USDA Hemp Descriptor and Phenotyping Handbook, Version 3

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ABOUT

Objectives

The USDA Hemp Descriptor and Phenotyping Handbook was undertaken with the following objectives:

- To assist breeders and researchers in identifying accessions with specific traits to facilitate germplasm selection within hemp (*Cannabis sativa* L.) improvement programs.
- To identify gaps in the existing hemp collections and help formulate strategies for future collection and conservation efforts.
- To designate and maintain a core collection of critical materials.
- To increase NPGS user utility and accessibility to hemp germplasm and associated data.
- To identify duplicate accessions and reduce costs of hemp genetic resource conservation.

The methods and protocols are based on peer-reviewed literature and/or crowd-sourced from the hemp community. Robust, reliable, and high-dimensional data generated from these phenotyping efforts will empower conservation of hemp genetic diversity and aid selection of materials with unique trait combinations for breeding programs.

We have attempted to compile a list of standardized characterization and evaluation methods to capture passport information and to quantify morphology, horticultural and agronomic quality, pathogen resistance, and metabolic profile. This document can be used a reference to standardize phenotypic data collection across the broader pool of hemp germplasm and will be updated periodically as better methodologies emerge.

The information gained from these phenotyping efforts will be digitally stored and made publicly available within GRIN-Global alongside the hemp germplasm held within the Plant Genetic Resources Unit (PGRU) in Geneva, NY. An example of the germplasm that is held at PGRU can be seen here. Phenotypic summaries of PGRU hemp genetic resources can be accessed on GRIN here.

PGRU coordinates hemp germplasm collection and exchanges from domestic and foreign sources. Information related to plant genetic resources increases usefulness to diverse stakeholders. Phenotypic data can be collected either by the curator during routine multiplication or by collaborators during collection, germplasm screening, or breeding experiments. PGRU asks germplasm recipients or donors to provide as much data associated with these materials **as possible**.

Collected data can be stored in a spreadsheet using the `trait_name` as column headings and `PUID` as row names. Our lab prefers the conventions of "Tidy Data" 1,2. This document can then be emailed to the hemp germplasm curator (zachary.stansell@usda.gov) for inclusion into GRIN-Global. Please do not hesitate to reach out with any questions, comments, suggestions!

Versioning

This is **version 3.0** of the *USDA Hemp Descriptor and Phenotyping Handbook* published in June 2023. Here, we try to draw on new experience, publications, conversations since the publication of version 1.0 in September 2021.

- Small details are clarified throughout
- Expanded sections:
  - Pathology
  - Agronomic evaluation
  - Fiber quality
- More protocols are added:
  - Feral collection.
  - Seed threshing
  - Tissue culture
  - Pollen collection
- Additional citations

Editors

- Zachary Stansell (Hemp Curator, USDA-ARS, Plant Genetic Resources Unit; Project Lead, corresponding author ✉: zachary.stansell@usda.gov)
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- Anya Osatuke (Cornell Cooperative Extension; Editor/Literature Summary)

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Faith Sparks, George Stack, Jeffrey Steiner, Conor Stephen, Alan Taylor, Jacob Toth, Daniela Vergara, and Don Viands.

The drawing on the front cover is used with permission by Anya Osatuke. Kadie Britt provided many primary source images and text for the invertebrate section. Craig Carlson provided original figures, methods, and many ideas. Jacob Toth, Joshua Havill, Savanna Shelnutt, Brian Campbell, Shelby Ellison, and Jeffrey Carstens provided many helpful comments, references, protocols, and edits. We have tried to acknowledge everyone who's helped with this work, but any omissions are solely Zachary Stansell's fault.

This work has drawn heavily on input from the Cornell Hemp Stakeholder Survey. Please take the survey if you have not already done so.

Please contact zachary.stansell@usda.gov with any questions, comments, remarks, or ideas.

Boxes

Throughout this document there are special colored text boxes:

Phenotype/Descriptor

`trait_name` [datatype; units]

`elevation_meters` [decimal; m]
Elevation of collecting site above sea level.

🧪Phenotyping Protocol🧪

Seed germination

- 10 waterproof trays
- Sterile water-holding material (cotton wool, paper towels)
- 200 seeds, stored between 4 to 56 weeks…

📐Equation📐

Percent moisture may be calculated as:

$$\frac{(wet - dry)}{wet} \times 100\%$$

📜List📜

Invertebrate pests

- *Acherontia atropos*
- *Aculops cannabicolus*
- *Aecidium cannabis*
- …

📚Additional References📚

One of the earliest publications lauding hemp was “The Praise Of Hemp Seed” by John Taylor -Taylor (1620).

Keywords

- hemp
- *Cannabis sativa* L.
- germplasm
- phenotype
- trait
- characterization
- evaluation
- USDA-ARS
- NPGS
- PGRU

Language

GRIN-Global supports displaying data in multiple languages for system-level data. That is, if the system requires text to be displayed that is not actual GRIN-Global data, that text should be in the appropriate language for the current user. This is accomplished by using a table ending with **`_lang`** as a child table.

Data types & units

Units
All units are SI unless otherwise indicated.

`datetime`
A datetime data type that can handle time in nanoseconds and has a year range extending from the year “0001” to “9999.”

`decimal`
The decimal data type can store a maximum of 38 digits, all of which can be to the right of

the decimal point. The decimal data type stores an exact representation of the number; there is no approximation of the stored value.

int
The integer data type is stored as a 4-byte integer; numeric values can range from -2^{31} through $2^{31}-1$.

nvarchar
An nvarchar field can store a string of text characters (maximum 4,000). The "n" in nvarchar means uNicode. "varchar" is an abbreviation for variablelength character string. Essentially, nvarchar is variable text field that supports two-byte characters, therefore capable of handling non-English symbols.

PASSPORT

An accession consists of seed or plant material representing a sample of a single species, collected at a single time and location. An accession may be a sample of multiple plants found at the same location at the same time, or it may be collected from a single individual. By default, NPGS will retain different samples of a putative cultivar/population as discrete inventories nested within the Plant Introduction accession.

Accession

taxonomy_species_id [nvarchar]
Scientific name of accession linking the accession record to its taxonomy parent (genus / species). Modified from GRIN-Global. Subtaxon may be included:

- 'subsp.' (subspecies)
- 'var.' (variety; not the same as the breeder's named variety [uniform & stable product of breeding] or cultivar.)
- 'f.' (form)
- 'group' (botanical variety not cultivar name)

PUID [nvarchar]
If persistent, unique identifier has been previously assigned, report. Assigned to one accession to be unambiguously referenced at the global level, with associated information aggregated via automated means. Genebanks not applying a true PUID should use a combination of Institute Code, Accession Number, and the Genus as a globally unique identifier. Modified from Bioversity International, FAO (2015).

improvement_status [nvarchar]

Short paragraph. If known, elaborate on material improvement status, e.g., wild, landrace, breeding material, hybrid, founder stock, colonal selection, mutant, polyploid, mapping population, transgenic, etc.

plant_name [nvarchar]

Top name assigned to display (sometimes referred to as the top name), typically given by farmer, breeder, seed-saver. Cultivar name is a possible type of top name. If in non-Latin alphabet, provide original spelling alongside a Latin-alphabet transliteration in remarks. Modified from GRIN-Global and Bioversity International, FAO (2015).

accession_pedigree [nvarchar]

Description of plant pedigree, if known, e.g.:

- "Selection from 'Carmagnola'"
- 'Beniko'/'Carmagnola'//'Futura 75'///'Carmaleonte'/'Felina 32'//'Futura 75' * "Mutation found in 'Beniko'"

ploidy [int]
Record ploidy if known. If mixoploid or other, elaborate in **passport_remarks**. See Adriel Garay and Sabry Elias (1998).

accession_ipr [nvarchar]
State PVP registration status, if applicable. U.S. Link.

Varieties may also be protected by a U.S. Plant Patent (e.g. `CW2A`) or Utility Patent and/or Plant Breeder's Right from a UPOV country; e.g., Canada.

- US patents search
- UPOV requires account to search Variety Database.
- Canada

crop_use [nvarchar]

Explain crop use(s); e.g., oil, fiber, secondary metabolite, ecosystem services.

Germplasm source

source_cooperator_id [nvarchar]
Field associating the cooperator (person or organization) who was the source of the germplasm. See Appendix.

collector_cooperator_id [nvarchar]
Indicating the individual collecting sample. See Appendix.

developer [nvarchar]
List the name of the organization (or person) that bred the material.

Sampling & location

Modified from S-1084 Collection Protocols, GRIN-Global, personal conversations with hemp researchers (Shelby Ellison and Jeffrey D. Carstens), and Bioversity International, FAO (2015) standards. See Appendix.

number_plants_sampled [int]
Number of plants sampled to collect the accession material (S-1084 Collection Protocols). See Appendix.

source_date [datetime]
Date when germplasm is collected from source material (S-1084 Collection Protocols).

geography_id [nvarchar]
The internal geographic identifier indicating the cooperator's country and state (S-1084 Collection Protocols).

elevation_meters [decimal; m]
Elevation of collecting site above sea level (S-1084 Collection Protocols).

latitude & **longitude** [decimal]
Latitude and longitude in decimal degree format. The format is 10 integers and 8 decimals. Positive values are east of the Greenwich Meridian; negative values are west of the Greenwich Meridian (S-1084 Collection Protocols).

coordinate_method [nvarchar]
Georeferencing method used (e.g.; GPS, map, estimated). Modified from Bioversity International, FAO (2015).

uncertainty [decimal; m]
Maximum coordinate uncertainty radius.

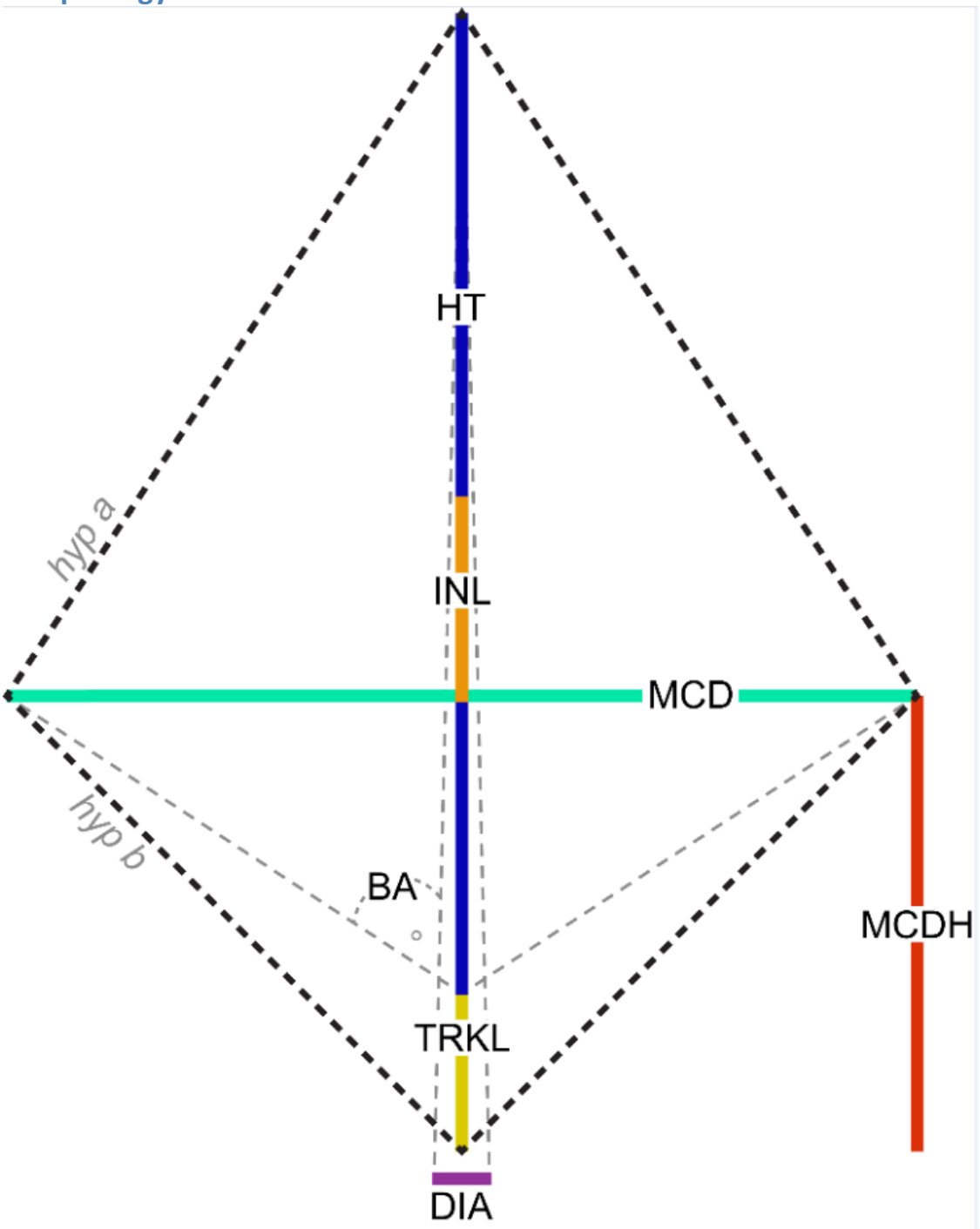
georeference_datum [nvarchar]
Geodetic datum/spatial reference system; WGS84 datum is preferred.

accession_inv_voucher_note [nvarchar]
If applicable, include additional voucher information.

ARCHITECTURE

Unless stated otherwise, measure plant architecture traits as the mean of 10 unpruned plants during week of sampling. Samples submitted to NPGS will be evaluated by a USDA-ARS laboratory using similar protocols as described below.

