Cryopreservation of *Mentha* sp. germplasm

At the NCGRP, cryopreservation of *Mentha* (mint) shoot tips was based on three methods: vitrification without cold acclimation (30 accessions); vitrification with cold acclimation (9 accessions), and encapsulation-dehydration (5 accessions.) The project started 1998 and ended in 2006 resulting in 44 accessions successfully placed in liquid nitrogen with 40 to 94% viability. Several accessions were cryoprocessed at the National Clonal Germplasm Repository at Corvallis, Oregon and placed in the NCGRP cryotanks for long-term storage. The cryostored material includes 19 *Mentha* species and hybrids: *M. aquatica, M. arvensis, M. australis, M. cervina, M. cunninghamii, M. diemenica, M. gattefossei, M. haplocalyx, M. japonica, M. longifolia, M. pulegium, M. requienii, M. spicata, M. suaveolens ssp., M. x maximilianea, M. x. piperita, M. x smithiana, M. x verticillata and M. x villosa*.
Plant material in the form of tissue culture used for cryopreservation at the NCGRP was obtained through collaboration with the Corvallis repository.

In vitro* culture of *Mentha

Mentha plantlets were established in Magenta® GA7*culture vessels (Magenta Corp., Chicago, IL*) on Murashige and Skoog (MS) growth medium and subcultured every 4 to 12 weeks depending on the growth rate of an individual accession. The growing density was 13 plantlets per culture vessel. A sub- cultured section consisted of two axillary nodes. All cultures were kept in a growth room (see supplemental information).

***In vitro* culture of nodal sections**

Nodal sections of *Mentha* were harvested from 4 week old *in vitro*-grown plantlets and plated on MS growth medium for 2-3 days. Each section consisted of two axillary nodes (~ 3 mm).

Three methods used to cryopreserve *Mentha* shoot tips

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

Method 1: Vitrification without cold acclimation

I. Shoot tip isolation and pretreatment (Day 1 and 2)
   A. Shoot tip isolation
      Excise shoot tips from 2-3 day old nodal sections or from axillary nodes of 4-12 week old *in vitro*-grown plants. Shoot tips consist of the apical dome and 2-3 leaf primordia (0.5-1.0 mm).

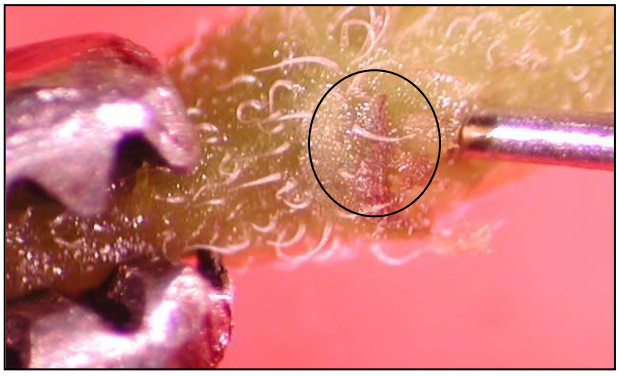

Image 1. Excise shoot tips from nodal sections or from axillary nodes (circled) of *in vitro*-grown plants.
Photo by NCGRP

B. Pre-culture
   1. Place shoot tips in a Petri dish containing MS+0.06 M sucrose medium (25 shoot tips/2.5 ml medium) and place in growth room for 24 hours.
   2. After 24 hours, replace the solution in the Petri dish with MS+0.3 M sucrose medium (25 shoot tips/2.5 ml medium) and place in the growth room for an additional 24 hours.

II. Loading, dehydration and vitrification (Day 3)
   A. Loading

      After 24 hours, replace the solution in the Petri dish with MS+2 M glycerol+0.4 M sucrose medium (25 shoot tips/2.5 ml medium). Allow shoot tips to soak for 2 hours.

   B. Dehydration
      1. After 2 hours, remove the sucrose solution from the Petri dish and replace with Plant Vitrification Solution 2 (PVS2) (25 shoot tips/2.5 ml solution). Allow shoot tips to soak for 20-30 minutes (exposure time varies by accession).
      2. Within the last five minutes, use a pipette to transfer shoot tips to sterile aluminum foil (2x5 mm). Each drop should contain 1 shoot tip plus 2-3 µl of PVS2 (12-13 drops/foil strip).
      3. In the last minute, use a pipette to remove the PVS2 liquid from the foil strip leaving the shoot tips. Fold tips. Fold the foil strip over the shoot tips and insert into a 1.2 ml cryovial (1 foil strip with 12-13 shoot tips/cryovial).

