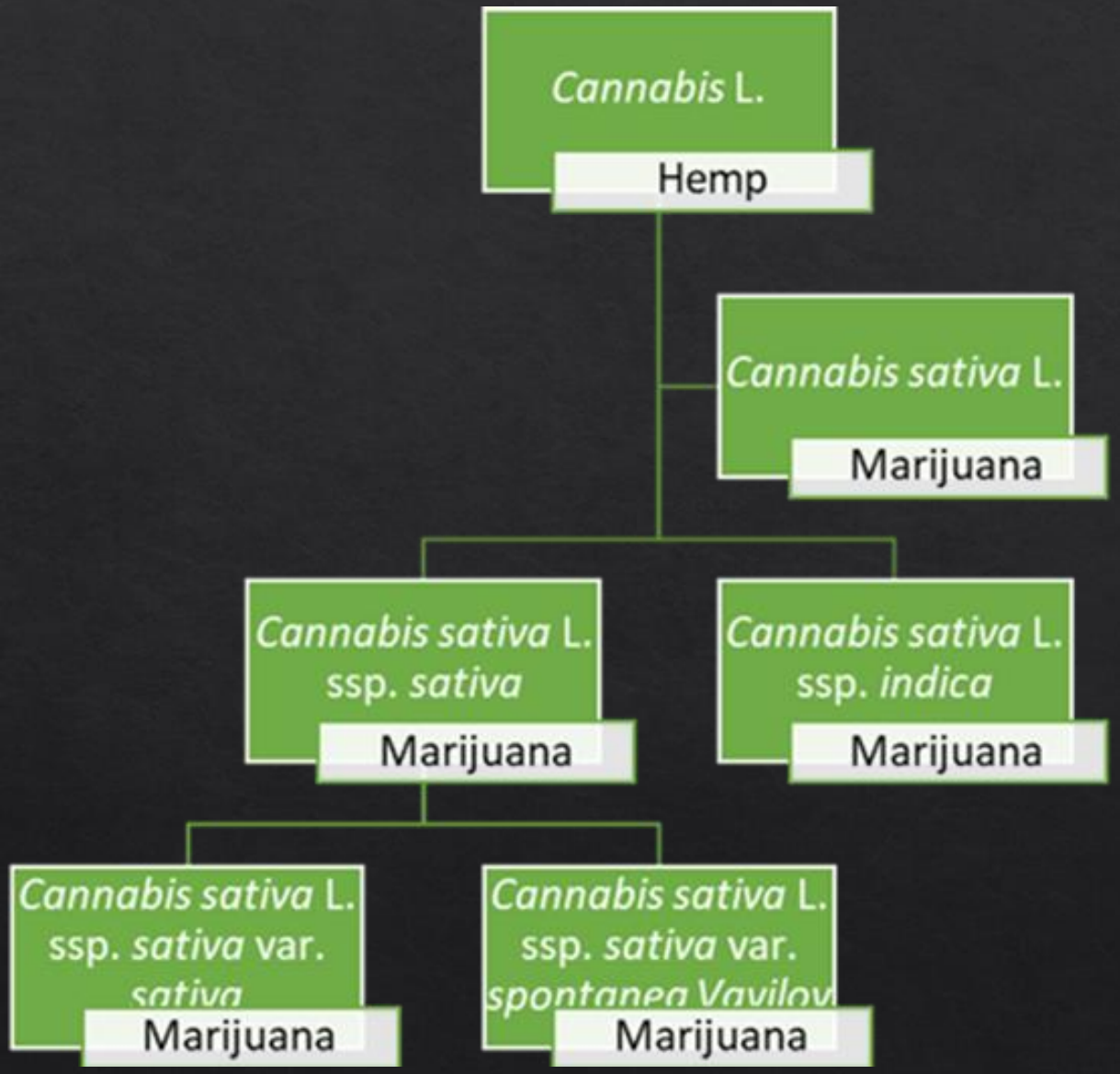
Research Gridlock



Cannabis research at academic institutions is significantly limited by the DEA Schedule I status.
This causes a "research gridlock" where more research is needed, but policy restricts research opportunities.



Taxonomy according to USDA Plants database Classification Report 2021.

Listed from top to bottom: Genus, Species, Subspecies, and Varieties of Cannabis.

Naming Issues/Lack of Best Practices

C. *Sativa* (1753)
Sativa=Cultivated

- Carl Linnaeus

C. *Indica* (1785)
Indica=India

- J. Baptiste-Lamarck

Basic Genetic Inheritance Principles (1865)

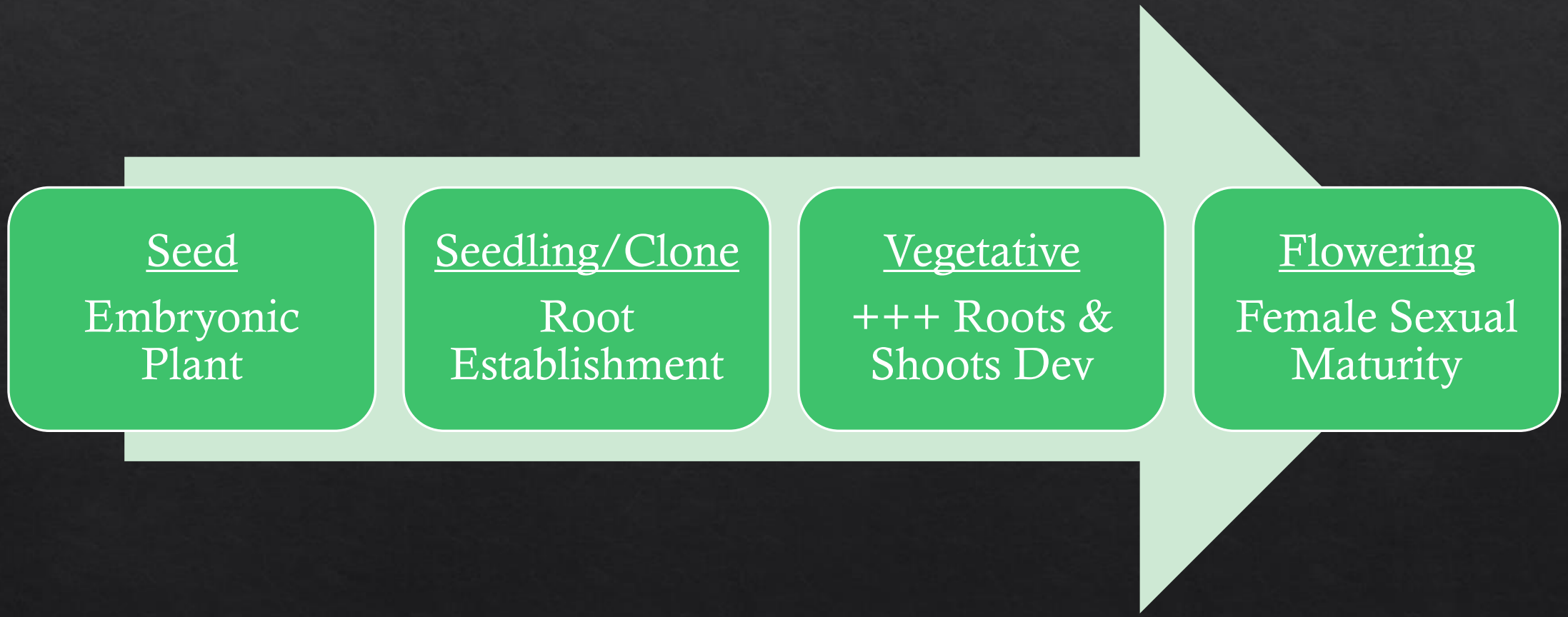
- G. Mendel

Strains names and indica/sativa designation are not consistently and reliably representative of secondary metabolite profiles

