Cryopreservation of *Humulus* germplasm

At the NCGRP, cryopreservation of *Humulus* (hop) shoot tips is based on an encapsulation-dehydration method adopted from Martinez et al. (1999) with a single modification of a multiplication medium that is supplemented with Sequestrene™* iron (Reed and Aynalem, 2005). Plant material, in a form of tissue culture used for cryopreservation at the NCGRP was obtained through collaboration with the National Clonal Germplasm Repository (NCGR) Corvallis, Oregon. The NCGR screened all accessions for virus infection 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 three weeks. After, plants were subcultured on *Humulus* growth medium II (HGII) and grown for an additional three 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).

Procedure for cryopreservation of *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, previously multiplied, to a fresh HGII medium and move the culture 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°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).
2. 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, Tames RS, Revilla MA. 1999. Cryopreservation of in vitro-grown shoot-tips of hop (*Humulus lupulus* L.) using encapsulation/dehydration. Plant Cell Reports 19:59-63.

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

Reed BM. 2004. Shoot-tip cryopreservation manual. National Clonal Germplasm Repository-Corvallis, OR, USA. Pp 39.

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

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