Image 2. Vitrification without Cold Acclimation. Use a pipette to transfer shoot tips to sterile aluminum foil strips. Photo by NCGRP

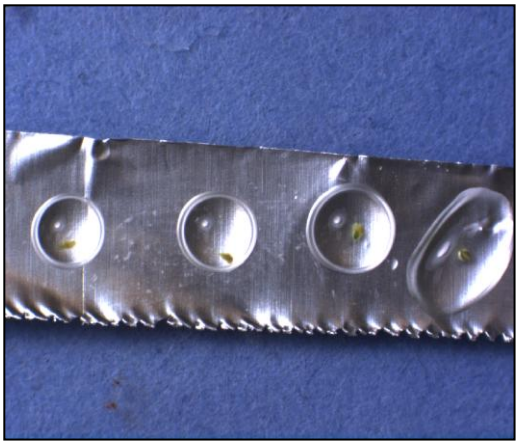

Image 3. Vitrification without Cold Acclimation. Each drop should contain 1 shoot tip plus 2-3 µl of PVS2. Photo by NCGRP

   C. Vitrification

      Immediately, plunge cryovial into LN.

III. Rewarming and recovery (Day 3)

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

A. Rewarming

1. Submerge the LN-cooled cryovial in a 40 °C water bath for 3-5 seconds.

2. Uncap cryovial and add 1 ml MS+1.2 M sucrose medium. Immediately remove the foil strip from the cryovial and transfer to a Petri dish containing fresh 1.2 M sucrose medium for 20 minutes (3 ml medium/foil strip).

B. After 20 minutes, pour off sucrose solution and shoot tips onto sterile filter paper to drain.

C. Recovery

1. Transfer shoot tips to a Petri dish containing solid MS+BA+IBA recovery medium. Place Petri dish in the dark for 48 hours followed by dim light for 72 hours and after, transfer to full light. Viability can be assessed 4-6 weeks post-thaw.

Method 2: Vitrification with cold acclimation

In vitro cultures of *Mentha* are placed in a cold acclimation chamber (see supplemental information) for 1-6 weeks prior to shoot tip isolation.

I. Shoot tip isolation and preculture (Day 1 and 2)

A. Shoot tip isolation

1. Excise shoot tips from 2-3 day old nodal sections or from the axillary nodes of 4-6 week old *in vitro*-grown plants. Shoot tips should consist of the apical dome and 2-3 leaf primordia (0.5–1.0 mm).

B. Pre-culture

1. Place shoot tips in a Petri dish containing MS+5% DMSO medium (25 shoot tips/2.5 ml Petri dish) and move to a cold acclimation chamber for 48 hours.

II. Loading, dehydration and vitrification (Day 3)

A. Loading

After 48 hours, transfer shoot tips to a Petri dish with pre-chilled (0°C) MS+2 M glycerol+0.4 M sucrose medium (25 shoot tips /2.5 ml medium). Keep Petri dish on ice for 20 minutes.

B. Dehydration

1. After 20 minutes, remove the sucrose solution from the Petri dish and replace with PVS2 (25 shoot tips/2.5 ml solution).

2. Immediately transfer shoot tips from Petri dish to a pre-cooled 1.2 ml cryovial containing pre-chilled (0 °C) **PVS2** (1 ml solution /cryovial). Place cryovial with shoot tips on ice for 20, 25 or 30 minutes (exposure time varies by accession).

3. In the last minute, remove 0.75 ml of PVS2 from the cryovial.

C. Vitrification

Immediately plunge cryovial into LN.

III. Rewarming and recovery (Day 3)

A. Rewarming

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

1. Submerge LN-cooled cryovial in a 45°C water bath for 1 minute followed by 1-2 minutes in a 25°C water bath.

2. Uncap cryovial and add 1 ml MS+1.2 M sucrose medium. Allow shoot tips to soak for 20 minutes; refresh the sucrose medium twice during that time.

3. After 20 minutes, pour off sucrose medium and shoot tips onto filter paper to drain.

B. Recovery

Transfer shoot tips to a Petri dish containing solid MS+BA+IBA recovery medium. Place Petri dish in the dark for 48 hours followed by dim light for 72 hours and after, transfer to full light. Viability can be assessed 4-6 weeks post-thaw.