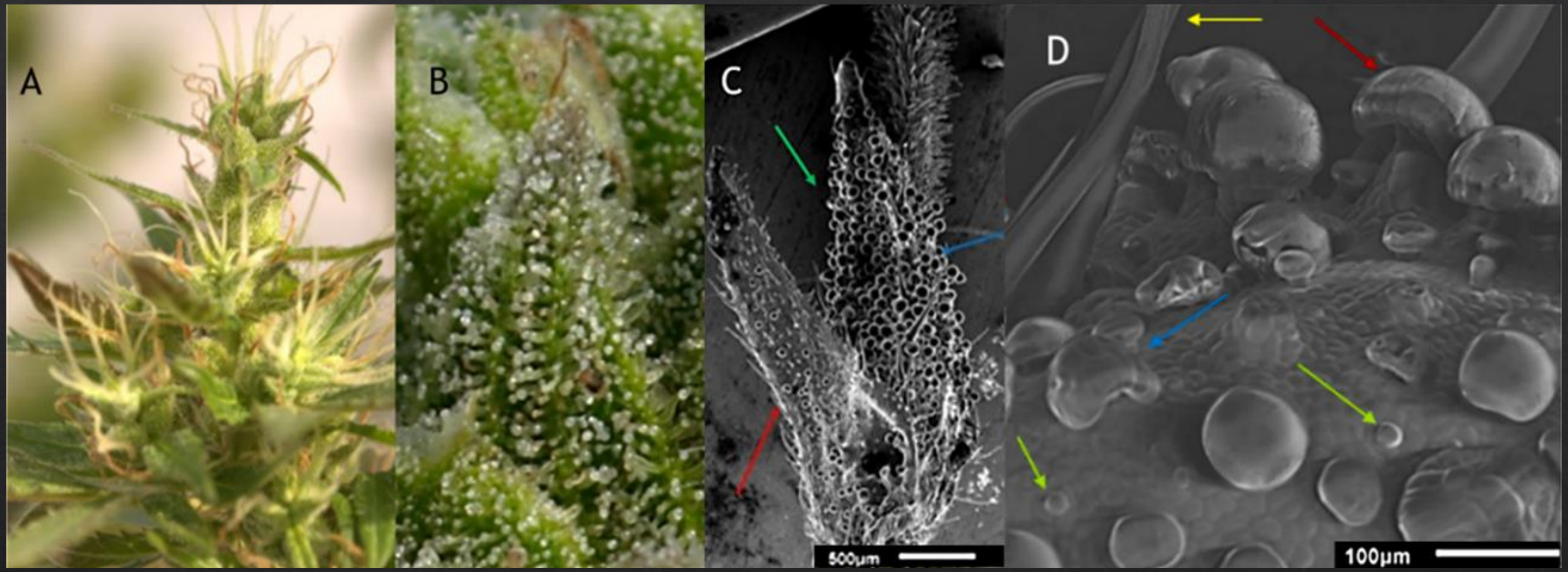
Problem

- Lack of Federal Best Management Practices
  - Field Plan
  - Cultivation
  - **Harvest**
  - **Post-Harvest & Storage**
  - *Protection for heirloom and landrace cultivars
- Industry Standard of Trichome Analysis (Subjective Analysis Method)
  - Light Microscope/Jewelers Loupe
  - Inconsistent insight to Cannabinoid Concentration risk non-compliance
- Lack of insight into factors affecting secondary metabolite production (pre & post harvest)
  - Genetics
  - Nutrients
  - Environment
- Cost effective in-house R&D
  - Lack of affordable and reproducible insight into cannabinoid acid levels in living plants prior to harvest and decarboxylation
  - Risk non-compliance
- Inconsistent quality products for end consumers
  - Decrease in demand, surplus in product, destabilizes market

Basic Life Cycles



Fiber, seed, and grain cannabis are not grown for trichome production. Male floral plants used for breeding.



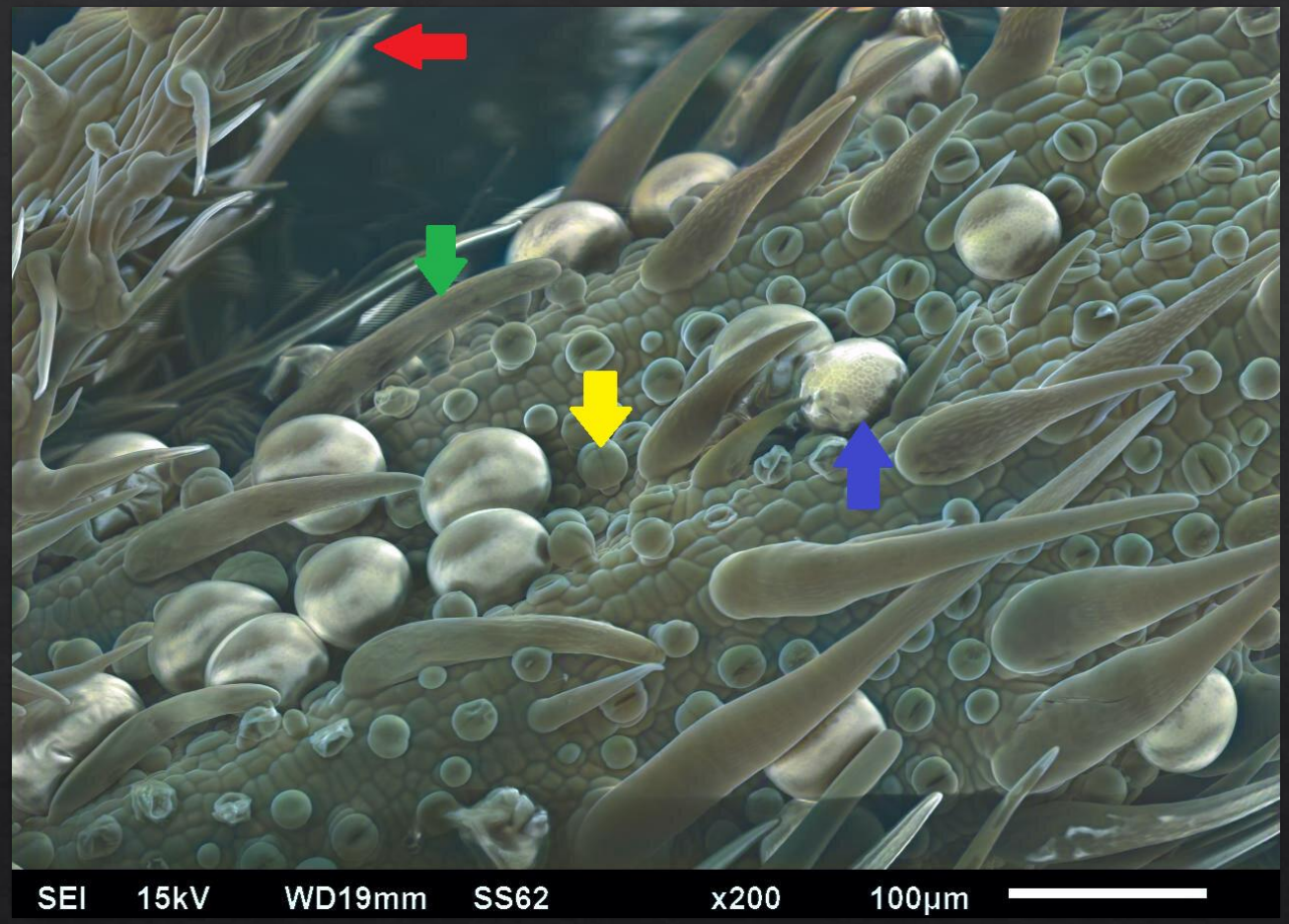
(A) Complete inflorescence (Stalk, leaves, and flowers) of sexually mature female Cannabis sativa L. (B) Zoomed image of trichome covered bract with amber style protruding. (C) Scale bar 500 µm; Red: Bract, Green Calyx, Blue: Stigma covered style. (D) Zoomed imaged of various trichomes covering calyx surface. Scale bar 100 µm; Red: Stalked Glandular Trichome, Blue: Sessile Glandular Trichome, Green: Bulbous Glandular Trichome, Yellow: Non-Glandular Trichomes.

Trichomes

Non-glandular ≠ Metabolites
- Physical barrier

Glandular = Metabolites
- Glandular Head (Specialized cells)
  - Synthesis
  - Storage

- Bulbous, Sessile, Stalked
  - Position
  - Morphology
    - Gland size
    - Gland Cell Count
    - Stalk/No Stalk
  - Mono/Sesquiterpene



Red: Style with protruding stigma
Green: Non-glandular trichome
Yellow: Bulbous glandular trichome
Blue: Sessile-glandular

- Trichomes purpose is to provide physical and/or chemical means of resistance and/or assistance to both biotic and abiotic environmental stressors

- The growth and development of glandular trichomes as well as the production of secondary metabolites are highly influenced by the environment

Cannabis Grows Like a Christmas Tree

Unlike other plants cultivated for flowers or fruit, morphological uniformity is highly desirable

Hypothesis

1. Density of trichomes will vary significantly across floral clusters retrieved at various nodes of the same plant.
2. Cannabidiol acid (CBDA) concentration and quantity (density) of trichomes will vary significantly across floral clusters retrieved at various nodes of the same plant.