Morphology



Architectural traits *modified from Carlson et al. (2021).*

ht [decimal; cm]
Height of the stem from the ground to tip apical inflorescence, modified from (2021).

mcd [decimal; cm]
Maximum canopy diameter (**mcd**) as width of plant at widest set of branches (2021). Measured from widest tip to tip without stretching branches. Include flowering tissue in measurement.

mcdh [decimal; cm]
Height evaluated at maximum canopy diameter (**mcdh**) from ground to max canopy diameter (2021).

trkl [decimal; cm]
Trunk length (**trkl**) is evaluated as distance from ground to first branch (2021).

inl [decimal; cm]

Average internode length (**inl**) is calculated between internodes along the primary stem (50 cm max, see diagram) (2021).

$$inl = \frac{ht - trkl}{branches}$$

hyp_a and **hyp_b** [decimal; cm]

Calculated (2021).

$hyp.a = \sqrt{(ht - mcdh)^2 + \frac{mcd^2}{2}}$.

$$hyp.b = \sqrt{mcdh^2 + \frac{mcd^2}{2}}$$

kite_perimeter [decimal; cm]

Calculated (2021).

$$kite.perimeter = 2 \times (hyp.a + hyp.b)$$

kite_area [decimal; cm^2]

Calculated (2021).

$kite.area = \frac{ht \times mcd}{2}$.

ba [decimal; (0-180°)]

Kite branch angle **ba** is calculated from the lower kite triangle, using the difference of maximum canopy diameter height and trunk length (2021).

$$ba = \arctan \frac{mcd}{2(mcdh - trkl)}$$

nodes | nodes_opp | nodes_alt [int]
Number of internodes (**nodes**) per plant; **nodes** are by definition 2 × **branches** (2021). Number of opposite internodes (**nodes_opp**) per plant. Number of alternate internodes (**nodes_alt**) per plant. When grown from seed, branching is initially opposite, transitioning to alternate as the plant matures. Plants propagated from cuttings generally have alternate branching in the whole plant Stack et al. (2021).

branches [int]

Number of **branches** per plant (2021). When grown from seed, branching is initially opposite, transitioning to alternate as the plant matures. Plants propagated from cuttings generally have alternate branching in the whole plant Stack et al. (2021).

$$branches = n.opp * (2 + n.alt)$$

kite.circularity [categorical]

A continuous scale of apical dominance can be derived (2021):

$$kite.circularity = \frac{4\pi \cdot kite.area}{kite.perimeter^2}$$

dia [decimal; mm]
Diameter of the stem at soil level using calipers, forestry or fabric measuring tape, modified from Carlson et al. (2021).

pith_diameter [decimal; mm]
Diameter of the pith in the stem cross section at stem midpoint, modified from International Union for the Protection of New Varieties of Plants (2012).

Uncrewed aerial vehicle evaluation

uav_xxx [TBA]
See Carlson et al. (2021).

Remarks

architecture_remarks [nvarchar]
If possible, report date of measurement [days from sowing], sex average, minimum, and maximum height and width observed in a planting (cm).

📚Additional References📚

- Anderson (1980)
- Werf, Haasken, and Wijlhuizen (1994)
- Werf et al. (1995)
- Meijer and Keizer (1996)
- Ranalli (1999)
- Mishchenko and Lajko (2016)

- Magagnini, Grassi, and Kotiranta (2018)
- Backer et al. (2018)
- Spitzer-Rimon et al. (2019)
- Carlson et al. (2021)
- Danziger and Bernstein (2021)
- Stack et al. (2021)
- Vergara et al. (2021)

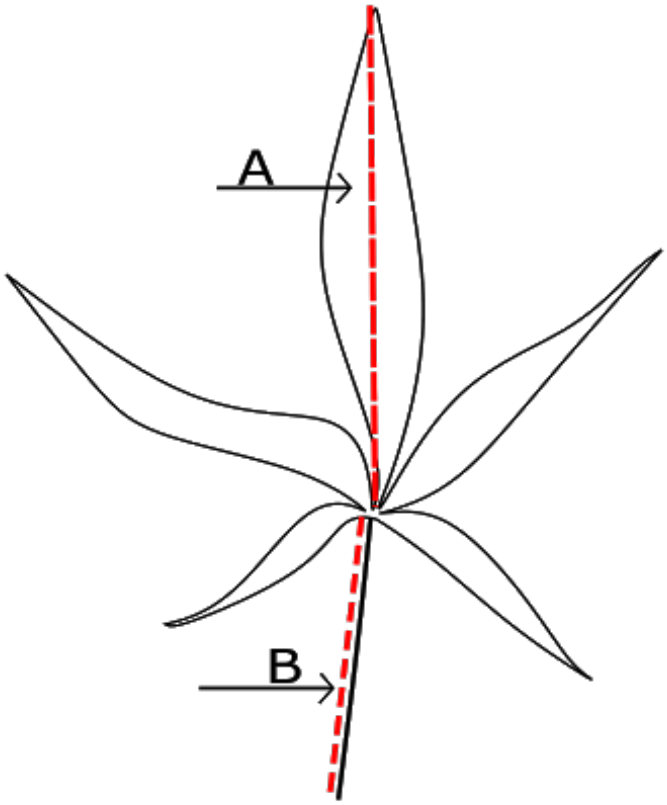
LEAF

Unless otherwise noted, gather leaf data from the uppermost set of mature leaves, as mean of 5 leaves gathered from each of 10 different plants immediately before onset of flowering.

Morphology

`petiole_length` [decimal; cm]
`central_leaflet_length` [decimal; cm] **`central_leaflet_width`** [decimal; cm]

Leaf is flattened and measured from tip until start of rachis; petiole is flattened and measured from base of rachis until petiole base, modified from -International Union for the Protection of New Varieties of Plants (2012) and -Anderson (1980).



*(**A**) **`central_leaflet_length`** and (**B**) **`petiole_length`** measurement.*

Imaging

`leaf_color_L` [decimal]
`leaf_color_a` [decimal]
`leaf_color_b` [decimal]

The average color of uppermost set of mature leaves, collected before flowering, measured with a colorimeter, modified from -International Union for the Protection of New Varieties of Plants (2012). A RHS color chart may also be used, but values should be converted to (L*a*b*) before addition to GRIN. From Wikipedia (accessed 2023-01-11): The CIELAB color space, also referred to as L*a*b*, is a color space defined by the International Commission on Illumination [...] in 1976. It expresses color as three values: L* for perceptual lightness and a* and b* for the four unique colors of human vision: red, green, blue and yellow. CIELAB was intended as a perceptually uniform space, where a given numerical change corresponds to a similar perceived change in color.

There are many programmatic solutions to convert colors (I use R for everything: 1,2,3) as well as many online tools (e.g., Colormine).

Consider printing a label to include in the scan as well. PGRU germplasm imaging and scans typically include accession ID, species, and plant id name (e.g. 'FIN-314'). PGRU uses a small

color wheel in the corner of our templates (DOWNLOAD), but that might not be necessary for you since you are measuring color with a colorimeter.

`leaf_variegation` [nvarchar; Y/N]

Indicate whether not variegation has been noted or is present.
Variegated leaves are most likely *not* virus based, but it might be worth investigating.

`leaf_scan_protocol` [.jpg or .png]

🧪Scan protocol🧪

Equipment

- Flatbed scanner
- Desktop monitor
- Black cloth, ideally velvet, of equal dimensions to scanner bed.

Protocol

1. Gather one mature leaf from a representative sample of 10 plants a week before the onset of flowering. Retain petioles. Keep on ice if wilting is a concern.
2. Scan leaves within the hour of collection using a scanner with the lid open, draping black fabric over the leaves to absorb background light. Include scale or ruler (cm) and **`puid`**.
3. Convert the scanned leaf image into .png file and save.

Leaf imaging setup at PGRU

Leaf scan example from routine phenotyping at PGRU

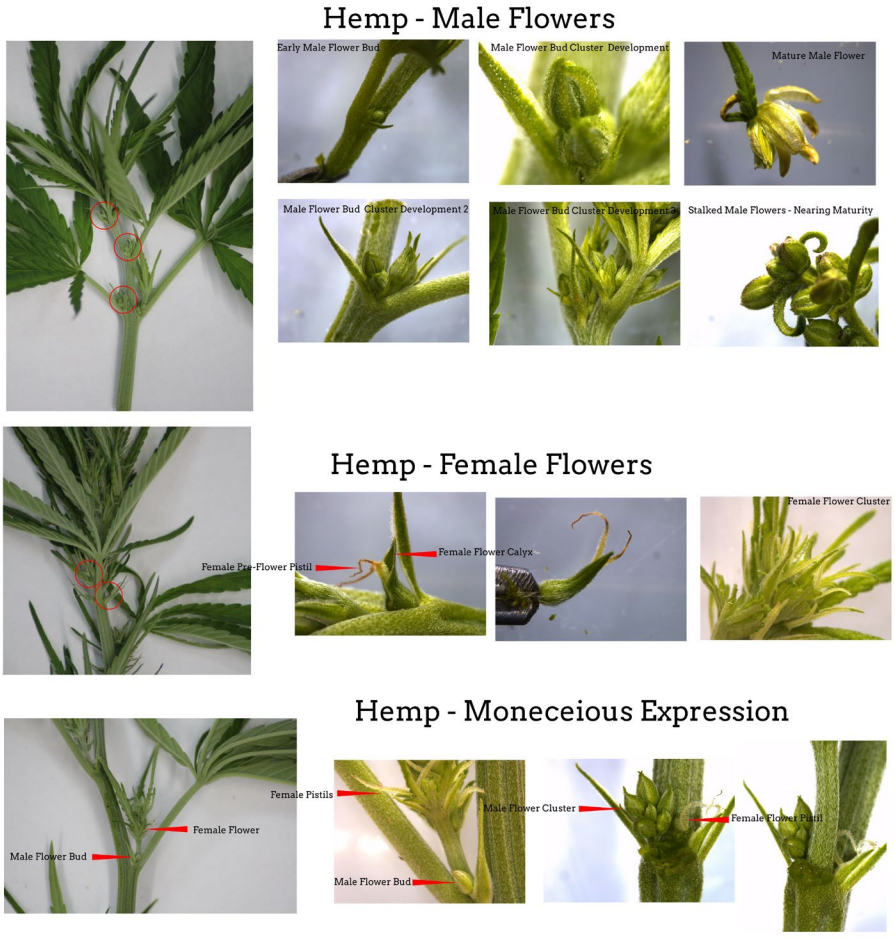
📚Additional References📚

- Haney and Kutscheid (1975)
- Dayanandan and Kaufman (1976)
- Anderson (1980)
- Meijer and Keizer (1996)
- Amaducci et al. (2008)
- Hall, Bhattarai, and Midmore (2012)
- Mishchenko and Lajko (2016)
- Magagnini, Grassi, and Kotiranta (2018)
- Spitzer-Rimon et al. (2019)
- Carlson et al. (2021)
- Stack et al. (2021)
- Vergara et al. (2021)

SEX & INFLORESCENCE

General remarks

Phenotyping sex characters is (in our experience) more complex than it might seem initially. At PGRU, we have evaluated phenotypic sexual expression at both the plot level and by measuring many individual plants. Some groups have simply recorded the sex of 100 preselected individuals at harvest. Both approaches should produce the same phenotype. In our 2022 field trials in Geneva, NY, we recorded the exact first flowering date (of pistillate and staminate flowers) of > 2,400 individuals and then calculated (by population) the mean percent female, male, and monoecious. Specify your approach in **`sex_remarks`**, as well as sowing/transplanting date, field or greenhouse conditions, and photoperiod.



Hemp flowering guide produced by XXX with permission from

Sex ratio

sex_ratio [nvarchar]
Sex ratio during flowering.
Target a sample of 100 individuals in the field before flowering begins. Label and number sample plants clearly. Record F:M:O; as the ratio of female, male, and monoecious (individuals with male and female flowers) individuals, respectively.
If plants are wild-collected, 100 plants may not be available; measure as many as possible (See S-1084 Collection Protocols).

Phenology

date_flower_female [datetime]
date_flower_male [datetime]
date_flower_monoecious [datetime]

Pre-terminal date when axial flowers with shortening internodes and terminal pistils (clusters of flowers at shoot termini) were observed Carlson et al. (2021). Record sowing/transplanting date in **sex_remarks**. See also Faux et al. (2013); Shams et al. (2020).

days_2_female [integer; d]
days_2_male [integer; d]
days_2_monoecious [integer; d]

days_2_female = **date_flower_female** - sow date

days_2_male = **date_flower_male** - sow date

days_2_monoecious = **date_flower_monoecious** - sow date

maturity [int; d]
Days from germination to commercial maturity, calculated as harvest date - sowing date.

day_neutrality [nvarchar]
Describe flowering behavior as critical day length and/or day neutral response. Calculate as percent of plants in population exhibiting day neurtrality. **A more precise definition is required to define this phenotype quantitatively**.

Inflorescence

inflor_length [decimal; cm]
Length of inflorescence cluster at the uppermost branch, excluding leaves. Remove leaves with more than 1 leaflet. Retain stems and seeds; inflorescence may be retained as clusters.

inflor_weight [decimal; cm]
Measure weight after drying at room temperature for >48 hr.

inflor_yield [decimal; $kg \cdot ha^{-1}$]
Combine inflorescence dry weight data with planting density to calculate kg/ha.

inflor_color [nvarchar; L*a*b*]
Measured with a colorimeter at commercial maturity. A RHS color chart may also be used, but values should be converted to (L*a*b*) before addition to GRIN.

