Pycnanthemum

Work in Progress

There are currently **32** successions of *Pycnanthemum* in long term storage at the National Center for Genetic Resources Preservation (NCGRP), Fort Collins, Colorado. All plant material was originally screened for diseases and sent to the NCGRP by the personnel at NCGR. Procedures for cryopreservation of *Pycnanthemum* were adopted from *Ribes* and *Pyrus* protocols and the paper from Benson et al (1996). Predicted moisture content was determined to be between 18 – 22% moisture. Encapsulation/dehydration procedures have been successful with all *Pycnanthemum* species thus far at the NCGRP with viabilities from 60 – 100%.

Mountain Mint (*Pycnanthemum sp*.)

	Culture	Recovery	Cold acclamation
Photoperiod	**16h light/8h dark**	**16h light/8h dark**	**8h light/16h dark**
Light Intensity	**87µmol/m^2/s**	**87µmol/m^2/s**	**87µmol/m^2/s**
Temperature	**25°C constant**	**25°C constant**	**20°C light/-1°C dark**
Transfer interval	**3 weeks**	**2 weeks**	**2 weeks**
Vessel used	**Magenta GA7 culture vessel**	**60mm x 15mm Petri dish**	**Magenta GA7 culture vessel**

In vitro culture of *Pycnanthemum*

Plants are multiplied on MS multiplication medium and grown in Magenta GA7 culture vessels (Magenta Corp., Chicago, IL). Cold acclamation occurs with 3 week old plants in Magenta GA7 culture vessels.

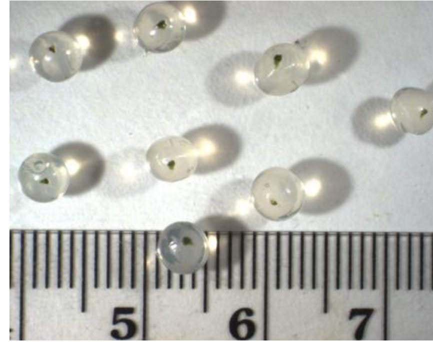
Procedures for cryopreservation of *Pycnanthemum sp*. Shoot tips

All steps take place at room temperature (22-25°C)

I. Create Blank Alginaic Beads (Day 1)

1. Using a plastic pipette with an opening of about 1 mm, drop 30 individual blank beads (no shoot tips) into 100mM $CaCl_2$ medium. Allow to set for about 30 minutes to solidify.
2. Remove all 30 beads from the $CaCl_2$ and place them into a flask of 75ml of 0.5M sucrose solution. Place flask on shaker for 18-20hrs at approximately 100rpm.

II. Blank beads (Day 2)



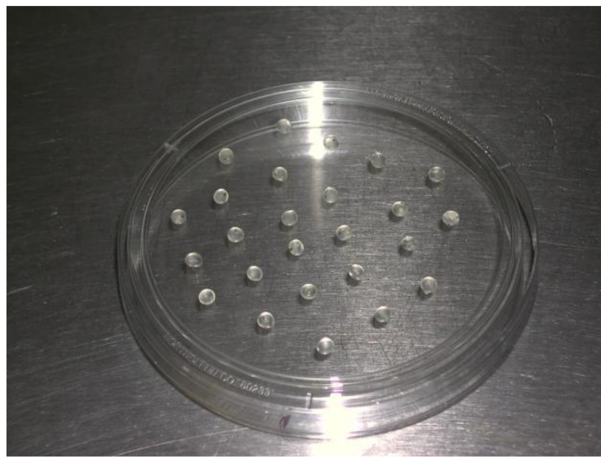
1. Remove blank beads from the 0.5M sucrose solution and put the beads in a solution of 0.75M sucrose for 18-20hrs on a shaker at about 100rpm.

III. Blank beads, Shoot tip isolation, and Encapsulation (Day 3)

1. Take the blank beads out of the 0.75M sucrose and roll them around on sterile filter paper. Place the 30 beads into three pre-weighed aluminum weigh boats. Immediately weigh the wet beads and weigh boats then place in oven at 90°C for 18-22hrs.
2. Isolate shoot tips from cold acclimated plants. A shoot tip consists of 1-2 leaf primordial plus the apical dome (approximately 0.8-1mm). Transfer shoot tips to a 60mm x 15mm Petri dish containing liquid MS medium (with no calcium) until enough shoot tips are cut.
3. Remove liquid MS from Petri dish leaving shoot tips in the dish. Pour alginate solution into the dish to cover all shoot tips. Using a modified plastic pipette (opening cut to approximately 1mm), pipette out one shoot tip at a time and drop into 100mM $CaCl_2$ (at the same time form 30 blank beads). Allow the beads with the shoot tips to sit in the calcium solution for about 30 minutes to form solid beads (usually beads turn opaque). In one 60mmx15mm Petri dish pour alginate and use this dish to drop 30 blank beads (no shoot-tips).
4. After beads are formed, remove the beads from the calcium solution and place 25-30 beads in a flask containing 75ml of 0.5M sucrose solution. Place all flasks into a container and cover with aluminum foil. Place container on shaker for 18-20hrs at approximately 100rpm.
5. Drain each flask of the 0.5 M sucrose and replace with 0.75M sucrose. Place the flask back on the shaker for an addition 18-20hrs. (day 4)

IV. Bead dehydration (Day 5)

1. Remove beads from flask, roll beads on sterile filter paper in a Petri dish to remove excess moisture and place 25-30 beads with shoot tips on the



bottom of a 100mm x 15mm plastic Petri dish. Place 10 blank beads on the bottom of a 100mm x 15mm Petri dish that have been pre-weighed. Weigh the dishes with the blanks recording that weight.