HYPOTHESIS	Null	Alternative
Trichome Density	Significant variation of trichome density	No variation of trichome density
Cannabinoid Concentration	Significant variation of CBDA concentration	No significant variation of CBDA

*Local variation refers to the 3 sample retrieval locations per plant (Apical meristem, 50% of apical meristem height, and the lowest flowering node)

Sample Collection

7 Cultivars Sampled (1 Died)

- Greenhouse-Grown Federally Compliant Hemp

Sample Collection Date

- Nov 9 (Week 9 of Flowering)

3 Sample Location per Cultivar

- Tallest Flowering Stem
- 50% of Tallest Flowering Stem
- Lowest Flowering Stem

2 Samples Collected per Location

- Same Location; Adjacent Floral Clusters

*Cultivars intentionally anonymized. Each cultivar is represented by Plant ID. Sample represented by (1,2,3)

Research Layout

On-site floral cluster cannabinoid an analysis of samples

Cultivars Labeled: Plant A-G

2 individual calyx dissected from floral cluster

Calyces fixed to brass block, submerged in liquid nitrogen, and placed in SEM

Images saved to be analyzed for trichome morphology and ratio

Images uploaded into ImageJ

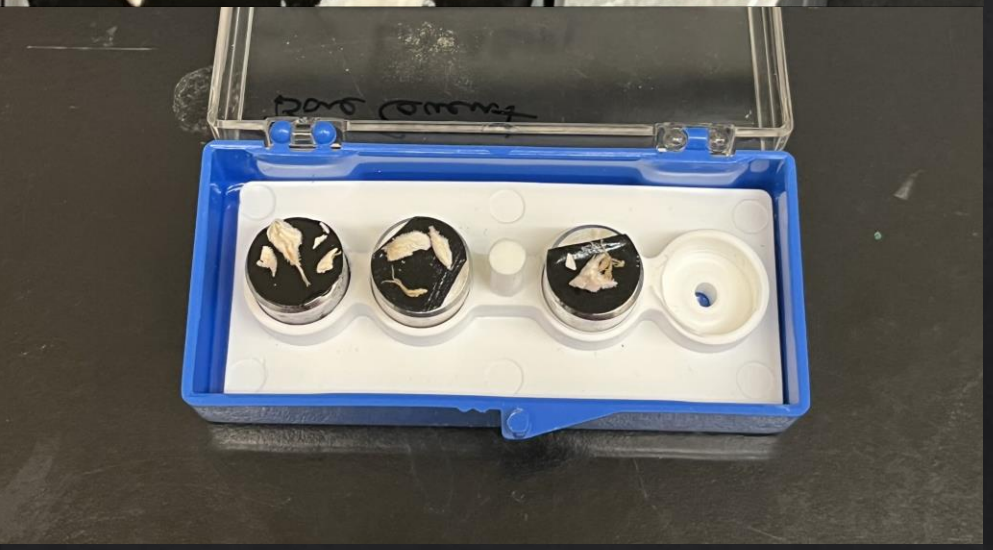
Trichome manually counted by morphology

Data entered in Excel

Excel data imported to DATAtab statical analysis software

Aims

- Aim 1: Compare Methanol-Ethanol fixation to Liquid Nitrogen Fixation
- Aim 2: Use Cryo-SEM to image individually dissected cannabis calyx.
- Aim 3: To analyze flower cluster samples for cannabinoid concentration and compare them to trichome density*.
  - *Cyro-SEM images were used to quantify glandular trichomes, measure calyx length, width, and area in samples collected from the apical meristem, 50% of the apical meristem, and lowest flowering node of sexually mature female cannabis plants



Post ME wash Calyx prior to imaging

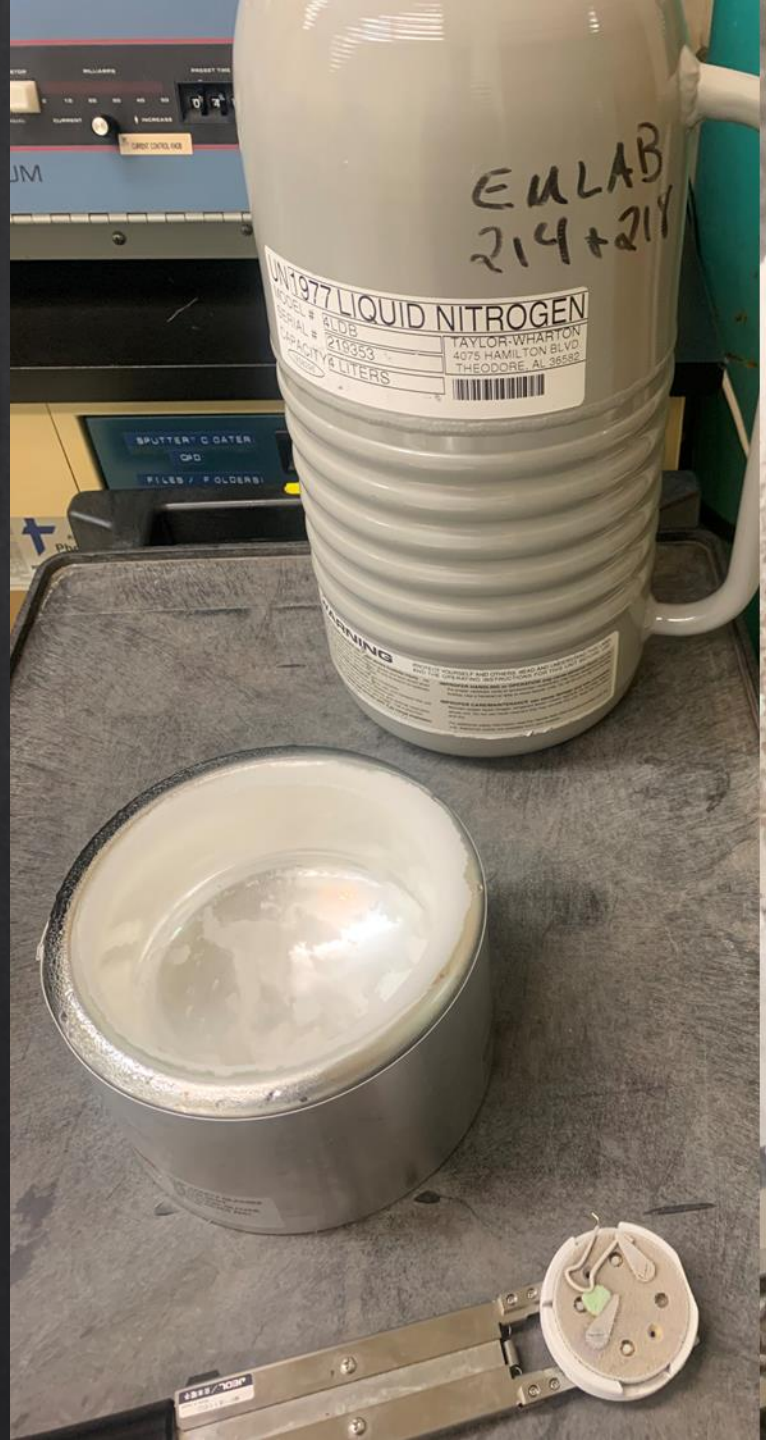
Aim 1 Methanol-Ethanol (ME)