Remarks

sex_remarks [nvarchar]
Short paragraph, if possible/applicable include:

- If plants were grown in field, greenhouse, or growth chamber.
- Planting density (e.g., $\frac{seeds}{m^2}$) if applicable.
- Female and male bract, stigma, and flower color.
- Date of collection.
- Day length (hh:mm) on the date of the first observed open female flower.

📚Additional References📚

- Schaffner (1921)
- Grishko, NN and Levchenko, VI and Seletski, VI (1937)
- Werf, Haasken, and Wijlhuizen (1994)
- Mandolino et al. (1999)
- Ranalli (1999)
- Lisson, Mendham, and Carberry (2000)
- Shao, Song, and Clarke (2003)
- Pahkala, Pahkala, and Syrjälä (2008)
- Cosentino et al. (2012)
- Hall, Bhattarai, and Midmore (2012)
- Faux et al. (2013)
- O. V. Razumova (2014)
- Lynch et al. (2016)
- Olga V. Razumova et al. (2016)
- Vergara et al. (2016)
- Punja, Rodriguez, and Chen (2017)
- Zhang et al. (2018)
- Eichhorn Bilodeau et al. (2019)
- Salentijn, Petit, and Trindade (2019)
- Kovalchuk et al. (2020)
- Punja and Holmes (2020)
- Toth et al. (2020)
- Adal et al. (2021)
- Danziger and Bernstein (2021)
- Dowling, Melzer, and Schilling (2021)
- Hurgobin et al. (2021)

- Stack et al. (2021)

SEED

Samples submitted to NPGS will be evaluated by a USDA-ARS laboratory using similar protocols as described below.

General

`hundred_seed_weight` [decimal; g]
Record mass of 100 seeds.

`thousand_seed_weight` [decimal; g]
Record mass of 1000 seeds.

`seed_image` [.jpg or .png]

🧪SEED IMAGE🧪

Equipment

- Flatbed scanner
- Desktop monitor
- Black cloth, ideally velvet, of equal dimensions to scanner bed.
- Transparent, flat-bottomed tray or transparency film to protect scanner bed from scratches.

Protocol

1. Gather a sample of 20 grains.
2. Scatter samples across tray or transparency film.
3. Convert the scanned seed image into .PNG file and save.

Seed image. Photo credit Alan Taylor,Michael Loos, and Masoume Amirkhani

`seed_size_length` [nvarchar]
`seed_size_width` [nvarchar]

Size of the largest, smallest, and median seed in the lot (mm) as L:S:M using using Tomato Analyzer, Smart Grain, Photoshop, GNU Image Manipulation Program, or other imaging software to make these calculations based on a scanned image of 20 seeds.

`seed_moisture` [%]
Dry seeds in a single layer in a constant temperature oven held at 105 ˚C for 20 h.

$**`seed_moisture`** = \frac{(wet - dry)}{wet} \times 100\%$

Germination/viability

`percent_germ` [%]

🧪SPROUT TEST🧪

Equipment

- 10 waterproof trays
- Sterile water-holding material (cotton wool, paper towels)

- Water source
- 200 seeds, stored between 4 to 56 weeks after harvest in dry conditions between 16 - 25˚C.

Protocol

1. 4 sub-samples of 50 seeds are isolated.
2. Each sub-sample is spread evenly on trays lined with water-holding material. Seeds may be placed on top of water holding material and in between rolls.
3. Each tray is saturated with water and placed in a temperature-controlled room: 16 hours of dark at 20˚C, 8 hours of light at 30˚C. Trays are kept moist.
4. Live, normal, sprouted seeds are counted by hand, sprouts are then removed from the germination tray.
5. After 7, 14, and 21, days the number of live, normal, sprouted seeds is counted by hand. The duration of experiment may be extended if dormancy is an issue (record in **`germ_remarks`**)
6. Germination percentage for each tray is calculated as: $\frac{sprouted}{total} \times 100\%$ and averaged per tray.
7. Consider executing TZ tests for lots with high dormancy to verify they truly are dormant and not dead.

Seed germ testing (photo credit: Alan Taylor, Michael Loos, and Masoume Amirkhani).

Modified from Seed Laboratory Oregon State University (2018), NPGS protocols, and communication with Alan Taylor, Jeffrey Carstens, and Joshua Havill.

germ_remarks [nvarchar]
Record any dormancy issues and stratification methods used. Note: dormancy in feral/naturalized populations may also be important to characterize.
Cold stratification may be the norm in feral specimens with a temperate origin.

Seed threshing

See Appendix

Yield

grain_yield [decimal; kg/Ha]
Subsample larger strip trials (transect method) at seed maturity. Subsamples are chosen depending on the size of the field/plot to estimate kg/hectare.

shattering [nvarchar; L,M,H]
Retain subsample of field planting for shattering evaluation and report as low, medium, or high. **A more precise definition is required to define this phenotype quantitatively**.
See Planteome ontology.

Oil

oil_content [decimal; %]
The percentage of oil constituting the whole, unhulled seed mass calculated as:

$$\frac{extracted.oil}{ground.sample} \times 100\%$$

Indicate whether measurement was made using Nuclear Magnetic Resonance (NMR) imaging, or using a Soxhlet extractor in `seed_remarks`.

🧪Soxhlet evaluation🧪

- Seeds are ground to a particle size of 0.5 mm ± 0.1.
- Place 10 g of ground seed into an extractor thimble, extracting with 100 mL hexane for 24 h at 70 ˚C.

If a different protocol is used, record the sample weight (g), solvent name (IUPAC) and volume (mL), extraction time (h) and extraction temperature (˚C).

Adapted from Devi and Khanam (2018).

🧪NMR evaluation🧪

- To ensure accurate NMR evaluation of hemp seed, seed moisture content must be below 8%.
- Subsample 10-15 seeds after evaluation of moisture content.
- The sample is evaluated with a 40 mm diameter sample probe.

- Prior to being placed in a sample box, seeds are to be further temperature-conditioned in the drying oven or a heating block at 40 ˚C for 90 min.

- Instrument settings:
  - 5 mHZ operating frequency
  - 4 scans
  - 1 second re-cycle delay
  - 40 ˚C magnetic box temperature.
- A calibration curve is constructed by comparing several varieties with a range of oil concentrations.

- A linear calibration curve is constructed against the peak area of the NMR resonance signal normalized against sample mass.
- The y-axis of the plot reports normalized peak area, and the x-axis reports % oil concentration.
- Validate oil concentration from seeds of the same batch using Soxhlet extraction .
- Indicate the % oil concentration of seed and the R2 value of the linear equation.
- If a different NMR setting is used, list the weight of seeds sampled (g), seed temperature conditioning (˚C), operating frequency (mHZ) and line equation.

Adapted from Hutton, Garbow, and Hayes (1999). See also Shams et al. (2020); Yadav and Murthy (2016).

`seed_fatty_acid`

See ISO 12966-1:2014 gas chromatography protocols for determination of fatty acids, free and bound, in animal and vegetable fats and oils following their conversion to fatty acid methyl esters (FAMEs).

Protein

`combustion_analysis` [decimal]

🧪Protein combustion analysis🧪

- 150 mg seeds are frozen in liquid nitrogen and ground.
- Seeds are placed into a combustion analyzer.
- Combustion tube is at 960 °C, and oxygen is dosed for 80 s at 170 mL.

Adapted from Schultz et al. (2020).

🧪Kjeldahl method🧪

Based on an adaption of AOAC Official Method 991.20 by Thiex et al. (2002), where

`Kjeldahl N %` = (`[std.acid sample (mL)]` - `[std.acid blank (mL)]`) x (`[HCl]`) x 14.01) / (`weight (g)` x 10)

Crude protein % = % Kjeldahl N x 6.25

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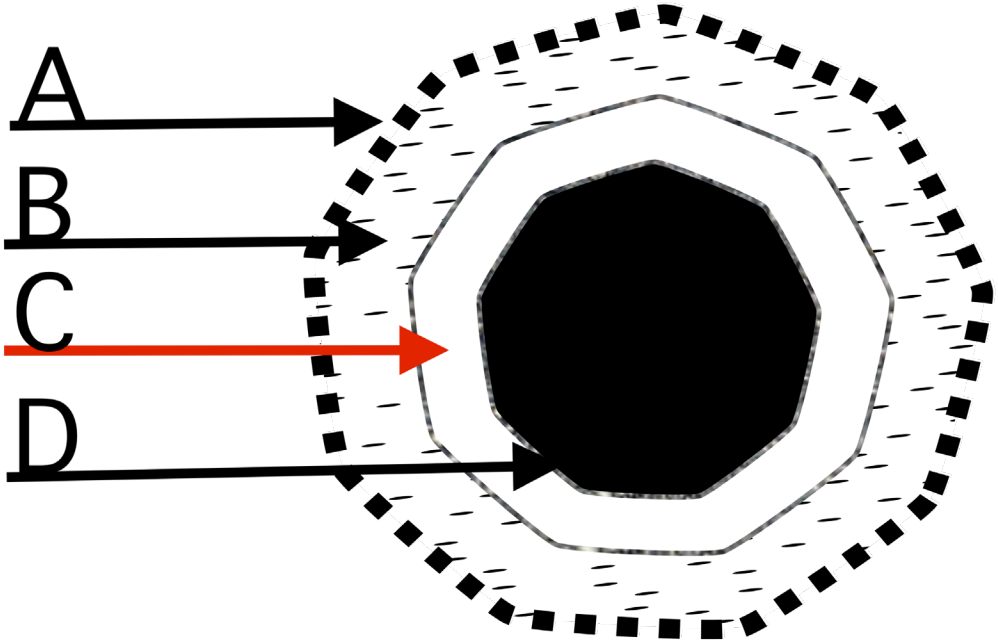
FIBER

Samples submitted to NPGS will be evaluated by a USDA-ARS laboratory using similar protocols as described below.

Yield



***Hemp stem cross section**. **A** = epidermis; **B** = bast and cortex; **C** = pith, also known as hurd, **D** = inner core, usually hollow but not at joints.*

`fiber_yield` [decimal; $\frac{kg}{Ha}$]
Fiber yield; modified from Serkov (2015). See also Backer et al. (2018). Harvest 10 plants, remove top and bottom 15 cm, defoliate run sample through a chipper, and measure mass. Calculate **`fiber_yield`** based on planting density.

`wet_fiber_biomass` [decimal; g]
Harvest stems 15 cm from base, remove top 15 cm, and defoliate. Wet biomass is average weight per stem without inflorescences from a sample of 10 plants Modified from Carlson et al. (2021).

`dry_fiber_biomass` [decimal; g]
Use sample from **`wet_fiber_biomass`** measurements. Dry biomass is average weight per stem dried at 65 ˚C until brittle from a sample of 10 plants. Modified from Carlson et al. (2021).

`bast` [decimal; %]
Stems collected from a sample of 10 plants, dried until brittle, and separated into bast (bark) and hurd (core) using a flax breaker, or by hand. Record as fraction of oven-dried mass.

`hurd` [decimal; %]
Stems collected from a sample of 10 plants, dried until brittle, and separated into bast (bark) and hurd (core) using a flax breaker, or by hand. Record as fraction of oven-dried mass.

📐Bast & hurd content📐

$\texttt{bast} = \frac{bast}{dry} \times 100\%$

$\texttt{hurd} = 100\% - bast$

`bast_soluble` & **`fiber_solubility`** [decimal; %]
Soluble materials in bark after evaluation with sodium hydroxide evaluation.

🧪Sodium hydroxide evaluation🧪

Protocol

- 10 - 15 g isolated hurd samples isolated from dry stems, or 10 - 15 g isolated bast samples from dry stems.
- Pulp dispersion apparatus, consisting of a variable speed motor and a stainless steel stirrer with a shell.
- Thermometer
- Vacuum source
- Filtering flasks, 100 mL
- Graduated cylinder, 100 mL
- Watch glasses
- Stirring rods
- Tall, 200 mL beakers
- Water bath with cover and holes to securely submerge the bottoms of tall, 200 mL beakers.
- Filtering crucibles, 30 mL, 10 - 15 µm maximum pore size.
- 1000 mL sodium hydroxide solution, 1.0 % ± 0.1 NaOH (0.25 N)
- 1000 mL acetic acid, 10%.

1. Grind hurd or bast sample into a fine meal.
2. Dry filtering crucibles before use in an oven at 105° ± 3 °C for 60 min, cool in a desiccator, and record weight.
3. Heat water bath to 97 - 100 ˚C
4. Adjust speed of the motor and the angle of the blades so that no air is drawn into the pulp suspension during stirring.
5. Place 10 g meal into 200 mL beaker and add 100 mL 1% NaOH and stir with glass rod.
6. Cover beaker with watch glass and place into water bath for 1 h. Keep water level above level of alkali solution in the beaker at all times.

7. Stir the meal with a rod for about 5 s at 10, 15, and 25 min after placing into the bath.
8. At the end of 1 h, transfer the material to a tared filtering crucible and wash with 100 mL of hot water.
9. Add 25 mL of 10 /% acetic acid and allow to soak for 1 min before removal. Repeat this step once again.
10. Wash material with 100 mL hot water three times to remove acid.
11. Dry crucible and contents in the oven at 105 - 108 ˚C until constant weight.
12. Cool in desiccator.

📐% Soluble materials📐

$$\frac{meal.before - meal.after}{meal.before} \times 100\%$$

`non_fiber` [TBD]
Protocol for evaluating stem non-fiber components not determined.

Quality

There is little modern research on hemp fiber quality evaluation protocols. This work will be conducted in collaboration with the Southern Regional Research Center (SRRC).