2. Place all Petri dishes with shoot tips in a row about three inches from the back of a laminar flow hood (the hood should be on) with the blank beads evenly dispersed among the dishes that have beads with shoot tips.
3. During the dry down, periodically remove the Petri dishes with the blank beads and weigh the dishes and the beads to determine the wet weight for the formula to calculate moisture content.
4. When the beads reach moisture content of 18 to 22%, place 10 beads with shoot tips into 1.2ml cryovials. Submerge vials into liquid nitrogen.

V. Recovery of frozen beads (Still Day 5)

1. After at least 1 hour, remove two vials from liquid nitrogen and open the vial. Let the vials stand open in a laminar flow hood at room temperature for 15 minutes to thaw.
2. After the beads have thawed, place enough liquid MS solution into the vials to cover all the beads and let the beads rehydrate in the solution for 10 minutes.
3. Remove the beads from the vial and place beads in a 60mm x 15mm Petri dish containing *Pycnanthemum* recovery medium. Place in subdued light at 25°C for 24hrs.

VI. Evaluation of viability

1. After the shoot tips have grown on recovery medium for 2 weeks remove the shoot tips from the beads and plate them in a GA7 vessel containing growth medium. Place GA7 on lighted shelf (87μmol/m^{2}/s^{-1}) in a controlled room (25°C with a 16 hour photoperiod).
2. Allow the shoot tips to grow for 3 weeks. Count the number of plants that have formed a stem and leaves, divide by the number of shoot tips thawed and plated, multiple by 100 to determine the viability of your shoot tips.

References

Benson, E.E., B.M. Reed, R.M. Brennan, K.A. Clacher, and D.A. Ross. 1996. Use of thermal analysis in the evaluation of cryopreservation protocols for *Pycnanthemum nigrum* L. germplasm. Cyro-Letters 17:347-362.

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

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

Media Recipes for *Pycnanthemum* (Note: all recipes make 1 liter)

0.5M SUCROSE PRETREATMENT

1. One Package of M-519[1]
2. Add Reagent Grade Sucrose 171.16g
3. Bring to volume
4. Adjust pH to 5.7
5. Dispense 50ml into 125ml flasks
6. Cap with foil and autoclave

0.75M SUCROSE PRETREATMENT

1. One Package of M-519[1]
2. Add Reagent Grade Sucrose 256.72g
3. Bring to volume
4. Adjust pH to 5.7
5. Dispense 50ml into 125ml flasks
6. Cap with foil and autoclave

LIQUID MS FOR BEAD REHYDRATION

1. One Package of M-519[1]
2. Add Reagent Grade Sucrose 30g
3. Bring to volume
4. Adjust pH to 5.7
5. Dispense 20ml into test tubes
6. Cap and autoclave

100mM $CaCl_2$ MEDIUM

1. One Package of M-519[1]
2. Add Reagent Grade Sucrose 30g:
3. Add addition $CaCl_2$ 7.3g
4. Bring to volume
5. Adjust pH to 5.7
6. Dispense 100ml into a 250ml flask
7. Cap with foil and autoclave

***PYCNANTHEMUM* GROWTH MEDIUM**

1. Macronutrients
   a. KNO_3 **Stock Solution** 6mls (30% of normal MS)
   b. $NHNO_3$ **Stock Solution** 6mls (30% of normal MS)
   c. $MgSO_4$ **Stock Solution** 10mls
   d. $CaCl_2$ **Stock Solution** 10mls
   e. KH_2PO_4 **Stock Solution** 10mls
   f. Iron **Stock Solution** 20mls
2. Micronutrients **Stock Solution** 10mls
3. MS Vitamins **Stock Solution** 10mls
4. Vitamins Ascorbic Acid 0.05g
5. Add Glucose 20g
6. Plant Growth Regulators
   a. GA3 (5.8μmol) 0.2mg/L
   b. BA (4.4μmol) 0.1mg/L
7. Bring to volume
8. Adjust pH to 5.7
9. Gelling Agent
   a. Sigma[2] A7002 Agar (0.35%) 3.5g
   b. Gelrite (0.145%) 1.45g
10. Dispense into Magenta Cubes
11. Autoclave

***PYCNANTHEMUM* MERISTEM MEDIUM**

<u>Macronutrients</u>
KNO_3 6mls (30% of normal MS)
$NHNO_3$ 6mls (30% of normal MS)
$MgSO_4$ 10mls
KH_2PO_4 10mls
<u>Iron</u>
10mls
<u>Micronutrients</u>
10mls
<u>MS Vitamins</u>
10mls
<u>Vitamins</u>

Ascorbic Acid 0.5g
Disaccharides
Glucose (2%) 20g
Bring to volume
pH 5.7

PYCNANTHEMUM RECOVERY MEDIUM

Macronutrients
KNO_3 6mls (30% of normal MS)
$NHNO_3$ 6mls (30% of normal MS)
$MgSO_4$ 10mls
$CaCl_2$ 10mls
KH_2PO_4 10mls
Iron
20mls
Micronutrients
10mls
MS Vitamins
10mls
Vitamins
Ascorbic Acid 0.5g
Disaccharides
Glucose (2%) 20g
Plant Growth Regulators
GA3 (5.8µmol) 0.2mg/L
BA (4.4µmol) 0.1mg/L
Polyvinyl Pyrrolidone 1g
Bring to volume
pH 5.7
Gelling Agent
Agar (.25%) 2.5g
Gelrite (.145%) 1.45g