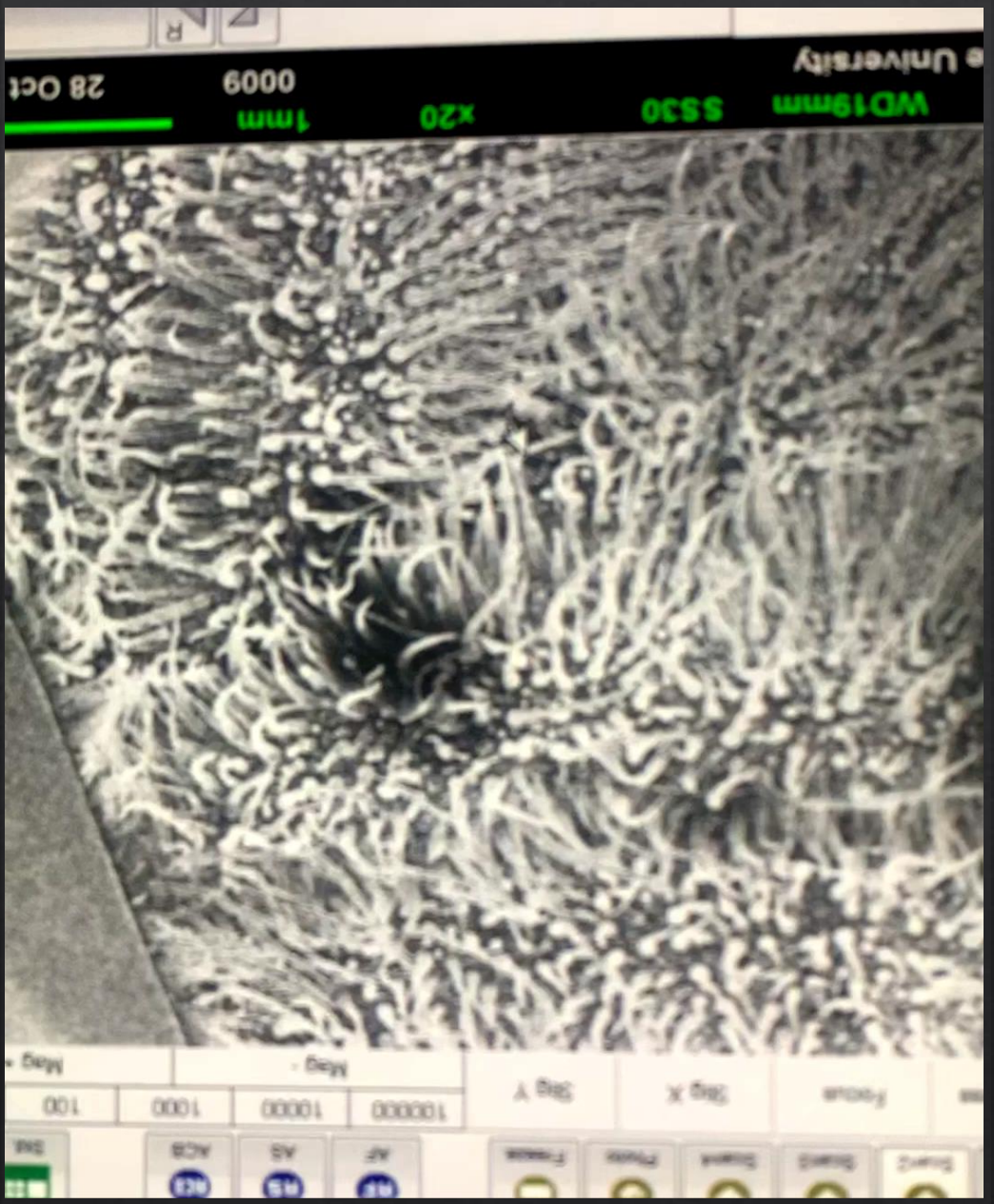
1. Individual calyx were placed in a vial submerged in 100% methanol for 10 minutes.
2. Samples were then removed from methanol and transferred to a vial containing 100% dry ethanol for 30 minutes followed by a 30 additional minutes in 100% dry ethanol.
3. To complete the drying process, each sample was subjected to critical point drying using the Bal-Tec CPD 030 Critical Point Dryer.
4. Individual calyx were then secured to a brass stub, secured to the SEM stub holder, and inserted into SEM for imaging.

Aim 1 Liquid Nitrogen

1. Calyx fixed to brass stub
2. Individual Calyx cryogenically frozen using liquid nitrogen
3. Brass stub inserted into SEM
4. Samples Imaged

*Image of single *Coleus ambonicius* for test run

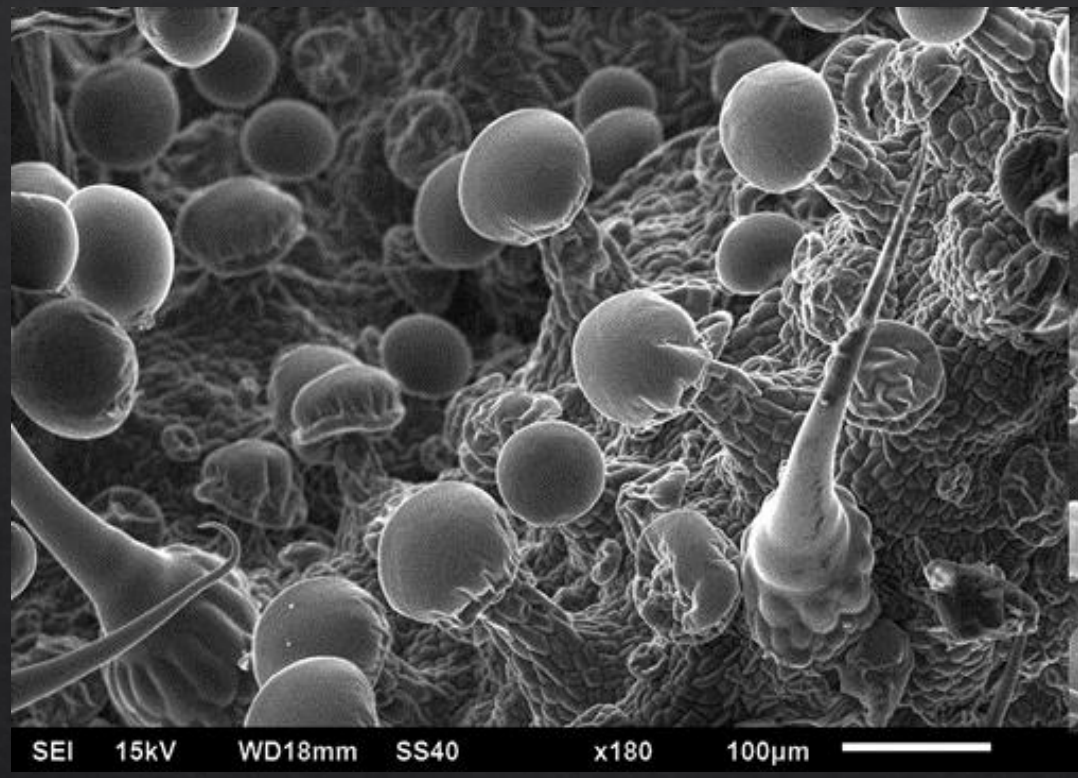




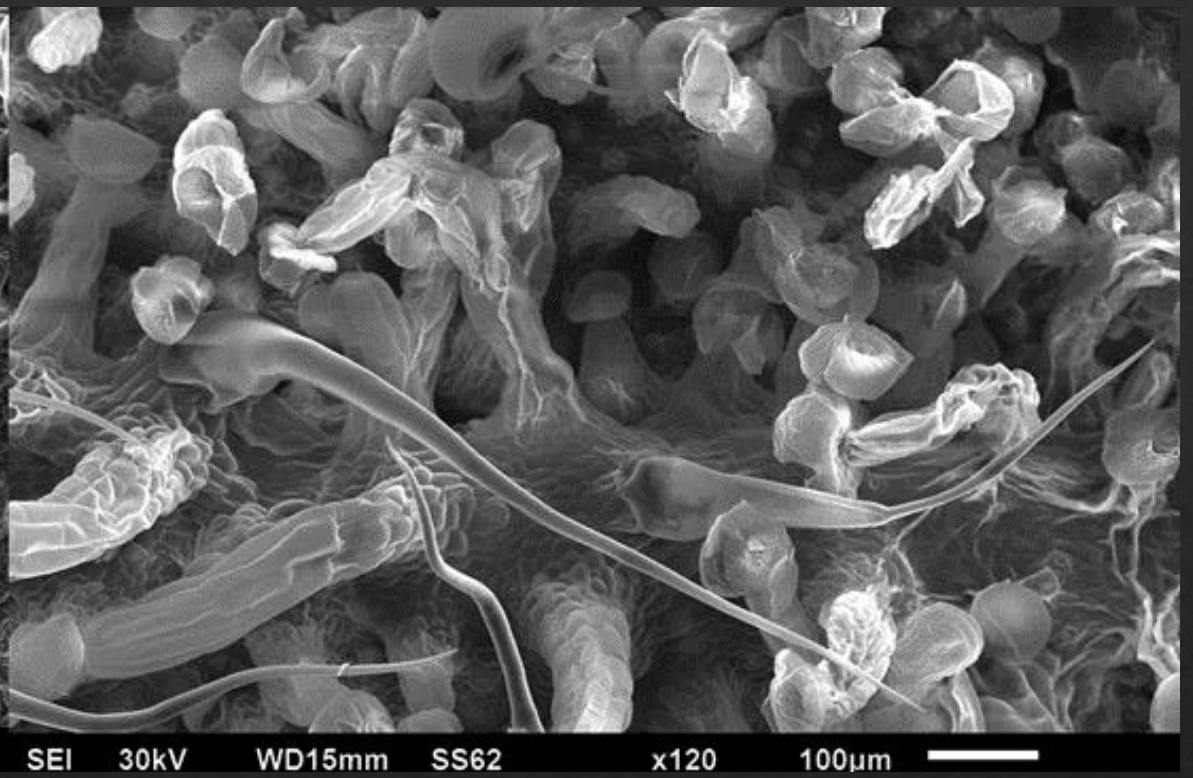
Navigating the SEM

- SEM Microscopes operate using electrons instead of light.
- SEM must operate under a vacuum; sample preparation required to remove water
- SEM is an extremely useful tool in analyzing trichome quantity and morphology
- However, SEM is an artform that requires practice to hone sample preparation technique