📜Candidate fiber traits📜

- fiber length
- fiber strength
- fiber flexibility; see Werf, Haasken, and Wijlhuizen (1994)
- fiber length/diameter ratio; see Ranalli (1999)
- tensile strength; see Ranalli (1999)
- brittleness; see Ranalli (1999)
- crystallization/cellulose crystallinity Rongpipi et al. (2019)
- cross linking
- elasticity; see Ranalli (1999)
- ease of decortication
- mechanical vs microbial retting
- cellulose:lignin ratio

`fiber_remarks` [nvarchar]

Remarks are used to add notes or to elaborate on fiber descriptors

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SECONDARY METABOLITES

Unless otherwise stated, collect from a sample of 10 plants at harvest. Samples submitted to NPGS will be evaluated by a USDA-ARS laboratory using similar protocols as described below.

Chemotype

chemotype [int; 1-6]
Note that living tissues synthesize acid forms of most cannabinoids (i.e. THC-a, CBD-a) and these are often decarboxylated to non-acid forms during evaluation (i.e. THC, CBD). Chemotype is largely driven by segregation of alleles at the *B* or *O* loci Toth et al. (2020).

1 = Mostly THC(A)
2 = ~1.5:1 CBD(A):THC(A)
3 = Mostly CBD(A)
4 = Mostly CBG(A)
5 = Low overall cannabinoid content
6 = Other

chemotype_segregation [nvarchar]

To not mask chemotype, we recommend measuring cannabinoids from 10 individual plants rather than pooling samples. If multiple individuals are evaluated for chemotype and differing chemotypes are observed in the same population, indicate the percentage of each chemotype using the `chemotype` scale (1:#;2:#;3:#,4:#,5:#).

Cannabinoids

cannabinoids [decimal; $\frac{\mu g}{mg}$]
Cannabinoid content is evaluated either by UPLC or HPLC. THC is a ''sticky'' compound and residues will persist on laboratory equipment and analytical vessels following cleaning. THC carryover onto subsequent samples may erroneously increase the reported THC content. We strongly advise against re-using sample vials for UPLC and HPLC cannabinoid evaluation.

UPLC uses a shorter run time per sample, however HPLC provides better resolution for minor cannabinoids. Both methods are sufficient for quantifying total THC in compliance with the **2021 USDA-AMS Hemp Final Rule**.
10 samples collected from 10 individual plants. Each sample should be analyzed in triplicate. Cannabinoid content is variable across the height of the plant. THC-a decarboxylates into THC following exposure to heat. We advise against decarboxylating cannabis samples prior to instrument evaluation, as this process introduces error through the volatilization of a variable percentage of the total cannabinoid content.

Instead, we recommend combining the THC-a and THC content of unheated plant samples to calculate the total THC content. The formula for this is:

📐 THC & Cannabinoid analytes 📐

$$THC_{total.potential}(\mu g/mg) = THC + 0.877 \cdot THC_a$$

$$Cannabinoid.analyte = C_e \frac{V_f}{W_s}$$

where:

C_e: Conc of the sample in the extraction solution ($\frac{\mu g}{\mu L}$)
V_f: Final volume of the sample (μl)
W_s: Weight of the sample (mg)

🧪UPLC cannabinoid evaluation🧪

Sample run time: 10 min.

Equipment

- Sonicator
- Thermo Scientific UltiMate 3000 controlled by Chromeleon software
- IntertSustain C18 3 µm chromatographic column (2.1 x 100 mm)
- 5 mM aqueous ammonium formate
- 0.1% formic acid
- 95% formic acid
- Acetonitrile
- Volumetric flasks: 1.5 L, 50 mL.
- 0.45 µm membrane filter.

Standards:

- Cannabidivarinic Acid (CBDVA; CAS #: 31932-13-5)

- Cannabigerovarinic Acid (CBGVA; CAS #: 64924-07-8)
- Cannabidivarin (CBDV; CAS #: 24274-48-4)
- Cannabivarin (CBV; CAS #: 33745-21-0)
- Cannabidiolic Acid (CBDA; CAS #: 1244-58-2)
- Cannabigerolic Acid (CBGA; CAS #: 25555-57-1)
- Tetrahydrocannabivarin (THCV; CAS #: 31262-37-0)
- Cannabigerol (CBG; CAS #: 25654-31-3)
- Cannabidiol (CBD; CAS #: 13956-29-1)
- Tetrahydrocannabivarinic Acid (THCVA; CAS #: 39986-26-0)
- Cannabichromevarinic Acid (CBCVA; CAS #: 1628112-69-5)
- Cannabinol (CBN; CAS #: 521-35-7)
- Cannabinolic Acid (CBNA; CAS #: 2808-39-1)
- Δ9-Tetrahydrocannabinol (THCd9; CAS #: 1972-08-3)
- Δ8-Tetrahydrocannabinol (THCd8; CAS #: 5957-75-5)
- Δ9-Tetrahydrocannabinolic acid A (THCA or THCAA; CAS #: 23978-85-0)
- Cannabicyclol (CBL; CAS #: 21366-63-2)
- Cannabichromene (CBC; CAS #: 20675-51-8)
- Cannabichromenic Acid (CBCA; CAS #: 185505-15-1)

- Cannabicyclolic Acid (CBLA; CAS #: 40524-99-0)

Standard preparation

- All standard solutions are to be prepared at a concentration of 100 µg/mL.
- Cannabinoid acid standards are prepared in acetonitrile. Decarboxylated cannabinoids are prepared in methanol.

Reagent preparation

- 1 L reagent for Mobile Phase A: Combine 0.21 mL 95% formic acid with 5 mM ammonium hydroxide (0.62 mL 30% ammonium hydroxide solution) with millipore water to 999 mL. Add 1 mL formic acid.
- 1 L reagent for Mobile Phase B: Combine acetonitrile with formic acid to 0.1% formic acid.

Protocol

1. Dry inflorescences at room temperature (20 °C) to < 10% moisture.
2. Grind samples.
3. Sieve through a number 20 sieve (850 µm).
4. Place 0.100 grams of ground hemp material in a 20 mL glass scintillation vial.
5. Prepare each sample in triplicate.
6. Calibrate the pipetter to ensure it is delivering the correct volume of 200-proof ethanol. Check that the weight of 20 mL of ethanol delivered is 15.78 g (density: 0.789 g/cm^3).
7. Add 20 mL of HPLC grade 200-proof ethanol to the sample.
8. Sonicate (40 kHz) in a sonication bath for 30 min at room temperature.
9. Remove from sonication bath.
10. Allow the sample to sit overnight (18 h) in the dark at room temperature.
11. The next day filter the supernatant through a 0.45 µm Nylon membrane filter into a sample vial for analysis.
12. Inject 1 µL sample volume.
13. Set flow rate to 0.60 mL/min.
14. UPLC gradient program: 0-2 min, 70% B (concave curve 9); 2-5.5 min, 75% B (concave curve 9); 5.5-6 min, 100% B (linear curve 5); 6-7 min, 100% B; 7-7.5 min, 65% B (linear curve 5); and 7.5-8, 65% B followed by 2.0 min of equilibration at 65% B between injections.
15. The autosampler chamber and column temperature are set to 8°C (if a temperature-controlled sample tray is available) and 50°C, respectively. .
16. Five-point standard curves are developed for the cannabinoids using a setting of 228 nm from the diode array detector.

Methods provided by Berhow and Gude (2021) and modified by Dr. Korey Brownstein in 2023.

Other metabolites

`terpenes` [decimal]

🧪Terpene evaluation🧪

Equipment

- GC/MS (Agilent 7890B GC, Agilent 5977B MSD, PAL 3) controlled by LabSolutions software
- HP-5MS UI, 30 m × 0.25 mm, film 0.25 µm column
- 0.45 µm filter
- Hexane

Standards

- β-bisabolol
- Bulnesol
- m-Camphorene
- p-Camphorene
- Δ3-Carene
- β-Caryophyllene
- 10-epi-γ-Eudesmol
- α-eudesmol
- β-eudesmol
- α-humulene
- Limonene
- Linalool
- β-Myrcene
- Plastochromanol-8
- α-Phellandrene
- α-Pinene
- cis-Sabinene hydrate
- γ-Selmene
- Selna-3,7(11)-diene
- α-Tocopherol
- β-Tocopherol
- δ-Tocopherol
- γ-Tocopherol
- β-Pinene
- Nerolidol
- Camphene
- Terpinolene
- Ocimene

- α-Terpinene
- γ-Terpinene

Protocol

1. Dilute essential oil standards with hexane to a concentration of 0.1 mg/mL.
2. Dry inflorescences at room temperature (20 ˚C) to > 10% moisture.
3. Grind samples.
4. Combine 20 mg plant material with hexane.
5. Pass through the 0.45 µm filter to extract terpenes.
6. Dilute filtrate 1:20 with hexane.
7. Inject samples into split injection port (1:5).
8. Instrument settings as follows:
    - Injection port held at 100 ˚C with an initial time of 4 min.
    - Inlet temperature fixed at 250 ˚C.
    - Detector temperature fixed at 280 ˚C.
    - Column held at 35 °C for 5 min, then raise to 150 °C at 5 °C/min. Then raise to 250 ˚C at 15˚ C/min. Hold time 90 min.
    - Helium serves as carrier gas, with flow rate 1 mL/min.

Adapted from Hanuš and Hod (2020).

See also hops methods

`flavonol_index` [decimal]

🧪Flavonol evaluation🧪

A handheld MPM-100 meter by ADC BioScientific can do this measurement instantly. (See)

- **Flavonoids**: Spectroscopic measurement of fluorescence at different wavelengths (F660 nm and F325 nm):
- **Flavonols**: (F660 nm/F325 nm).

`anthocyanins` [decimal]

🧪Anthocyanin evaluation🧪

Sample prep and extraction

- Samples are ground into a fine powder with a coffee mill and passed through a 60 mesh filter to collect the fine ground fraction.
- Batches of 250 grams of ground sample are extracted with 1% HCl in methanol with stirring overnight at room temperature.
- The liquid is decanted and filtered through Whatman 54 filter paper.
- The remaining solid material is extracted with only methanol a second time with stirring overnight and decanted and filtered.

- The extracts are pooled, and approximately 400 mL of water is added, and allowed to evaporate over 72 hours in the hood or rotovaped at 45 °C to remove the methanol.
- The concentrated extract is filtered gain to remove any remaining solid material.

Method A: Preparative Chromatography

- A Buchi (Newcastle, DE) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with SepacoreRecord 1.2 chromatography software is used.
- A 40 x 150 mm flash cartridge column with approximately 90 grams of preparative C18 reverse phase end capped bulk packing material (Silicycle SiliSep BUC18 (17%) 60 Angstroms, 40-63 um, Silicycle, Quebec, Canada) is used for the preparative separation.
- The columns are installed in the flash chromatography system, and new dry columns are initially washed with methanol and then equilibrated with 0.2% acetic acid in water for five minutes at a flow rate of 50 mL per min.
- After samples (50-100 mL) are loaded on the column, the column is developed with a binary gradient to 40% methanol over 10 minutes, then to 100% methanol over an additional 5 minutes. For anthocyanins, the effluent is monitored at 520 nm and 50 mL fractions are collected in the fraction collector by the software program.
- Fractions are concentrated by evaporation in the hood at room temperature or methanol is removed by rotovap at 45 °C.
- The fractions containing the DGA are pooled and freeze dried over 2-5 days to dryness.

Method B: HPLC Analysis

- Samples are run on a stand-alone Shimadzu 10A HPLC system (SCL-10A system controller, two LC-10A pumps, CTO-10A column oven, and SIL-10A autoinjector).
- Peaks are monitored using a Hewlett-Packard 1040A photodiode array detector running under the HP Chemstation software version A.02.05.
- The column used is an Inertsil ODS-3 reverse phase C-18 column (5 µM, 250 x 4.6 mm, with a Metaguard column, from Varian).
- The initial conditions are 2% acetonitrile and 0.5% acetic acid in water, at a flow rate of 1 ml per minute.
- The effluent is monitored at 520 nm on the PDA.
- After injection (typically 25 µL), the column is held at the initial conditions for 2 minutes, and then developed to 100% acetonitrile in a linear gradient over 60 minutes.
- Standard curves based on nanomoles injected are prepared from a pure standard of delphnidin-3-O-glucoside purchased from Chromadex (Irvine, CA).
- Extinction coefficients are calculated from a linear regression formula based on four different nanomole concentrations of anthocyanin standards (purchased from Chromadex) injected and their respective mAbs areas.

- The extinction coefficient for each anthocyanin is then used to calculate respective anthocyanin glycoside concentration in the samples by the following formula:

$$mAbs(area) \cdot extc.coef\left(\frac{nM}{mAbs}\right) \cdot \frac{1}{inj.vol(\mu L)} \cdot vol.extract(mL) \cdot MW_{anth.glucoside}\left(\frac{\mu g}{nM}\right) \cdot \frac{1}{sample(g)}$$

- Addition of a glycosyl groups to the anthocyanin has little effect on its absorption profile, so anthocyanin aglycones can be used to prepare standard curves for anthocyanin glycosides on a molar basis (Berhow 2002, Mabry 1970, Markham, 1982).

