Work in Progress

The NCGRP has successfully placed 3 accessions of *Humulus lupulus* in liquid nitrogen (LN) for long-term storage. The encapsulation-dehydration method used for the cryopreservation of *Humulus* accessions was adopted from Martinez et al. (1999) with a single modification. Based on research at the National Clonal Germplasm Repository (NCGR) *Humulus* is multiplied on a medium supplemented with Sequestrene™ iron (Reed and Aynalem, 2005). Viability of the *Humulus* accessions placed in long-term storage ranges from 50 to 80%.

Plant material was obtained in cooperation with the National Clonal Germplasm Repository (NCGR) Corvallis, Oregon. The NCGR screened all accessions for disease prior to their cryopreservation at NCGRP.

In vitro culture of *Humulus*

Shoot tips were multiplied on **Humulus growth medium I** (HGI) and grown in Magenta® GA7 culture vessels (Magenta Corp., Chicago, IL) for 3 weeks. After 3 weeks, plants were subcultured on **Humulus growth medium II** (HGII) and allowed to grow for an additional 3 weeks. A subcultured section consisted of one to two axillary nodes, with or without the shoot tip. All *in vitro* cultures were kept in a growth room (see supplemental information).

Method used to cryopreserve *Humulus* shoot tips: Encapsulation-dehydration

All steps take place under aseptic conditions and at room temperature (21 °C) unless noted otherwise.

I. Cold acclimation

Subculture *in vitro*-grown plants, multiplied previously for 3 weeks on **HGI** then 3 weeks on **HGII**, using fresh **HGII medium** and move to a cold acclimation chamber (see supplemental information) for 15 days.

II. Control beads (day 1)

Blank beads (without a shoot tip) are used to estimate the moisture content of non-control beads.

1. Using a plastic pipette with an opening of ~1 mm dispense 30 drops of **Ca-free MS+3% Na-alginate medium** into a flask containing **100 mM calcium chloride+MS encapsulation medium** (30 beads/50 ml medium). If successful, each drop will form a bead. Allow the blank alginate beads to remain in the encapsulation liquid for at least 30 minutes. As they polymerize, the beads change in appearance from transparent to whitish-opaque.

2. Once polymerized, transfer beads to a flask containing **0.75 M sucrose+MS medium** (30 beads/50 ml medium). Cover flask with sterile aluminum foil and place on a rotary shaker for 18-20 hours (100 rpm).

III. Determining dry weight (DW) of blank beads, shoot tip isolation and encapsulation (day 2)

A. Determining dry weight (DW) of blank beads

1. Remove blank beads from the sucrose medium and roll on filter paper to remove excess moisture.

2. Place beads into three pre-weighed aluminum weigh boats (10 beads/weigh boat). Immediately weigh the beads and then transfer to an oven set at 103 ^{o}C for 18–22 hours.

3. Remove beads from oven and weigh in weigh boats; average the three weights and record value as the **dry weight (DW)**. Discard beads.

B. Shoot tip isolation

1. Isolate shoot tips from cold acclimated plants and place into a Petri dish containing **Ca-free MS medium**. Allow shoot tips to remain suspended in the solution until the desired number has been isolated. Shoot tips consist of the apical dome and 1-2 leaf primordia (~0.8–1.0 mm).

C. Encapsulation

1. Remove medium from Petri dish leaving the shoot tips. Pour enough **Ca-free MS+3% Na-alginate medium** into the dish to cover all shoot tips. Using a modified plastic pipette (opening cut to ~1 mm), transfer one shoot tip at a time and drop into **100 mM calcium chloride+MS encapsulation medium**. Allow the beads with shoot tips to remain in the encapsulation medium for at least 30 minutes to polymerize.

2. Once polymerized, transfer beads to a flask containing **0.75 M sucrose+MS medium** (25 beads/50 ml medium). Cover each flask with sterile aluminum foil and place on a rotary shaker (100 rpm) for 18-20 hours.

D. Make additional blank beads

1. Make 30 blank beads (without a shoot tip) following encapsulation steps 1 and 2.

IV. Bead dehydration, calculating moisture content and vitrification (day 3)

A. Dehydration of beads with shoot tips

1. After 18–20 hours, remove beads with shoot tips from **0.75 M sucrose+MS medium** and roll on sterile filter paper in a Petri dish to remove excess moisture.

2. Place beads on the bottom of an overturned sterile Petri dish (25 beads/Petri dish). Place Petri dishes approximately three inches from the back of a laminar flow hood to dry.

B. Dehydration of blank beads and determining fresh weight (FW)

1. After 18–20 hours, remove blank beads from **0.75 M sucrose+MS medium** and roll on filter paper in a Petri dish to remove excess moisture.

2. Place blank beads on the bottom of three overturned, pre-weighed Petri dishes (10 blank beads/Petri dish). Weigh the dishes with the blank beads; Average the three weights and record value as the **fresh weight (FW)**. Return beads to flow hood after weighing.

3. Evenly disperse the dishes with the blank beads among the dishes with the shoot tips in the laminar flow hood.

C. Calculating moisture content using blank beads

1. While drying, periodically remove the Petri dishes with the blank beads; weigh each dish with beads. Determine the fresh weight by averaging the three weights. Calculate the moisture content:

$$[(FW - DW) / FW] \times 100 = \%$$

2. When the blank beads reach an average moisture content of 18-22%, the encapsulated shoot tips are ready for vitrification.

D. Vitrification

1. Place beads with shoot tips into 1.2 ml cryovials (10 beads/cryovial). Submerge vials into liquid nitrogen.

V. Rewarming and Recovery

Keep cryopreserved shoot tips in liquid nitrogen for at least one hour prior to rewarming.

A. Rewarming

1. Remove cryovial from liquid nitrogen, uncap and allow it to rewarm in a laminar flow hood at room temperature for 5 minutes, or until thawed.

2. Once thawed, remove beads from the vial and place in a Petri dish containing **Humulus recovery medium**. Move Petri dish to dim light for 7 days.

B. Recovery

1. After one week in dim light, excise shoot tips from beads and place on fresh **Humulus recovery medium**. Return shoot tips to dim light for an additional 7 days.

2. After 14 days in dim light, move shoot tips to full light in a controlled growth room (see supplemental information). Viability on rewarmed LN-treated shoot tips can be assessed 4-6 weeks post thaw.

Supplemental Information

In vitro cultures of *Humulus* were grown in an environmentally controlled growth room set at 25±3 °C with a 16-hour light/8-hour dark photoperiod. Light intensity was 55 µmol m^{-2} s^{-2}.

The cold acclimation chamber was programmed for 5 °C with 24 hours of darkness.

References and supplemental reading

Martinez, D., R.S. Tames, and M.A. Revilla. 1999. Cryopreservation of in vitro-grown shoot-tips of hop (*Humulus lupulus* L.) using encapsulation/dehydration. Plant Cell Reports 19: 59-63.

Murishige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plantarum 15:473-497.

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Reed, B. M. and H. Aynalem. 2005. Iron formulation affects in vitro cold storage of hops. Acta Horticulturae 668: 257-262.