Liquid Nitrogen vs Methanol-Ethanol



Liquid Nitrogen prepared SEM image

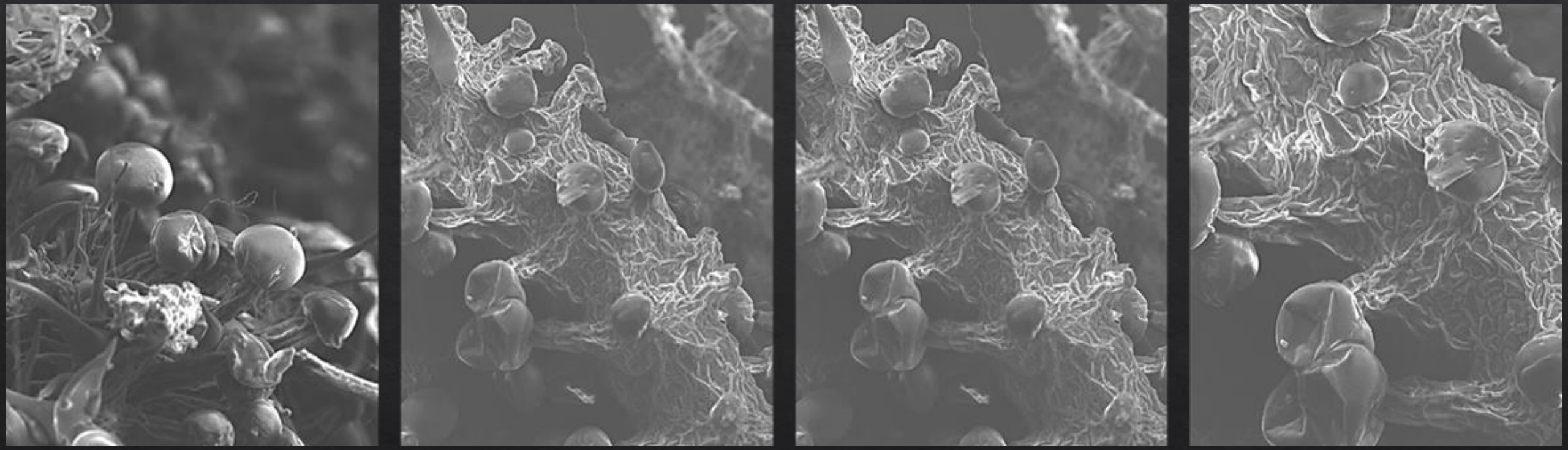


Methanol-ethanol prepared SEM image.

METHOD	FIXATIVE	DEHYDRATING AGENT	CRIT PT DRIED	PROS	CONS
Methanol-Ethanol	Methanol	Ethanol	Y	Sample can be saved/stored after imaging	Cell-Shrinkage Cell Tightening
Cryo	Liquid Nitrogen	N/A	N	Preserves plant cell shape/structures without water removal	Possible frost damage Sample destroyed after imaging

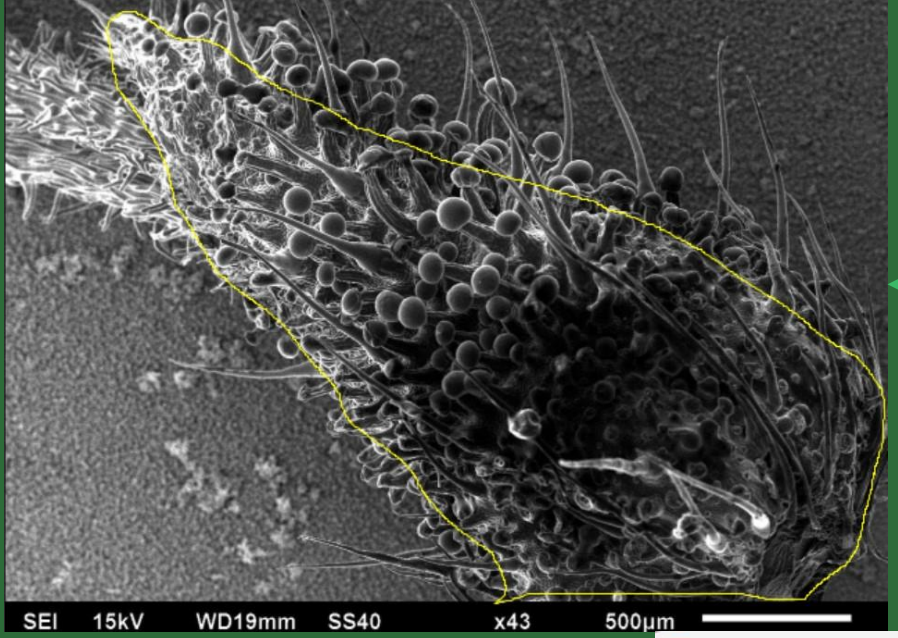
Table 1: Characteristics used to evaluate comparative methods

Industry Standard is to air dry prior to processing or packaging.



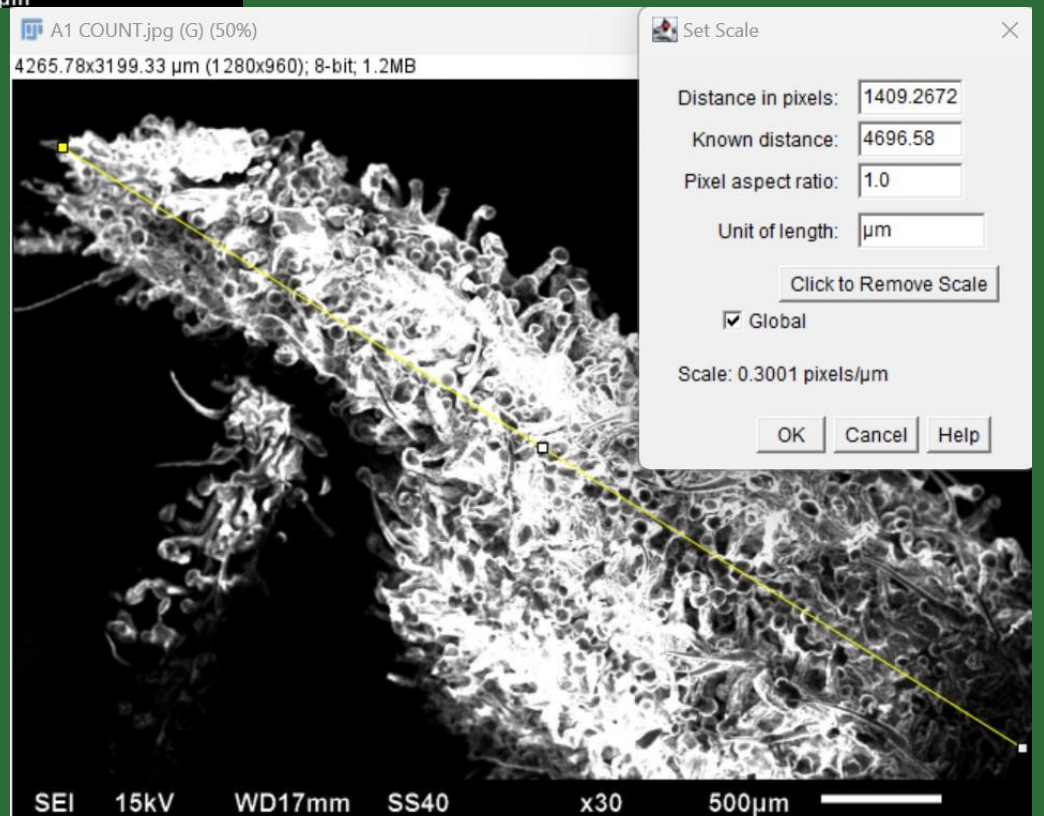
SEM Image showing visible morphological distortion of glandular trichomes in cannabis that has been dried. Glandular heads ap pear disturbed, resembling deflated balloons. Cell floor displays gross morphological changes.