Method C: LC-ESI-MS Analysis of Anthocyanins

- Samples are run on an Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer – a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a higher energy C-trap dissociation (HCD) cell attached – with an Ion Max electrospray ionization (ESI) source; a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump; ACCELA1 HTC cool stack autoinjector; and a ACCELA 80 Hz PDA detector); all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software.
- HPLC conditions: The column is a 3 mm x 150 mm Inertsil reverse phase C-18, ODS 3, 3 µ column (Metachem, Torrance, CA).
- For anthocyanin analysis, the initial solvent system is 10% methanol verses water with 0.1% formic acid at a flow rate of 0.25 mL per minute.
- After injection (1 µl or less) the column is held at the initial conditions for 2 minutes then developed with a linear gradient to 100% methanol and 0.1% formic acid over 50 additional min.
- The column effluent is monitored at 520 nm by the PDA detector.
- The MS is run with the ESI probe in the positive mode.
- The source inlet temperature is set to 300 ˚C, the sheath gas rate is set at 50 arbitrary units, the auxiliary gas rate is set at 5 arbitrary units and the sweep gas rate is set at 2 arbitrary units.
- The maximal mass resolution is set at 30,000, the spray voltage is set at 3.0 kV, the tube lens is set at 100 V.
- The MS is typically calibrated at least weekly with a standard calibration mixture recommended by Thermo Scientific and the signal detection optimized by running the autotune software feature as needed.
- Other parameters are determined and set by the calibration and tuning process.
- The software package will usually be set to collect mass data between 100-2000 AMUs.
- Generally the most significant sample ions generated under these conditions are $[M]^+$.

Methods provided by Berhow and Gude (2021), see also Giusti and Wrolstad (2001).

`phenolics` [decimal]

🧪Phenolic evaluation🧪

Sample prep and extraction

- Freeze-dry samples overnight and grind each to a fine powder.
- Weigh samples ~0.25 g and place in vial with 3 mL methanol:DMSO (1:1) solvent.
- Sonicate for 30 min, allow to stand overnight at room temperature.
- Filter extract through a 0.45 µM nylon 66 filter

Methodology

- We use a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSolutions version 1.22 chromatography software, Columbia, MD, USA) and an Inertsil ODS-3 reverse phase C-18 column (5 µm, 250 x 4.6 mm, GL Sciences, Torrance, CA).
- For phenolic compound analysis, the initial conditions are 10% methanol (or acetonitrile) with 0.25% trifluroacetic acid and 90% water with 0.25% trifluroacetic acid, at a flow rate of 1 ml per minute.
- The effluent is monitored at 280 and 340 nm on the VWD
- After injection (typically 25 µL), the column is held at the initial conditions for 2 minutes, then developed to 100% methanol with 0.25% trifluroacetic acid in a linear gradient over 50 additional minutes.
- Five-point standard curves are used for the evaluation of the concentration of the identified phenolics for the determination of extinction coefficients at 280 and 340 nm.

LC-ESI-MS Confirmation

- Samples are run on an Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer – a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a high energy collision (HCD) cell – with an Ion Max electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump, ACCELA1 HTC cool stack autoinjector, and a ACCELA 80 Hz PDA detector) all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software.
    - The MS is typically calibrated at least weekly with a standard calibration mixture recommended by Thermo Scientific and the signal detection optimized by running the autotune software feature as needed.
    - The MS is run with the ESI probe in the negative mode.
    - The source inlet temperature is 300 ˚C, the sheath gas rate is typically set at 50 arbitrary units, the auxiliary gas rate is usually set at 5 arbitrary units and the sweep gas rate is set at 2 arbitrary units.

  - The maximal mass resolution is set at 30,000, the spray voltage is set at 3.0 kV, the tube lens is set at -100 V.
  - Other parameters are determined and set by the calibration and tuning process.
  - For phenolic analysis, the initial solvent system is 10% methanol verses water with 0.25% formic acid at a flow rate of 0.25 mL per minute.
  - After injection (5 µl or less) the column is developed with a linear gradient to 100% methanol over 50 to 60 min.
  - The column effluent is monitored at 280 nm and 340 nm in the PDA detector. * The software package is set to collect mass data between 100-2000 AMUs. Generally, the most significant sample ions generated under these conditions are [M-1]- and [M+HCOO]-.
- Six mass spec "events" are programmed to run in sequence in the MS detection scheme.
  1) LTQ(IT)-MS full scan m/z 150 to 2000.
  2) LTQ(IT)-MS set to trap the most abundant ion above a threshold of 500 units and perform CID at 35% energy, with the resulting ions being detected by the IT-MS.
  3) FT-MS (Orbitrap) full scan m/z 150 to 2000.
  4) Mass-dependent MS/MS on the most abundant ion trapped by the IT-MS in Event 1 and perform HCD at 25% energy with the resulting fragmentation ions being detected by the FT-MS.
  5) Mass-dependent MS3 on the most abundant fragment ion generated from Event 2 and perform HCD at 25% energy with the resulting fragmentation ions being detected by FT-MS.
  6) Mass-dependent MS3 on the most abundant fragmentation ion generated from Event 2 and perform CID at 35% energy with the resulting ions being detected by IT-MS.
- For the evaluation of Xcalibur accurate mass data by the Cerno BioScience LLC MassWorks 5.0.0.0 software the FTMS is set to collect spectra at a resolution of 7500 and a range of m/z of 100 to 2000 and then evaluated by sCLIPS (self Calibrating Line-shape Isotope Profile Search) which enhances formula ID accuracy.

Remarks

`metabolite_remarks` [nvarchar]

Identification of other metabolites may be provided in the `metabolite_remarks` field.

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- Stack et al. (2021)

PATHOGEN/PEST

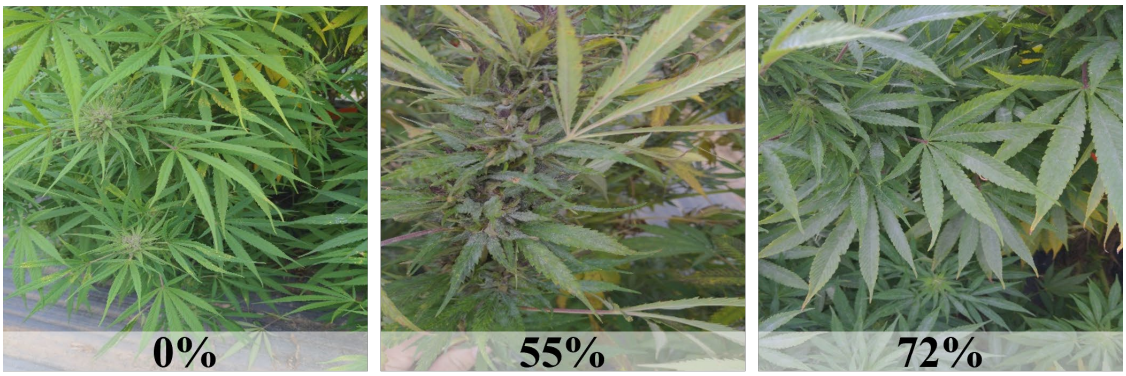
Diseases are scored by indicating% area of planting affected and whether crop failure occurred in conjunction with infection. If one data score is provided for multiple plantings, provide the highest area coverage observed. Ideally the hemp community should decide on a set of check-varieties to include in every trial to compare between seasons.

For consistent scoring of disease and stress data, it is recommended that the same individual be responsible for rating the entire planting.
Refrain from capturing data for a small set or singular accessions, instead prefer an actual growout of a replicated trial that contains numerous accessions in a single season.

Record other relevant information in the `disease_remarks` field. Specifically, it will be useful to distinguish between hop powdery mildew, caused by *P. macularis*, and cannabis powdery mildew, caused by *Golovinomyces ambrosiae*. These appear to possess varying degrees of aggressiveness and phenotyping remarks should include information about which pathogen species was used for phenotyping.

Fungal and oomycetes

Individual plants are visually inspected for powdery mildew severity based on a continuous scale of 0–100% plant area showing disease symptoms according to Stack et al. (2021). Three powdery mildew severity examples are shown below.



Powdery mildew on hemp; photo credit Tyler Gordon

`fungal_xxx` [decimal; %]
Known or suspected fungal/oomycete pathogens:

- *Alternaria* spp
- *Ascochyta* spp
- *Athelia rolfsii* or *Sclerotium rolfsii*
- *Bipolaris spp*
- *Bipolaris gigantea*
- *Boeremia*
- *Botrytis cinerea*
- *Botrytis pseudocinerea*
- *Botrytis porri*

- *Cercospora cf. flagellaris*

- *Chaetomium globosum*

- *Cladosporium* spp
- *Colletotrichum* spp
- *Curvularia* spp
- *Diaporthe eres/ D. subordinaria*
- *Exserohilum* spp
- *Fusarium avenaceum*
- *Fusarium brachygibbosum*

- *Fusarium chlamydosporum*

- *Fusarium equiseti*
- *Fusarium graminearum*
- *Fusarium lichenicola*

- *Fusarium oxysporum*
- *Fusarium proliferatum*

- *Fusarium solani*
- *Fusarium sporotrichiodes*

- *Fusarium tricinctum*

- *Globisporangium irregulare*
- *Globisporangium ultimum*

- *Golovinomyces ambrosiae*
- *Golovinomyces cichoracearum*
- *Golovinomyces spadiceus*
- *Lasiodiplodia theobromae*

- *Leveillula taurica*
- *Leptosphaeria*
- *Neofusicoccum parvum*

- *Penicillium* spp
- *Phoma* spp
- *Phoma multirostrata*

- *Phomopsis* spp
- *Phytophthora* spp

- *Podosphaera macularis* (syn. *Sphaerotheca macularis*)
- *Pseudocercospora* spp
- *Pythium aphanidermatum*
- *Pythium catenulatum*
- *Pythium dissotocum*
- *Pythium myriotylum*
- *Rhizoctonia solani*
- *Sclerotinia sclerotiorum*

- *Sclerotinia minor*

- *Sclerotium rolfsii*

- *Septoria* spp
- *Stemphylium* spp
- *Thielaviopsis basicola*
- *Uredo kriegeriana*
- *Verticillium dahliae*

Bacterial

`bacterial_xxx` [decimal; %]
Known or suspected bacterial pathogens:

- *Agrobacterium tumefaciens*
- *Pseudomonas koreensis*

- *Pseudomonas syringae*
- *Serratia marcescens*

- *Sphingomonas yanoikuyae*

- *Xanthomonas campestris* pv. *cannabis*

Virus/Viroid

`virus_xxx` [decimal; %]
Known or suspected virus/viroid/phytoplasma pathogens:

- Alfalfa mosaic virus (AMV)
- Arabis mosaic virus (ArMV)

- Beet curly top virus
- Cannabis sativa mitovirus 1
- Cannabis cryptic virus
- Citrus yellow-vein associated virus
- Cucumber mosaic virus (CMV)
- Curly top virus
- Lettuce chlorosis virus (LCV)
- Tobacco ringspot virus (TRSV)
- Tomato ringspot virus (ToRSV)
- Tobacco streak virus (TSV)
- Tomato mosaic virus (ToMV)

`viroid_xxx` [decimal; %]

- Hop latent viroid (HLVd)

phytoplasma_xxx

- *Candidatus phytoplasma trifolii*

nematode_xxx

- *Meloidogyne incognita* (Root knot nematodes)

Remarks

`disease_remarks` [nvarchar]

In a short paragraph, describe the disease. Please include the anatomy of the plant affected, the growth stage of the plant affected, symptoms of infection, patterns of spread in field or greenhouse, evidence leading to conclusion of the nature of the pathogen, and any other relevant observations.

Invertebrate

Key pests
Many insects and mites can be observed in hemp and the pest complex can differ depending on whether the crop is cultivated indoors or outdoors. While many arthropods can be found in hemp, some of the most often-seen pests include corn earworm, twospotted spider mite, cannabis aphid, and hemp russet mite.

Invertebrate pests are scored by indicating % area of planting affected and whether crop failure occurred in conjunction with infestation.

Refrain from capturing data for a small set or singular accessions, instead prefer an actual growout of a replicated trial that contains numerous accessions in a single season. Ideally the hemp community should decide on a set of check-varieties to include in every trial to compare between seasons.

Images and text were very generously provided by Kadie Britt -Kadie Britt (2021). We highly recommend John Michael McPartland, Clarke, and Watson (2000), W. Cranshaw et al. (2019), and Britt (2021) as excellent overviews.

Corn earworm

`helicoverpa_zea` [decimal; %]
Helicoverpa zea or corn earworm is the most damaging pest of hemp grown in outdoor environments as it targets marketable portions of hemp plants – floral regions of CBD and seeds of grain varieties -Kadie Britt (2021).

Young corn earworm larva. (Photo credit: Kadie Britt)

Bud rot in floral hemp bud from corn earworm feeding. (Photo credit: Kadie Britt

Adult corn earworm moth on hemp plant. (Photo credit: Katlyn Catron)

Hemp russet mite

`aculops_cannabicola` [decimal; %]
Aculops cannabicola or hemp russet mite is a microscopic, cannabis-specific mite that can be found in indoor and outdoor hemp. Mites have four legs on their white- to beige-colored, cigar shaped bodies and are not visible without the use of magnification Kadie Britt (2021) and John M. McPartland and Hillig (2003).

Hemp russet mites on underside of hemp leaf. (Photo credit: Kadie Britt)

Upward curling of hemp leaves. Can sometimes be a symptom of hemp russet mite feeding injury to hemp but depends on cultivar. (Photo credit: Kadie Britt)

Twospotted spider mite

`tetranychus_urticae` [decimal; %]
Tetranychus urticae or twospotted spider mite is a generalist mite pest that can be found indoors and outdoors. Feeding injury causes stippling marks on leaves (Figure 1.7) and webbing can sometimes be observed in apical portions of plants (Figure 1.8). This mite is small and oval in shape, has 8 legs, and can be orange/red or brown with two distinct dark spots on the body -Kadie Britt (2021).