Method 3: Encapsulation-dehydration

Fresh weight blanks and control beads are used in various steps with the goal of determining the moisture content of the non-control beads.

I. Nodal section isolation and encapsulation (Day 1)

A. Nodal section isolation

1. Harvest nodal sections from 3-4 week old *in vitro*-grown plants. Each section should consist of two axillary nodes (~ 3 mm).

2. Place nodal sections on solid Ca-free MS growth medium until the desired number have been harvested.

B. Encapsulation
   1. Transfer nodal sections to 3% alginic acid+0.2 M sucrose medium (25 nodal sections/50 ml medium).
   2. Next, cut the end of a disposable transfer pipette so the inside diameter measures 6 mm to make a bead size of ~ 4 mm. Using the modified transfer pipette, move nodes, one per droplet of alginate, to a flask containing 0.2 M sucrose+0.1 M calcium chloride medium (25 beads/75 ml solution). If successful, each drop will form a bead. Allow alginate beads to remain in the calcium chloride solution for at least 30 minutes. As they polymerize, the beads will change in appearance from transparent to whitish-opaque.

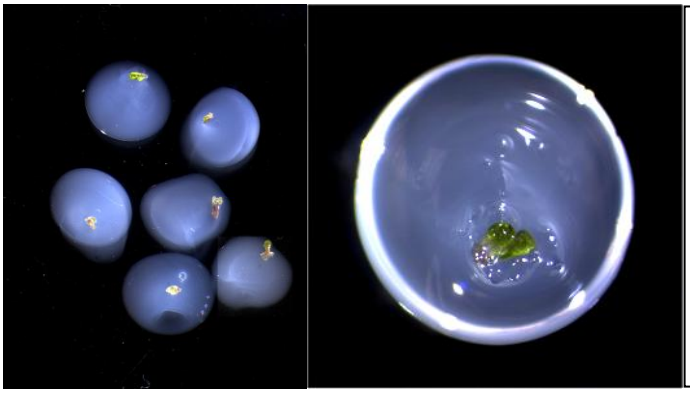

Image 4. Encapsulation-dehydration. As the alginate beads polymerize they change in appearance from transparent to whitish-opaque (a &b).
Photo by NCGRP

   3. Once polymerized, transfer non-control beads to a flask containing 0.2 M sucrose medium. Cover the flask with sterile aluminum foil and place on a rotary shaker for 16-24 hours (25 beads/75 ml medium).

C. Pretreatment of Fresh Weight (FW) blank control beads
   1. Following the encapsulation protocol, make 10 beads without nodal sections (blanks). These FW blanks will be used as controls to measure moisture content.

   2. Transfer the FW blanks to a flask containing 0.75 M sucrose medium, cover with sterile aluminum foil and place on a rotary shaker for 24 hours (25 beads/75 ml medium).

II. Pretreatment and fresh weight determination (Day 2)

A. Pretreatment
   1. After 24 hours, drain sucrose medium off non-control beads and replace with 0.5 M sucrose medium. Cover flask and place on a rotary shaker for 24 hours (25 beads/75 ml medium).

B. Drying and weighing Fresh Weight (FW) blank control beads

1. After 24 hours, transfer the 10 FW blank control beads to filter paper, blot dry and weigh control beads individually. Record weights.

2. Move blank control beads to aluminum weigh boat and place in an oven for 16 hours at 85 °C.

III. Loading and fresh moisture content determination (Day 3)

A. Loading Pretreatment

After 24 hours, drain 0.5 M sucrose medium off non-control beads and replace with 0.75 M sucrose medium. Cover flask and place on a rotary shaker for 24 hours (25 beads/75 ml medium).

B Determining Fresh Moisture Content (FMC) of Fresh Weight (FW) blank control beads:

1. After 24 hours, remove FW blanks from the oven. Weigh beads, individually, and use the resulting value to calculate Fresh Moisture Content (FMC):

(FW of blank control bead – oven dried weight of blank bead) / FW of blank control bead x 100

2. Calculate a combined average. Later, the resulting value of the blank beads' FMC will be used to calculate Predicted Weight (PW) of the non-control beads. Record the FMC and discard FW blank beads.

IV. Dehydration and predicted weight determination (Day 4)

A. Dehydration

1. After 24 hours, transfer the non-control beads from the 0.75 M sucrose medium to a filter paper and blot off excess liquid. Set aside 10 beads, to be used as controls in predicting the moisture content of non-control beads.