Aim 2: Image Quantification



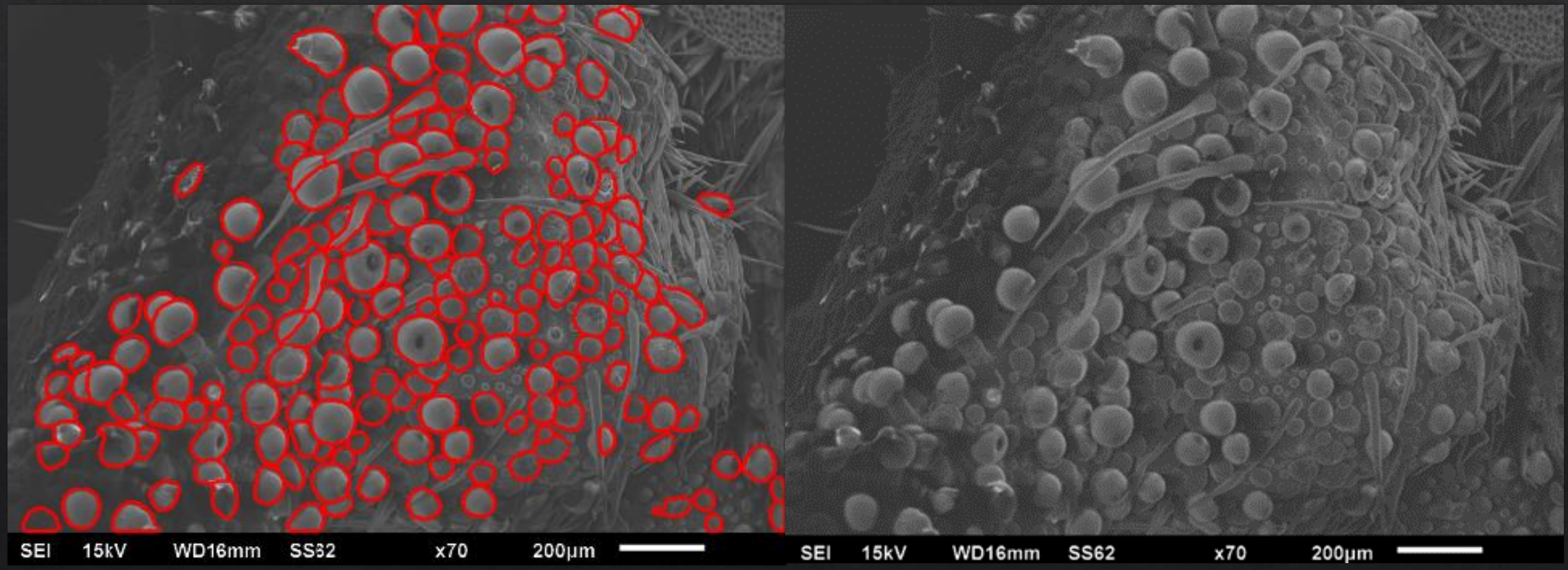
Calyx area calculated using known distance 500um scale bar

Image pixel scale ratio to um



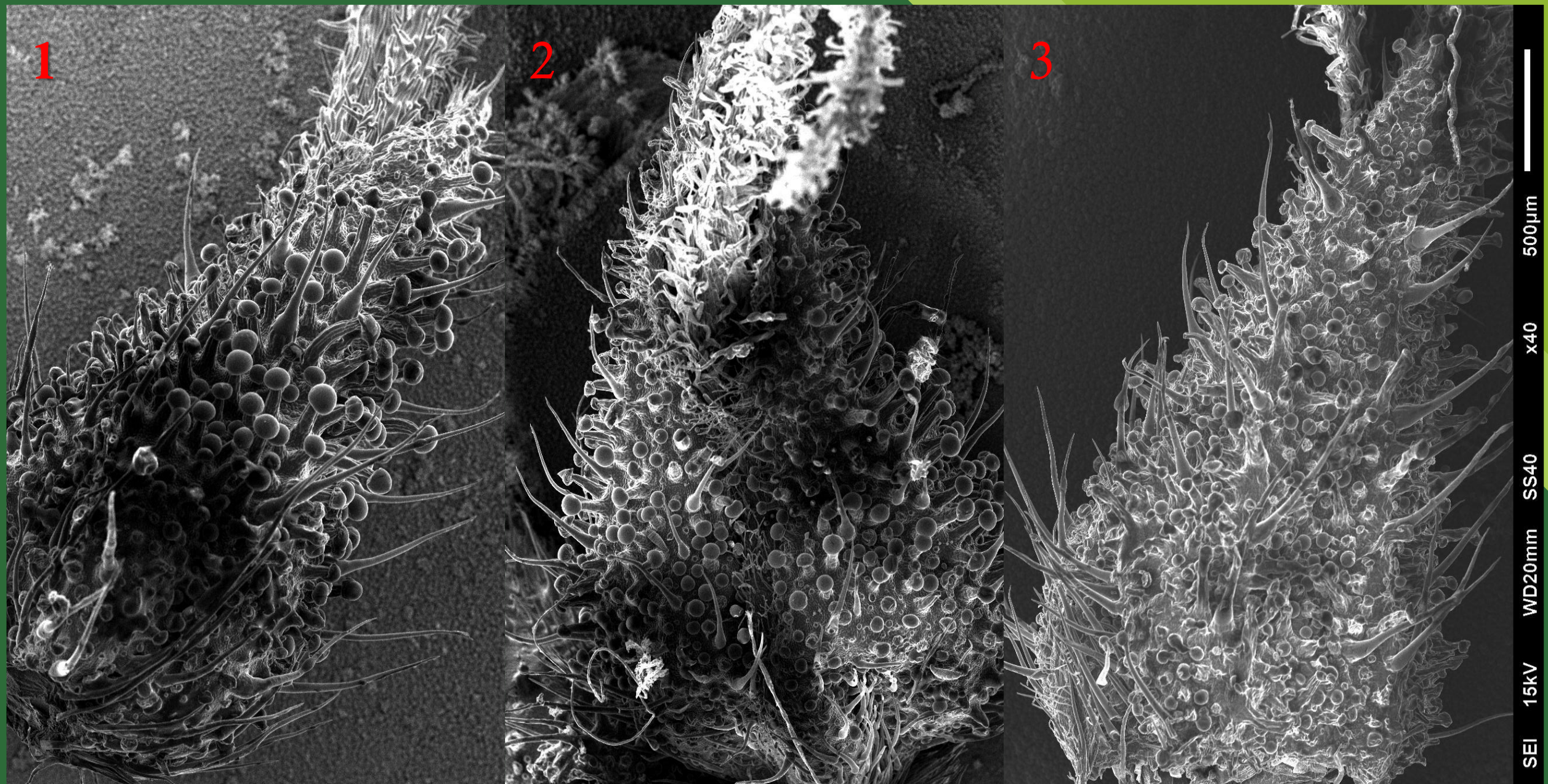
- Glandular Count: Total quantity of glandular trichomes counted per calyx
- Calyx Length: Measurement from the narrowest, most distal point to calyx base.
- Calyx Width: Measurement of the widest part of calyx.
- Trichome density: Total number of glandular trichomes divided by the surface area

Sample Analysis:
Adobe photoshop and ImageJ



Manual glandular trichome labeling and counting using ImageJ software.

Plant B Sample Location Images



Aim 3: The Differences

- To analyze samples for cannabinoid concentration, .20g (+/- .020g) of each floral cluster was homogenized, combined with 30mL methanol, and agitated for 5-8 mins. 4mL of solution was injected into Orange Photonics, Lightlab3 portable chromatography device for cannabinoid concentration.