Twospotted spider mite on hemp leaf. (Photo Credit: Kadie Britt)

Stippling on leaves due to twospotted spider mite feeding. (Photo credit: Kadie Britt)

Webbing in apical portion of plant due to twospotted spider mite populations. (Photo credit: Kadie Britt)

Cannabis aphid

phorodon_cannabis [decimal; %] *Phorodon cannabis* or cannabis aphid is a specialist, piercing-sucking insect that feeds exclusively on hemp. Populations can rapidly increase in favorable environments (Figure 1.10) as aphids can reproduce via asexual reproduction Kadie Britt (2021) and W. S. Cranshaw et al. (2018).

Cannabis aphid infestation on indoor hemp plant. (Photo credit: Kadie Britt)

Cannabis aphid skins caught in honeydew on surface of hemp leaves. (Photo credit: Kadie Britt)

Other pests

Known or suspected pests:

- *Acherontia atropos*
- *Aculops cannabicola*
- *Aecidium cannabis*
- *Agallia constricta*
- *Aglais urticae*
- *Agromyza reptans*
- *Aphis fabae*
- *Aphis gossypii*
- *Camnula pellucida*
- *Ceutorhynchus assimilis*
- *Chinavia hilaris*
- *Chloealtis conspersa*
- *Chlorochroa ligata*
- *Chlorochroa uhleri*
- *Chromatomyia horticola*
- *Cosmopepla lintneriana*
- *Bemisia tabaci*
- *Diabrotica undecimpunctata howardi*
- *Ditylenchus dipsaci*
- *Empoasca fabae*
- *Estigmene acrea*
- *Euschistus servus*
- *Frankliniella fusca*
- *Frankliniella occidentalis*
- *Graphocephala versuta*
- *Grapholita delineana*
- *Halyomorpha halys*
- *Helicoverpa zea*
- *Heterodera humuli*
- *Hysteroneura setariae*
- *Liorhyssus hyalinus*
- *Liriomyza cannabis*
- *Liriomyza strigata*
- *Loxostege sticticalis*
- *Mamestra configurata*
- *Melanchra picta*
- *Melanoplus bivittatus*
- *Melanoplus femurrubrum*
- *Melanoplus lakinus*
- *Melanoplus differentialis*

- *Microtechnites bractatus*
- *Micrutalis calva*
- *Miridae*
- *Nezara viridula*
- *Oebalus pugnax*
- *Ostrinia nubilalis*
- *Pentatomidae*
- *Peridroma saucia*
- *Phorodon cannabis*
- *Phyllophaga tristis*
- *Phyllotreta pusilla*
- *Podosphaera macularis*
- *Polyphagotarsonemus latus*
- *Popillia japonica*
- *Prionus*
- *Pseudoperonospora cannabina*
- *Pseudoperonospora humuli*

- *Psylliodes attenuata*
- *Rhopalidae*
- *Rhopalosiphum abdominalis*
- *Spilosoma virginica*
- *Spissistilus festinus*
- *Spodoptera exigua*
- *Spodoptera ornithigalli*
- *Strymon melinus*
- *Systena blanda*
- *Systena elongata*
- *Tetramorium caespitum*
- *Tetranychus urticae*
- *Thamnurgus caucasicus*
- *Thrips tabaci*
- *Thyanta custator*
- *Trichiocampus cannabis*
- *Uredo kriegeriana*
- *Vanessa cardui*
- Known European species

Additional References

- H M G Van der Werf, W C A van Geel, and M Wijlhuizen (1995)

- Punja, Rodriguez, and Chen (2017)

- W. Cranshaw et al. (2019)
- Eric Anderson (2019)
- Campbell et al. (2019)
- McKernan et al. (2020)
- Szarka et al. (2020)
- Farinas and Peduto (2020)
- Thiessen et al. (2020)
- J. Hu, Masson, and Dickey (2020)

- Stack et al. (2021)

APPENDICES

Feral Hemp Collection

Feral hemp collection protocol by Dr. Shelby Ellison and Dr. Ademola Aina at University of Wisconsin Madison. Germplasm collected from this work COLLECTION, PRE-CHARACTERIZATION, AND PRESERVATION OF AMERICAN HEMP GERMPLASM will be donated to the Plant Genetic Resources Unit.

🧪Feral Hemp Collection🧪

Materials needed:

- Paper bags #10 (6.5 x 4 x 13.25”) (50 per population)
- Reflective vests
- Waterproof folders/bags for datasheets
- Datasheets
- Sharpies
- Pens

Tools required:

- Gloves
- Tape measure
- Camera (Phone’s camera is okay)
- Loppers
- Pruning shears

Protocol

1. Secure state or federal licensing to possess industrial hemp in your jurisdiction.

2. Secure written authorization from your regulatory agency (state department of agriculture) and the University or institutional legal counsel (if required) to collect and hold feral hemp that has not been certified as < 0.3% $$9-THC. Maintain copies of permits, licenses, and/or written authorization during fieldwork and with collected material through all subsequent steps in the protocol.

3. Investigate locations identified by citizen scientists (either through iNaturalist or word of mouth) as potential feral hemp populations. Avoid cultivated fields of industrial hemp (with potential proprietary genetics) and cultivated marijuana, be it state-registered or clandestine and illegal. Endeavor to cover the broadest range of ecoregions that can feasibly be accessed during fieldwork.

4. Secure verbal and/or written permission from the landowner/tenants to collect plant material.

5. Collect GPS coordinates and take pictures of the plants while capturing their immediate surroundings using a smartphone. Upload new coordinates along with pictures on iNaturalist for future collection purposes. Record a brief description of the site – drainage patterns, surrounding vegetation, elevation, and soil classification from the web soil survey on the provided datasheet.

    a. Preferred population size > 50 female individuals, at a minimum of 10 females.

    b. Populations separated by at least 5 miles (8km) are preferred to increase the likelihood of pollen isolation.

    c. Give each population an ID code in this scheme: state (WI), year (23), collector's initials (SE), two-digit sequential program number (01), and plant number (01) -i.e. WI-23-SE-01-01.

    d. Upload photos to a shared computer drive for backup

6. Locate up to 50 female (seed-bearing) plants. For each plant, label a large paper bag with an ID code. Chop down at the base of plant with the loppers, lay on the ground, and measure length (primary meristem to base of plant) with a tape measure, record the ID code and length in the nearest inch on the datasheet. Use the pruning shears to cut the terminal and lateral seed-bearing branches into 6-8" sections and fill the bag up to ¾ of its height, allowing enough space to fold the top of the bag over. Do not collect more material than would fit a single bag. Collecting each plant in a single, separate bag is essential. If there are more seed-bearing branches on a plant than fit the bag, you might prioritize the most heavily seeded branches but do not overstuff the bag and do not put material from multiple plants in the same bag.

7. Hot, moist conditions are detrimental to seed viability. Bagged material should begin drying within 12 hours of collection. At no point should the paper bags be

enclosed in plastic. Samples should be kept as cool as possible during transport from the field to a location where they can be dried.

8. Dry the bags at room temperature with a fan to move air for at least one week. Seed and plant material can be placed in a drying oven as long as temperatures are less than 35 °C (95 °F). Conditions exceeding 35 °C (95 °F) are detrimental to viability.

9. Once the plants are dried, hand-strip the branches to remove the bulkier stems from each bag. Stems may be discarded in compost or as waste. Fold the top of the bag over and staple or tape it closed.

10. Line in a shipping box with a plastic bag, place paper bags and the datasheet(s) inside the plastic bag, seal the box. Attach the shipping label provided and arrange for a Fedex pickup or deliver to a Fedex drop-off location. Refer to the contents only as "Dried plant material for scientific research". Not hazardous and of no commercial value.

Data collection sheet should have the following details:

- Collector's name
- Date (YYYY-MM-DD)
- Location (verbal)
- Latitude and Longitude (decimal)
- State
- Population #
- Site Description
- Column Headers: Plant, Length (cm)

Threshing

🧪Threshing🧪

Hemp has valuable seed and grain that can be difficult to harvest by hand, especially in large quantities.

Example sample from PGRU trials

PGRU runs fatty acid and protein analysis for all germplasm held in the repository using three small, electric Almaco BT 14 belt threshers.

Belt threshing rig at PGRU

To safely and efficiently remove and separate seed from hemp stalks, PGRU follows the following protocol:

Personal Protective Equipment (PPE)

Protective eye wear, ear plugs, N-95 mask, gloves, closed-toed shoes, long pants, long sleeve shirt.

Useful Items

Trash bin, pruning shears, working table, bins for seed collection and chaff, vacuum, air compressor.

Procedure

- Verify the room you are using is well ventilated, turn on system.
- Verify the machine was cleaned after last use. Ensure that the thresher has been properly maintained and the belts, chain, and electric connections are in good working condition.
- Move thresher into open space and let others know that you will be threshing (generating dust and noise).
- Set up a seed collection tray on the back end of the thresher under the seed collection compartment.
- Turn machine on and verify both belts are operating smoothly. The belt speed can't be adjusted. The distance between belts can be adjusted.

- The fan on the thresher has variable air flow and can be turned off depending on the application.
- Set up a line of bags and a trash bin on the side you will be threshing.
- Pull individual stalks from the harvest bag and inspect them for seeds/chaff, if no seed or chaff is observed, discard the stalks. If seed or chaff is observed, hold the base of the stalk, and place it on the tray of the thresher. Slide the stalk into the running belts of the thresher and let the stalk go through the thresher. Sometimes the thresher is unable to remove all the seed, if this happens manually remove the seeds from the stalk onto belt. When all the seed has been removed, discard the stalks. Continue this until all stalks have been removed from the bag.
- The harvest bag will have smaller stems, seed, and chaff after the larger stalks have been removed. Empty the remaining bag contents into the thresher.
- Remove any large stalks from the seed collection bin and empty the collected seeds and chaff back into the harvest bag.
- Roll or fold the bag down to a small size and place the bag in a cool dry room for further processing.
- Turn off the thresher and clean the area at the end of the workday.

Considerations

- Consider the max diameter of half an inch for stalks that can go through the belts
- Constant monitoring of your airflow can be achieved by checking for clean seed (lacks chaff and stalk)
- One full sized stalk should only take 10-30 seconds run through the belts.
- Hemp variety plays a key role in threshing efficiency. Make notes of any hemp lines that have unique characteristics (i.e., lack of shattered seeds before threshing).

Cloning

🧪Cloning🧪

Cuttings should be harvested from disease-free stock plants under non-stressed conditions. Collect turgid cuttings during optimal water conditions (non-wilted plants). Environmental conditions that increase propagation success are provided by an atmosphere that reduces water loss and maintains leaf turgidity, with optimal humidity between 75 and 90%, often achieved using humidity domes or “mini greenhouses”. Ample but not excessive light and clean, moist, and well aerated rooting media should also be provided (Casillas 2016). Ideal temperature should be around 25-27 °C (78-80 °F) with a root zone temperature of about 27-30 °C (80-85 °F). Once cuttings are taken, check for disease daily along with moisture content of media. If using humidity domes, monitor humidity levels and include fresh air daily.

Materials

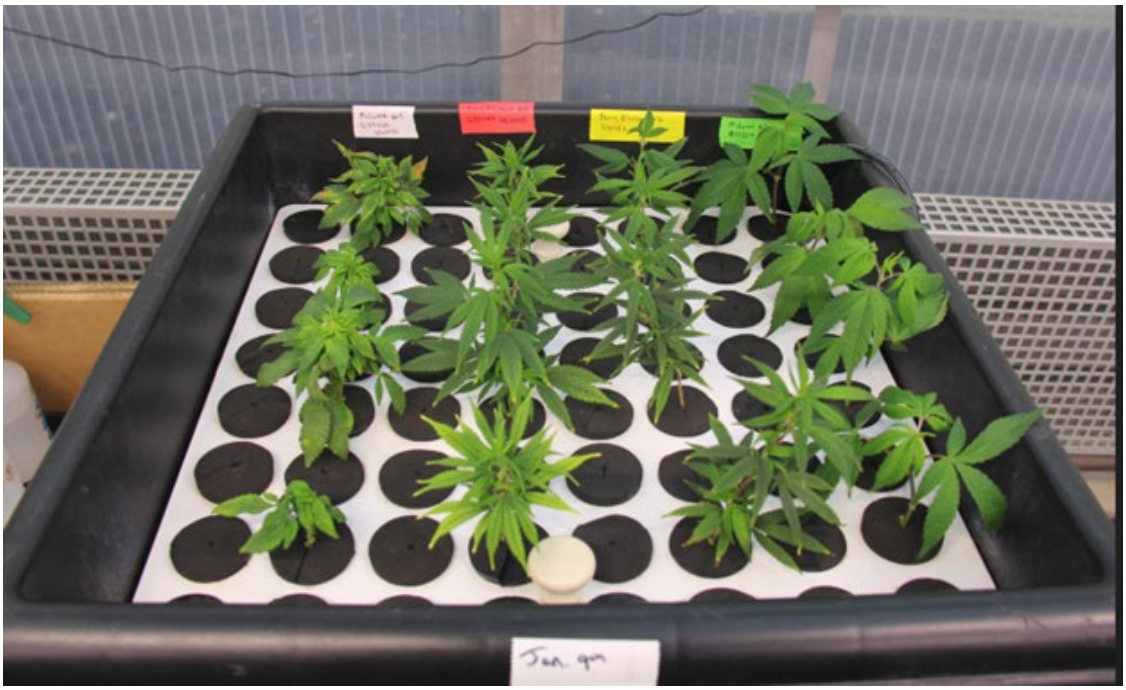
- Scissors/Pruning shears

- Lab Gloves
- IBA based rooting Hormone (we use Clonex™)
- Beakers/Cups
- Spray Bottle w/ water
- Sterile medium (potting soil/Oasis Cubes/Water)
- Humidity domes (if using potting soil + trays)
- 70% ethanol (alternative isopropyl alcohol)
- Paper towels
- Seedling flats
- Disease-free Cannabis plants (ideally in the vegetative state)
- Aeroponic apparatus
- Mist timer
- Heating mat