2. Move remaining non-control beads to the laminar flow hood. Place beads on a sterile Petri dish spacing evenly for uniform drying. Drying time can be affected by relative humidity, temperature and the air flow rate in the laminar flow hood.

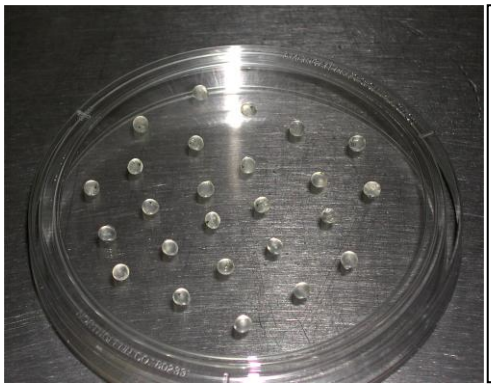

Image 5. Encapsulation-dehydration. Place alginate beads on a Petri dish in a laminar flow hood to dry. The optimal moisture content is 18-20%.
Photo by NCGRP

B. Determining Predicted Weight (PW) determination

The Predicted Weight (PW) is a value used to calculate Predicted Moisture Content (PMC). Control beads are dried and re-weighed and the PMC is calculated, at regular intervals, until the Optimal Moisture Content (OMC) is between 18 and 20%, has been reached. The PW, PMC and OMC of the control beads are used as approximations of the PW, PMC and OMC of the non-control beads.

1. Weigh the 10 control beads, all at once, and use the resulting value to calculate Predicted Weight (PW):

Weight of control beads x (1- FMC* of blank beads / 100) *from Section B, day 3

2. Record PW and calculate PMC. Move controls to the laminar flow hood spacing beads apart evenly for uniform drying.

3. Repeat weighing at hourly intervals. With each weighing calculate the Predicted Weight and the Predicted Moisture Content, until the Optimal Moisture Content (OMC) has been reached.

C. Predicted Moisture Content (PMC) determination

1. Calculate PMC of control beads:

(Sample weight of control beads – PW* of control beads / Sample weight of control beads) x 100 *section B, day 4

2. Discard the control beads once the PMC is between 18 and 20%. The non-control beads are ready for cryopreservation.

V. Vitrification

Move the non-control beads to 1.2 ml cryovials (12 to 13 beads/cryovial). Plunge cryovials into liquid nitrogen.

Image 6. Encapsulation-dehydration. Once the desired moisture content has been reached, move the beads to 1.2ml cryovials. Plunge cryovials into liquid nitrogen.
Photo by NCGRP

VI. Rewarming and Recovery (Day 4)

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

A. Rewarming

1. Remove cryovial from LN and allow it to re-warm at room temperature for 10 minutes.

2. Uncap cryovial and add 1 ml MS medium. Allow shoot tips to soak for 5 minutes.

3. After 5 minutes, pour solution and beads onto filter paper to drain.

B. Recovery

1. Transfer beads to Petri dish with solid MS+BA+IBA+recovery medium and move to growth room. Maintain Petri dish in the dark for 48 hours followed by dim light for 72 hours and after move to full light.

2. After a week, excise nodal sections from beads and place on fresh MS+BA+IBA+recovery medium and move to growth room. Assess viability after 4 weeks (see supplemental information for assessment procedure).

Supplemental Information

In vitro cultures of *Mentha* 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 16 hours of dark at 20°C, with a light intensity of 27 µmol m^{-2} s^{-1}, followed by 8 hours of light at -1.0°C.
Cryovials were pre-cooled in a cryorack previously frozen in a block of ice.

Viability on re-warmed LN-treated shoot tips was assessed after 4-6 weeks. A fully developed plantlet was considered as viable.

Summary of Formulas used in Encapsulation-Dehydration Method

1. Fresh Weight (FW):
   Weigh blank beads before drying in the oven

2. Fresh Moisture Content (FMC):
   (FW of blank beads – oven dried weight of blank beads)/ FW of blank beads x 100

3. Predicted Weight (PW):
   Weight of control beads x (1- FMC of blank beads/100)

4. Predicted Moisture Content (PMC):
   (Sample weight of control beads – PW of control beads/ sample weight of control beads) x 100

5. Optimal Moisture Content (OMC) = 18-20%

References and supplemental reading

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