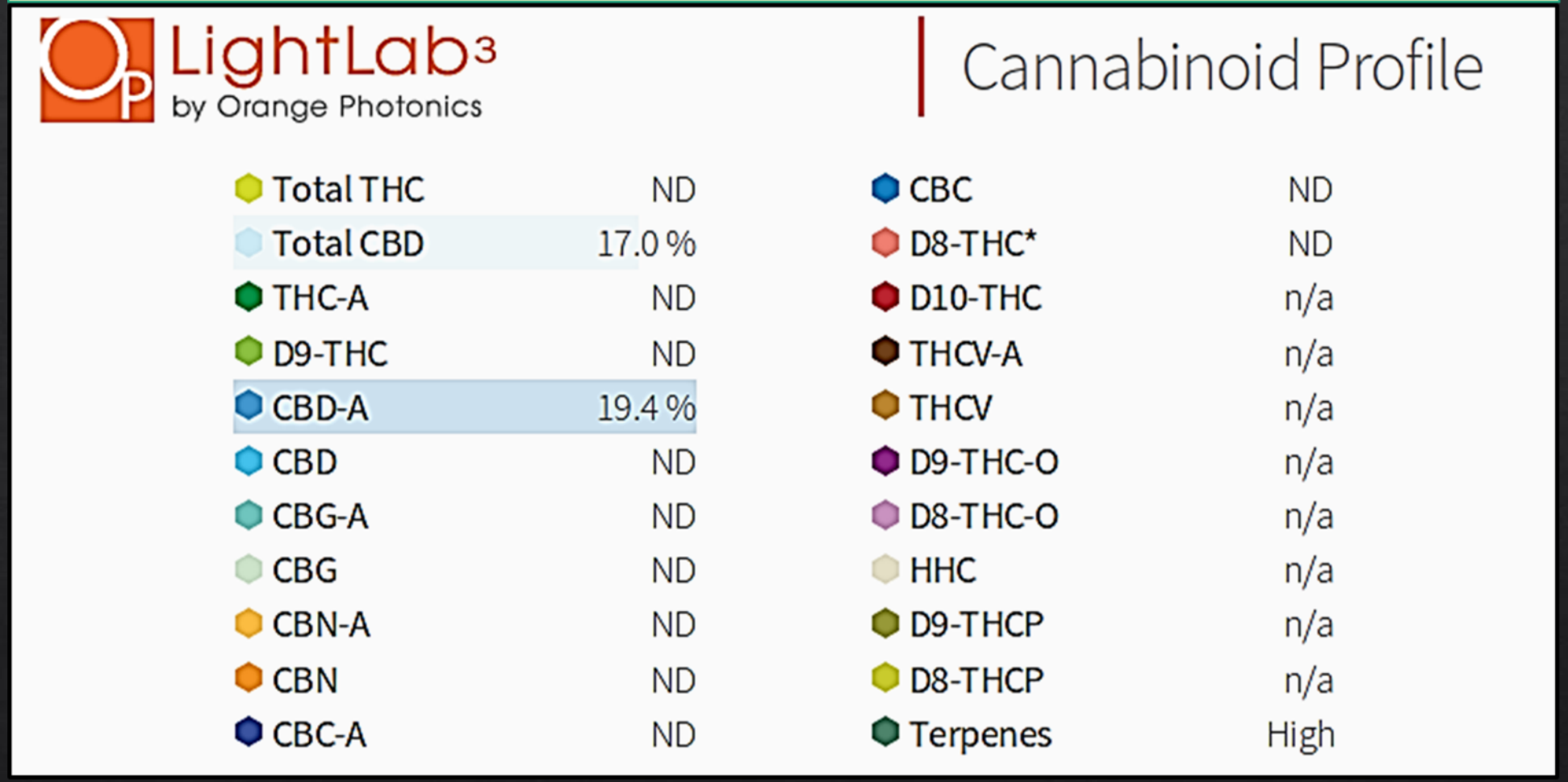
- The resulting CBDA concentrations were analyzed in comparison to the trichome density of their respective sample collection location.

Aim 3: Cannabinoid Concentration Analysis

*Note: Sample CBDA concentrations may appear higher than common industry sample analysis due to the isolation of calyx by removing all stems and leaves.

0.20g of the same cultivar, same sample, same branch. Different preparation method.