Common cloning tools and supplies

Methods (Aeroponic/Hydroponic)

1. Start with clean water (DI or tap) with no fertilizer
2. Cut lateral branch close to a node at a 45-degree angle
3. Gently scrape the stem around the cut to expose cambium layer
4. Remove any lower branches, keep approximately 3-5 nodes worth of leaves, leaving cutting looking like a “palm tree”
5. If taking many cuttings at a time, place cuttings in a beaker/cup of water until ready to dip into rooting hormone
6. Dip bottom 2 cm of cutting in rooting hormone, IBA (Clonex™)
7. Place cutting in aeroponic/hydroponic system
8. Let sit and watch for roots (7-14 days)
9. Use alcohol to disinfect tools before moving on to the next accession



Aeroponic cloning setup at PGRU

Methods (Potting soil/Oasis Cubes/Humidity Dome)

1. Cut lateral branch close to a node at a 45-degree angle
2. Gently scrape the stem around the cut to expose cambium layer
3. Remove any lower branches, keep approximately 3-5 nodes worth of leaves, leaving cutting looking like a “palm tree”
4. If taking many cuttings at once, place cuttings into beaker/cup filled with water until ready to dip into rooting hormone
5. When ready to root cuttings, emerge bottom 2cm of cutting in rooting hormone (Clonex™)
6. Place cutting with rooting hormone into seedling tray with potting soil or oasis cubes
7. Place humidity dome onto tray, mist water on inside of humidity dome
8. Let sit and watch for roots (7-14 days)
9. Use isopropyl alcohol to disinfect tools before moving on to the next accession

Typical humidity dome setup

Modified aeroponic rooting system protocol (Regas et al. 2021)

1. Generation of a mother plant for clonal propagation
    a. Select healthy, female mother plant that exhibits desirable traits
    b. Allow mother plant to reach the appropriate size (roughly 25 mature shoots) for clonal propagation
    c. Allow mother plants to remain in the vegetative growth stage (light:dark = 18h:6h) to promote shoot growth for future propagation
2. Construction and preparation of aeroponic system
    a. Begin by positioning the lid on top of the container. Drill desired number of holes into the lid while providing adequate space (~3cm) between each
    b. Position water pump in the center of the container
    c. Pour 7-8 L of distilled water into the container so that the pump nozzle remains roughly 2.3 cm above the waterline. NOTE: This ensures the submersible water pump can push water with enough force to spread across the container lid. Distilled water is recommended; however, regular tap water may also be used.
    d. Situate the appropriate amount of Oasis Cubes or media of choice into each slot. Turn on the pump and allow it to run for 24 h on a set timer.
3. Selecting and excising appropriate shoots
    a. Collect shoots near the apical meristem using a sterilized scalpel or scissor. Cuttings are ~10 cm in length, ideally with several nodes. NOTE: Cut the stem at a 45° angle. Cutting at a 45° angle increases the surface area of the basal portion of the cutting, allowing more space for root development.
    b. Remove all foliage except foliage present on the top three nodes

c. Dip the newly excised cutting into the rooting solution containing indole-3-butyric acid (IBA) ~ 2-5 cm up from the base of the stem for ~5 s
d. Insert the cutting into the center of an Oasis cube positioned in the aeroponic system. NOTE: the cutting insertion depth is to remain ~1-2 cm from the bottom of the Oasis Cube
e. Mist the unrooted cuttings with the water every 100 seconds for 20 seconds
f. Grow the cuttings with 18-24 h of light per day with a photosynthetic photon flux density (PPFD) of 100 µmol/m2/s at 24-29°C and 40-60% relative humidity.

4. Aeroponic system maintenance and propagule health
   a. Replenish the system with water at a pH between 5.0-6.0 every 2-5 days
   b. Lightly mist the cuttings every 100 seconds for 20 seconds
   c. Add 5 mL of each nutrient solution to the reservoir every 3-5 days
   d. Add 15 mL of the algae and bacteria cleaning solution containing hypochlorous acid (0.028%) per 10 L of water every 5 days
5. Transplanting propagules
   a. Select the cuttings with long, white, fibrous roots. NOTE: Avoid cuttings with brown, slimy, and short root systems as this is an indicator for the presence of root rot and will usually take longer to acclimate to the new growing medium and can bring unwanted diseases.
   b. Place cutting into potting soil media, transplant propagules to 4 L nursery pot filled with a nutritious soil mix. NOTE: Watering immediately is recommended to prevent the roots from drying out.
6. Cleaning and storage of aeroponic system
   a. When the system is no longer in use, wash with water and clean with 70% ethanol or another disinfectant (i.e Greenshield)
   b. Remove the filter from the water pump and rinse with water to remove debris
   c. Dry the system by wiping it down with paper towels or washcloth
   d. Place pump inside the tub with the lid on and store until it is needed.

Tissue Culture

🧪Tissue Culture🧪

PGRU is extremely grateful to Faith Sparks for the development of this protocol.

Protocol Overview:

1. Explant Collection, Preparation, and Induction
2. Sterilization
3. Media Composition & MS Vitamins Recipe Templates
4. Acclimatization
5. Best Practices

6. In-Vitro to Ex-Vitro Workflow chart
7. References

Abbreviations:

- CaCl: Calcium Chloride
- ddH_2O: deionized, distilled H_2O
- DLI: Daily light integral
- EDTA: Ethylenediaminetetraacetic acid
- FeEDTA: Iron + EDTA (chelated iron)
- KPO_4: Potassium Phosphate
- $MgSO_4$: Magnesium Sulfate
- µmol $m^{-2}s^{-1}$: micromole per meter squared per second
- MS: Murashige and Skoog
- NH_4NO_3: Ammonium Nitrate
- RH: Relative humidity
- UV: Ultra violet

Explant Collection, Preparation, and Induction:

Figure I, Stock plants kept under long day photoperiod of 18 hours of HPS light at 25-28°C and 65% RH.

- Choose unlignified, young, vegetative, preferably axial nodal tissue as explants for in-vitro propagation (*Fig. I*).
- Selectively cut the stem on an angle above a node to preserve structural integrity of the stock plant.
- Take cuttings from lateral branches and leave the most apical shoots on the stock plant.
- Be sure to consider the direction of the apical node left on the stock plant to encourage growth in the direction most conducive to your growing environment (Fig. I).

Figure II, Cuttings after surface sterilization (a), A cutting being processed to remove excess tissue prior to sterilization(b), explants ready to be placed in sterile media (c), Example of under-the-hood organization for aseptic technique (d).

- Be sure to take cuttings that have at least three-six meristematic nodes and are 4-6" in length (*Fig. II.a*).
- Always use clean and sharp shears.
- Work from cleanest facilities to most infected to reduce pathogen spread.
- Clean shears with ethanol if possible before moving on to the next plant.

- Avoid taking cuttings from any visibly infested plants. Modify sterilization procedure to accommodate pest pressure.
- Remove all fan leaves at least ½-1 inch from the stem leaving the length of the petiole (this will serve as a protective measure for the meristematic tissue against the destructive effect of the ethanol, bleach, and mechanical agitation used in the sterilization process (*Fig. II.b*)
- Place basal end of the cutting in clean water. Enclose the container if possible. Return nodal tissue to the laboratory as quickly as possible.
- When transporting fresh cuttings, it is helpful to place the vessels in a cooler with ice to maintain the cuttings' freshness as well as to halt bacterial growth at the cut sites prior to sterilization and in-vitro induction but this is not necessary for successful explant initiation.
- Always ensure the cuttings' basal ends are placed in vessels containing water for transport back to the laboratory. Plants will not survive the sterilization procedure if they have lost too much turgor pressure.

- It is best to sterilize and induct cuttings in-vitro within hours of sample collection, but it is possible to hold the fresh cuttings at 4°C with ample relative humidity overnight if necessary. Do not sterilize cuttings prior to storage if they are to be held at 4°C but do remove all foliage to reduce transpiration and to conserve space.
- Sterilize cuttings directly proceeding in-vitro induction after overnight storage.
- After sterilization, further separate meristematic groupings and remove damaged tissue with a sterile sharp scalpel so that each explant has about two meristematic buds per 2" of stem under a flow hood in a sterile environment with sterile tools (*Fig. II.c*).

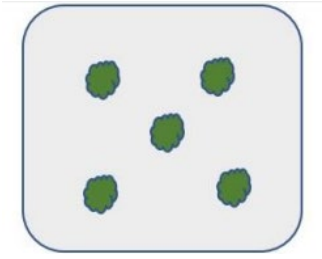


Figure III, A graphical view of a culture vessel from above with a suggested explant placement for invitro micropropagation of hemp.

- Place explants in media in the pattern shown in (*Fig. III*).
- Replace vessel lid and place vessel in low light environment at 28 ◦ C and 24-hour light.
- Gradually increase the PAR to 50-90µmol $m^{-2}s^{-1}$ over the course of a week, (*Fig. IV*).

Figure IV, Differing arrangements or vessels may be used depending on the environment of demands of the cultivation system. Pictured are standard GA-7 Magenta-type boxes.

Sterilization:

- Spray down work surface with at least 70% ethanol and allow to dry
- If available, use a UV light to reduce the population of additional microbes. Some would say this is unnecessary, however, it is the technician's preference. UV light will damage plant tissue so do not leave plant tissue exposed to UV light.
- Sterilize all tools with flame or other heat source and use sterile glass plates for a cutting surface. The recommendation is to use 125mm glass Pyrex dishes wrapped with aluminum foil and lined with sheets of 125mm Whatman paper. Autoclave for 20 minutes with a 20-minute dry cycle or comparable cycle to ensure sterility.
- If desired or necessary, bring microscope into flow hood as cleanly as possible.
- Submerge cuttings in 70% ethanol and seal the vessel under the flow hood. Apply gentle agitation for a duration of 2-2.5 minutes. Be sure to provide wiggle-room for the cuttings to be adequately sterilized on all surfaces. It is recommended to use 50mL conical vials for these submersion steps.
- Drain away the ethanol and rinse the cuttings once with sterile ddH_2O.
- Submerge the cuttings in 0.75-1.25% sodium hypochlorite (dependent on hemp variety and level of pathogen/pest present on the stock plant tissue) supplemented with 1-2 drops of tween per 100mL of bleach solution as needed (i.e. If there was a recent spray in the greenhouse or if there is substrate present on the plant tissue). Seal the vessel and apply gentle (<50rpm) agitation for ten minutes.
- Immediately drain away the bleach/tween solution and rinse five or more times with sterile ddH_2O until the smell of bleach is no longer detected and there is no more sodium hypochlorite left on the plant tissue

Media Composition and MS Recipe Templates

- Excel Table (?)

Acclimatization

Figure V, A C. sativa L. plantlet from tissue culture ready to acclimate into the greenhouse.

- Remove the vessel lid and pour clean and/or sterile H_2O (approx. 50 mL) on the media.
- Replace the covering ajar.
- Change the water once or twice per day for three days and allow the vessel closure to be increasingly open each day (*Fig. V*).
- Pull plantlet out of the media and gently remove excess media on the roots.
- Plant the rooted plantlet in LM-111 premoistened soil mix and water in with clean water.
- Cover the tray with a tall dome and keep humidity high within the dome.
- Place in a cool (but not less than 25°C) spot of the greenhouse out of direct light for one week and maintain humidity and circulation consistently (avoid spikes and dips in RH and for photoperiod sensitive plants maintain vegetative growth with short nights and long days dependent on genotype).

Figure VI, Hemp plants from tissue culture after acclimatization

- Remove the dome when new growth begins to appear in approx. one week (*Fig. VI*)
- Plantlets have survived omissions of the first four steps in this section, but acclimatization success rate is higher when a gradual humidity acclimatization is performed.

Best Practices

- Upon tissue collection, try to keep the humidity high in the transfer vessel and avoid direct light or heat as these abiotic stressors may influence turgor pressure and make the cuttings more susceptible to unreconcilable damage.
- **Always**
  give newly inducted tissue a gradual introduction to high light environments. The reduction of shock is necessary after the sterilization procedure.

- Never leave visibly damaged tissue on the cuttings after sterilization.
- It is best to make media prior to tissue collection.
- Use best judgment when transferring tissue early in-vitro and allow time and labor to be spent removing dead and dying tissue.
- Always truncate cuttings to two-three nodes per explant, and discard the most apical node.