Cannabinoid Profiles

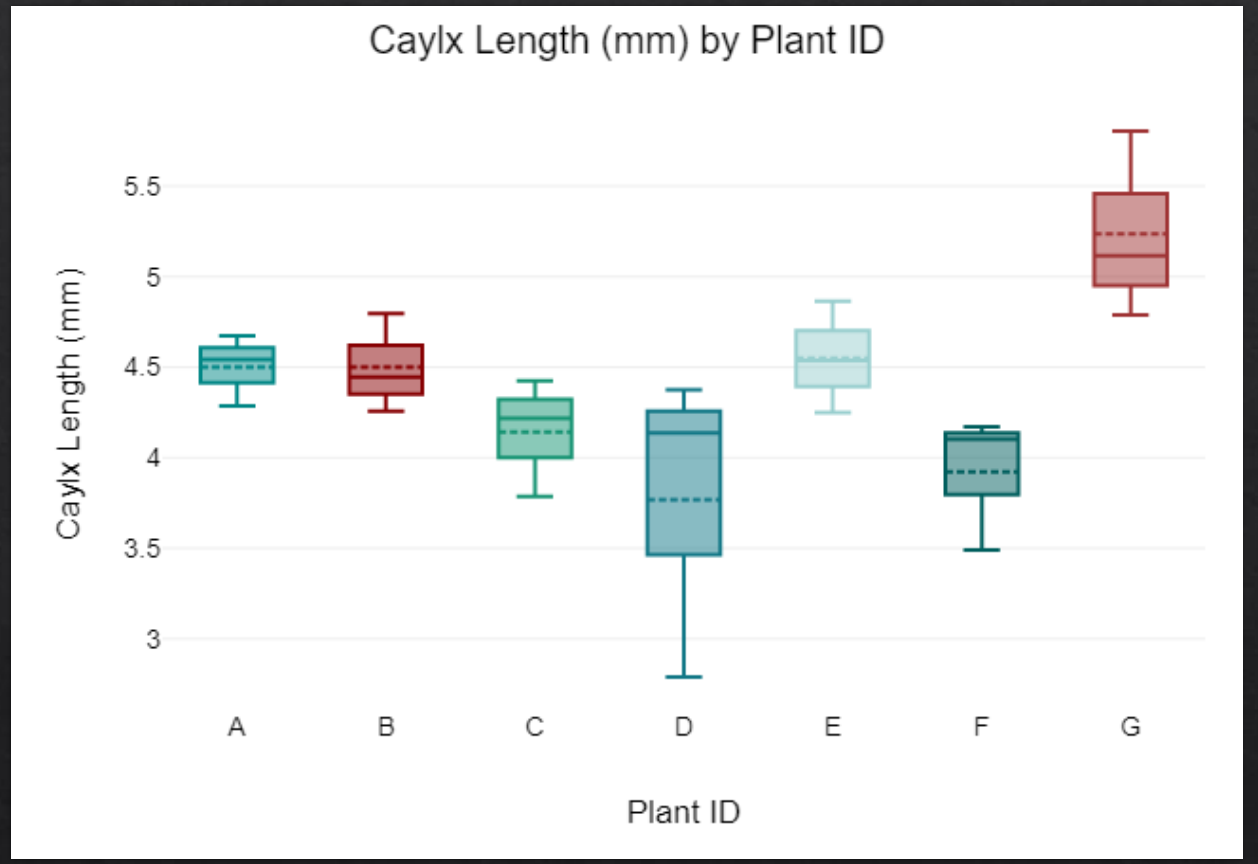


Sample Cannabinoid Profile from Floral Cluster

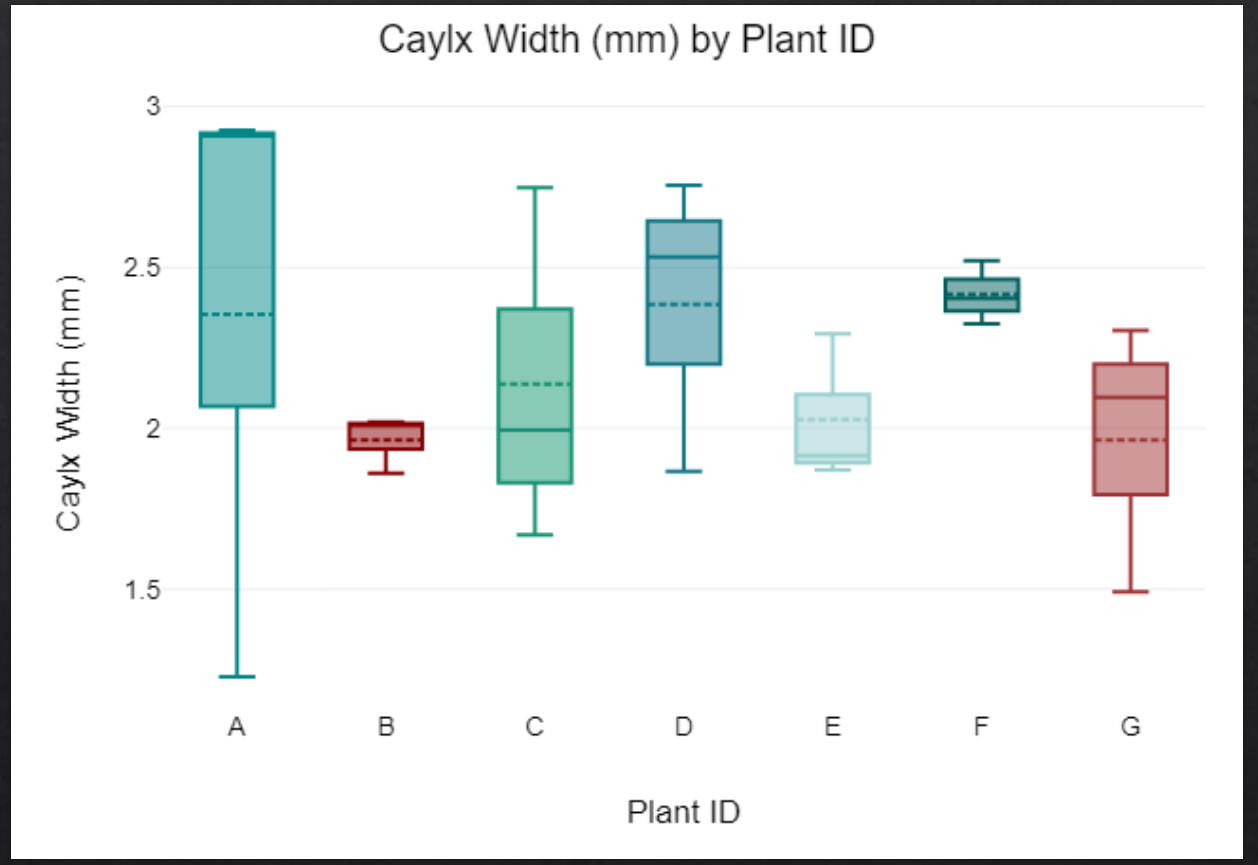
Metrics of Analysis

- All Cultivar Variables (All Cultivars; Same Locations)
  - Trichome density
  - CBDA concentration
- Intracultivar Variables (Same Cultivar; Different Locations)
  - Calyx Width vs Calyx Length
  - Calyx Area vs Trichome Density
  - Calyx Area vs Glandular Trichome Count
  - Glandular Trichome Count vs Trichome Density
  - Trichome Density vs CBDA Concentrations

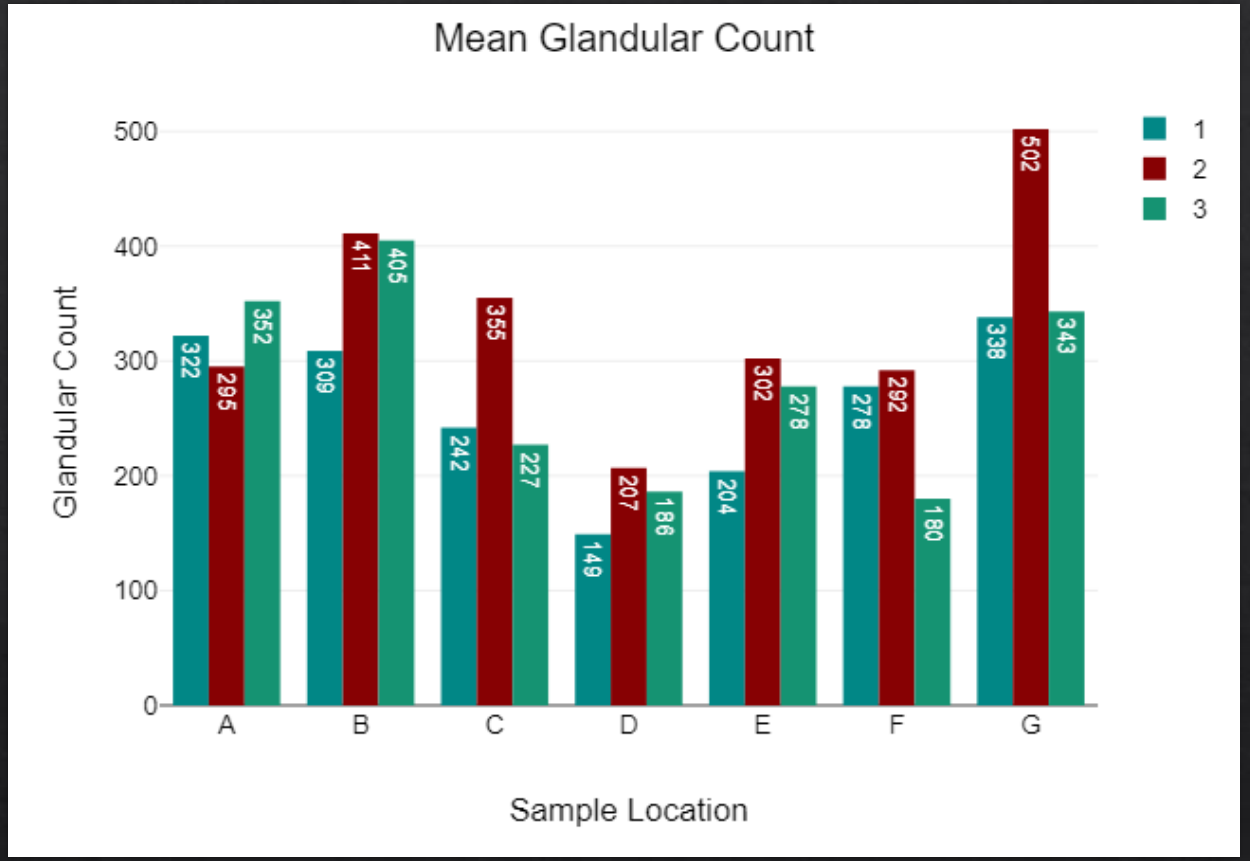
Cultivar Calyx Variation Length Box Plot



Cultivar Calyx Variation Width Box Plot



Average Glandular Trichome Count



Average CBDA Concentrations

Aim Preliminary Conclusions

Results revealed that submerging the sample in liquid nitrogen was more proficient at preserving gross trichome density and morphology without causing the distortion seen in the chemical fixation sample prep.

After this determination, I collected cryo-SEM images from the apical meristem, half of the apical meristem peak, and the lowest flowering nodes along the meristem of seven different flowering cannabis plants and used ImageJ to quantify trichome density.

Data collected from Cryo-SEM for visual trichome analysis and Orange Photonics Lightlab 3 cannabis analyzer for cannabinoid analysis suggest that both trichome density and cannabinoid concentration may vary significantly at different nodes of the same plant.

Summary of Preliminary Results

Mother

- Genetic potential
- Pollenated or Cloned???

Seed/ Clone

- Environment and Nutrients (Epigenetic affect on trichomes?)
- Vegetative growth (Root and Shoot development)
  - Non-glandular trichomes present; glandular trichomes not yet present

Flowering Female

- Glandular trichome production +
- Secondary metabolite production +

Unpollinated Flowering Female

- Senescence (Aging ultimately leading to death) of inflorescence
- Trichome metabolites visible to the unaided eye

Harvest

- Opinion: Clear, Cloudy, Amber=Variable metabolite concentrations
- Morphological trichome data, linear R&D for farmer (pre/post harvest), plant doesn't typically ripen (trichome + & PSM +) evenly
  - Trichomes are sensitive; Environment pre and post harvest may vastly affect quality of trichomes/quantity of metabolites

Compound Analysis

- What material is represented in the sample being tested? (Stem, fan leaf, sugar leaf, bract, calyx, pistil, seed)
- Is the post-harvest environment affecting trichome and secondary metabolite quality?

Acknowledgments

- My thesis advisor, Dr. Joyce Ache Gana
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