- Be sure your scalpels, forceps, and tool rests are all sterile and cooled to room temperature before allowing them to encounter sterile plant tissue, (hot tools cause unreconcilable damage to the delicate plant tissue and should not be used to handle the explants).
- The author suggests using #10 blades and developing a cutting technique that leaves cleaner cut sites
- If there is any doubt the sterile explant has touched an unsterile surface discard explant immediately and re-sterilize the tool it is not worth contaminating a whole box or the rest of the sterile plant tissue.
- Do not overcrowd explants in the vessel if you can avoid it or the shoots will develop etiolated tissue that cannot be easily acclimatized back to the greenhouse, it is best to provide adequate space for shoots to receive a sufficient DLI.
- Whenever possible, chose to use ventilated culture vessels. Providing sufficient gas exchange allows the tissue to develop more normally (i.e. not hyperhydric or vitrified) and saves time and resources throughout the process. Keep in mind ventilated lids make the cultures more susceptible to pathogen invasion. Adjustments to transfer schedule may be necessary depending on the system and biological demands.
- Sow initial cultures onto Hemp TC media for one week up to one month depending on the number of transfers that are necessary in early explant induction to eliminate persistent endogenous microbial populations or lingering contaminants.
- Long-night genotype cultures can be maintained on Hemp TC media indefinitely under a 24-hour photoperiod at approx. 50-90µmol $m^{-2}s^{-1}$ at 28°C without

flowering. Not all varieties of hemp are well suited to this environment, though and this may differ according to photosensitivity of the genotype, adjust lighting as necessary.

- Depending on contamination levels, cultures may require additional sterilization procedures throughout the cultures' in-vitro lifespans. It may be necessary to shorten the length of time between subcultures to reduce the pressure of the contaminant.
- In some instances, the most efficient course of action is discarding the contaminated cultures. To account for this potential loss it is recommended to take approx. 10% more tissue at explant induction and discard cultures that contain persistent contamination.

In-Vitro to Ex-Vitro Workflow Chart

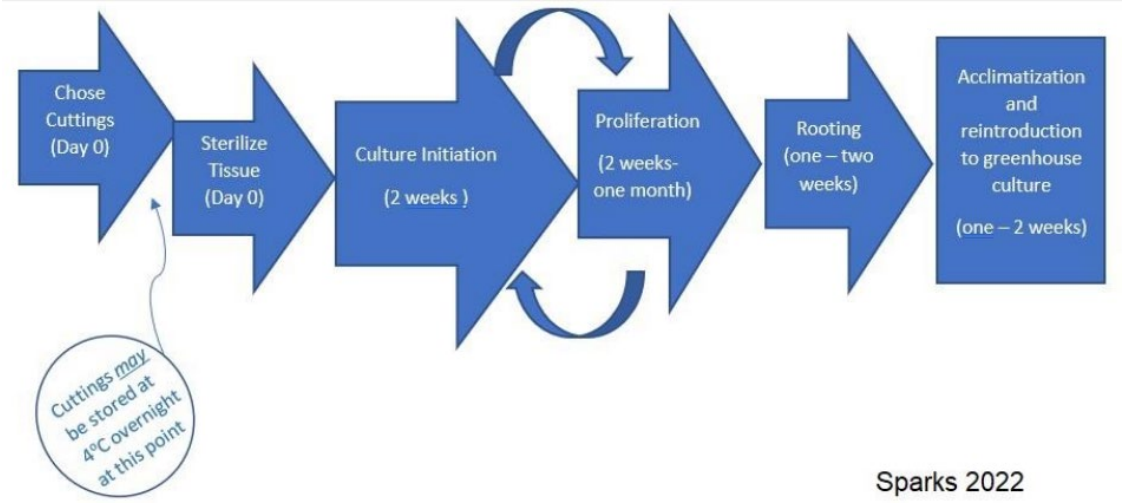


Figure VII, Workflow-chart of a typical hemp tissue culture project. Components should be adapted to the environment and genotype for healthiest propagules.

📚Additional References📚

- Hesami and Jones (2021)
- Lubell-Brand, Kurtz, and Brand (2021)
- Peterswald et al. (2023)
- Stephen et al. (2023)
- M.-R. Wang et al. (2022)

Pollen Collection Protocol

🧪Pollen Collection🧪

Created by: Daniel Meyers (last updated: March 30, 2023). Based on an unwritten protocol developed by Nicholas Genna during Summer 2022.

Background

Reliable pollen collection and storage methods are important for germplasm conservation, breeding efforts, and scientific inquiry. This protocol evolved during the collection and study of hemp (Cannabis sativa L.) pollen at the Plant Genetic Resources Unit (USDA-ARS) in Geneva, New York during the summer of 2022. The fine meshes were added to the existing components to eliminate the collection of insects and parts of flowers and leaves.

Materials

- Spore collection kit (Large Spore Cyclone, GRA-101, Tallgrass Solutions Inc.)
- Vacuum (Super Coach Pro 6, 107310, ProTeam)
- Glass vials (three included in above kit)
- 100 µm mesh, cut into squares (~12 cm x 12 cm)
- Rubber bands
- Air compressor or shop-vac with a blower port
- OPTIONAL: 10 µm mesh, cut into squares (~12 cm x 12 cm)
- RECOMMENDED:
  - Cooler with ice packs
  - Gloves
  - Ethanol (70%, spray bottle)
  - Face mask (for use if collecting for a long time, the author recommends N-95 or comparable)

Figure 1: Demonstration of pollen collection. Picture of Tony Barraco, by Anthony Rampulla.

Methods

1. Assemble the pollen collection apparatus and attach it to your vacuum (figs. 4-7). OPTIONAL: Place the 10µm mesh between the collection device and the vacuum. This is advised when collection of all pollen is the goal (e.g., if you are measuring total pollen produced at the individual level), as minimal pollen is lost into the vacuum, and some suction power is sacrificed.
2. Place a 100 µm mesh square on the nozzle of the pollen collection apparatus and fix in place with a rubber band. NOTE: As collection goes on, suction power will be lost

over time due to the mesh getting plugged up with a mixture of pollen and secondary metabolites. This issue can be resolved by moving the mesh over slightly and fixing it in place again, and eventually by replacing the mesh sheet. Mesh sheets are reusable after washing. The author recommends scrubbing with dish soap and, if the mesh is still sticky or stained, soaking it in 10% bleach for 24 hours, then rinsing with DI water.

3. Turn on the vacuum and collect the desired pollen by holding the pollen collection apparatus in one hand to an inflorescence and using the other hand to cup the inflorescence opposite the nozzle. Collect from the entire plant moving from inflorescence to inflorescence, and from lower branches to upper branches, attempting not to shake the plant during this process (and therefore lose pollen).
4. Turn off the vacuum, unscrew the collection vial and cap it. If you are not using the pollen or taking it back to be stored immediately, place it in a cooler with ice packs while you continue collecting.

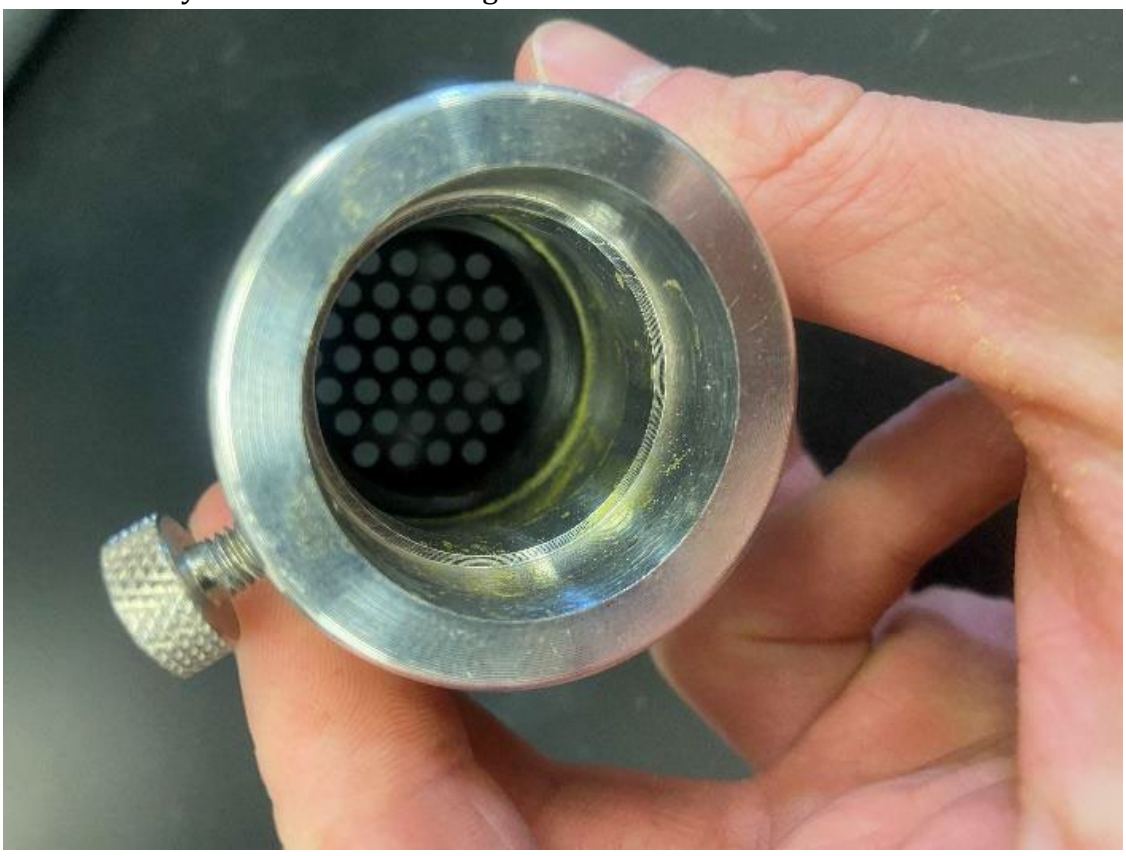
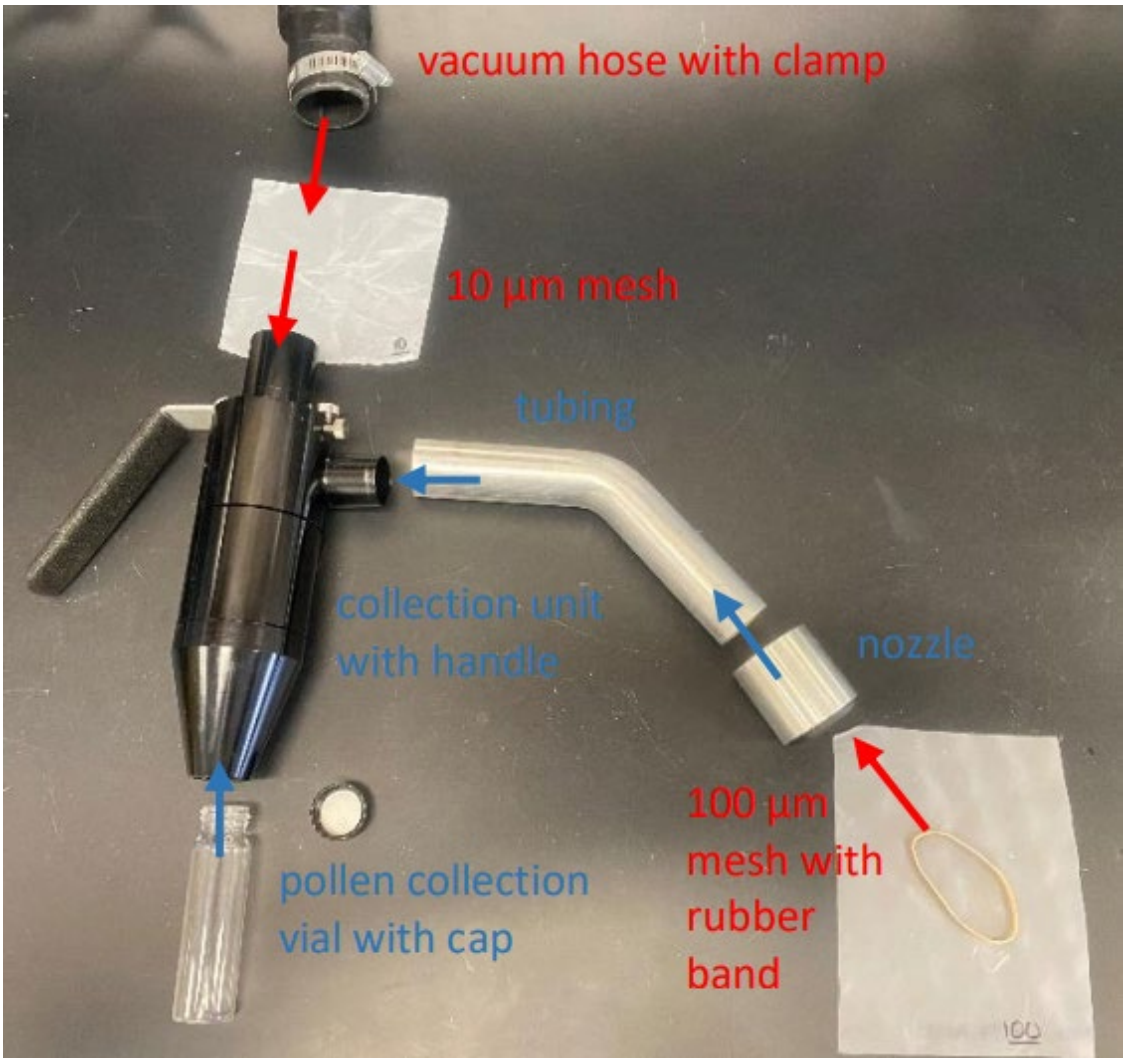


Figure 3: Pollen can collect along internal grooves and corners of the collection apparatus.

5. To minimize contamination, take the following steps between pollen collections:
    a. Replace all mesh used for the previous collection.
    b. Disassemble the pollen collection apparatus and blow it out using an air compressor or the blower port on a shop-vac. Make sure to clean out inside corners of the apparatus where pollen can collect (fig. 3).

c. Wear gloves, sterilize with 70% ethanol, and allow to dry.


vacuum hose with clamp
10 µm mesh
tubing
collection unit
with handle
nozzle
100 µm
mesh with
rubber
band
pollen collection
vial with cap

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