Work in progress

To date, NCGRP has successfully placed 27 accessions of garlic (*Allium sativum* L. and *A. longicuspis* Regel), in long-term storage using cryopreservation methods. All accessions were obtained through collaboration with the USDA Western Regional Plant Introduction Station, Pullman, WA. Field-grown bulbs were harvested and dried before they were shipped to the Center.

Two protocols are used concurrently for the cryopreservation of garlic shoot tips. One is based on a method developed by Niwata (1995) and uses Plant Vitrification Solution 2 (PVS2) as the cryoprotectant. A second method developed by Makowska, et al. (1999) uses Plant Vitrification Solution 3 (PVS3) as the cryoprotectant. The rationale for using both methods is that different garlic genotypes vary in their response, as measured by viability. Some genotypes respond better to PVS2, while others respond better to PVS3. Further, some genotypes respond equally well or equally poor to both methods. With no means in place to predict the response of different garlic genotypes it was decided that both methods would be used on all accessions. This strategy has proven highly successful.

Two different methods for cryopreservation of garlic shoot tips

Both methods follow the same protocol for day 1 (cubing and sterilization) and day 2 (shoot tip isolation and preculture). All steps take place under aseptic conditions and at room temperature (21 °C) unless noted otherwise.

Cubing and sterilization (day 1)

1. Cubing

    A. Break garlic bulbs into individual cloves and remove the dry skins. Pare down each clove, using a razor blade, to 1 cm cubes with the basal plate shaved free of debris but left intact (~1 mm thick). Place cubes in a Petri dish on moistened filter paper while cubing the remaining cloves.

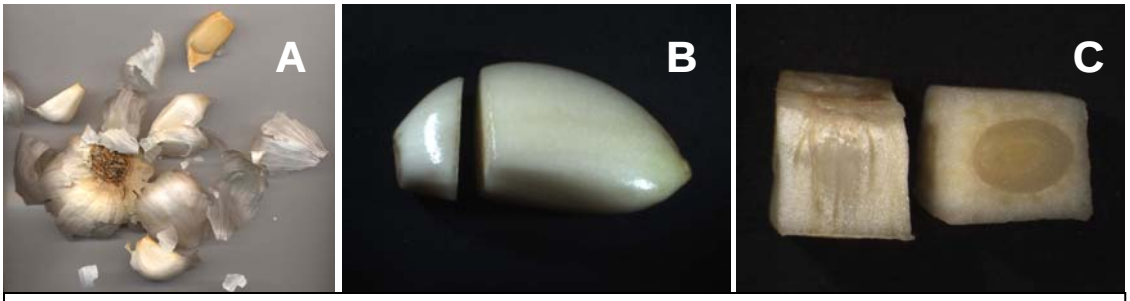


Image 1. Break garlic bulbs into individual cloves and remove the dry skins (A). Pare down each clove, using a razor blade, to 1 cm cubes with the basal plate shaved free of debris but left intact (B & C). *Photos by D. Ellis*

2. Sterilization

   A. To sterilize, place garlic cubes in a 70% ethanol solution, agitating gently, for 10 minutes.

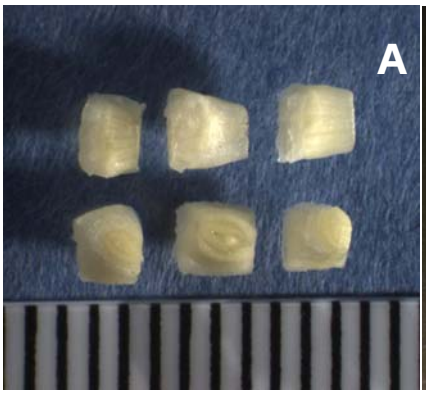
   B. Drain off ethanol and transfer cubes to a 20% commercial bleach (6.0% sodium hypochlorite) solution with one drop of Tween 20®, agitating gently, for 20 minutes.

   C. In a laminar flow hood, rinse cubes three times with sterile distilled water (15 minutes/rinse).

Shoot tip isolation and pre-culture (day 1-2)

1. Shoot tip isolation

   A. With the aid of a dissecting scope, excise shoot tips from the sterilized cubes. Shoot tips consist of an apical dome, 1-2 leaf primordia and a basal plate (~0.5 mm thick. Shoot tips should be 1-1.5x1-1.5 mm, ideally, not to exceed 2 mm. Place shoot tips in a Petri dish containing **Gamborg B5 basal medium+NAA+2iP**.



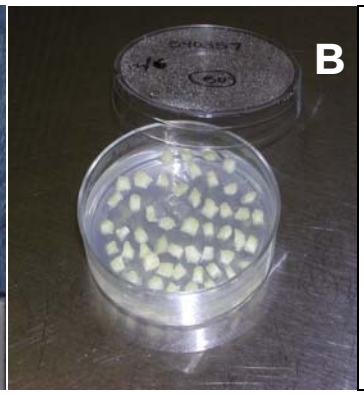


Image 2. Shoot tips consist of an apical dome, 1-2 leaf primordia and a basal plate and should not to exceed $2mm^2$ (A). Place isolated shoot tips in a Petri dish on solid medium (B).
Photos by D. Skogerboe

2. Pre-culture

   A. Place Petri dish with isolated shoot tips in the dark at 5 ^{o}C for 48 hours.

Method 1: Vitrification using PVS2 as a cryoprotectant (day 3)

I. Loading, dehydration and vitrification

1. Loading

A. Transfer precultured shoot tips to a Petri dish containing **2 M glycerol+0.6 M sucrose+1/2 Murashige & Skoog (MS) loading medium**. Allow shoot tips to soak for 20 minutes.

2. Dehydration

A. After 20 minutes, remove the loading solution with a Pasteur pipette and replace with chilled (0 °C) **Plant Vitrification Solution 2 (PVS2)**. Keep shoot tips in PVS2 solution, on ice, for 15 minutes.

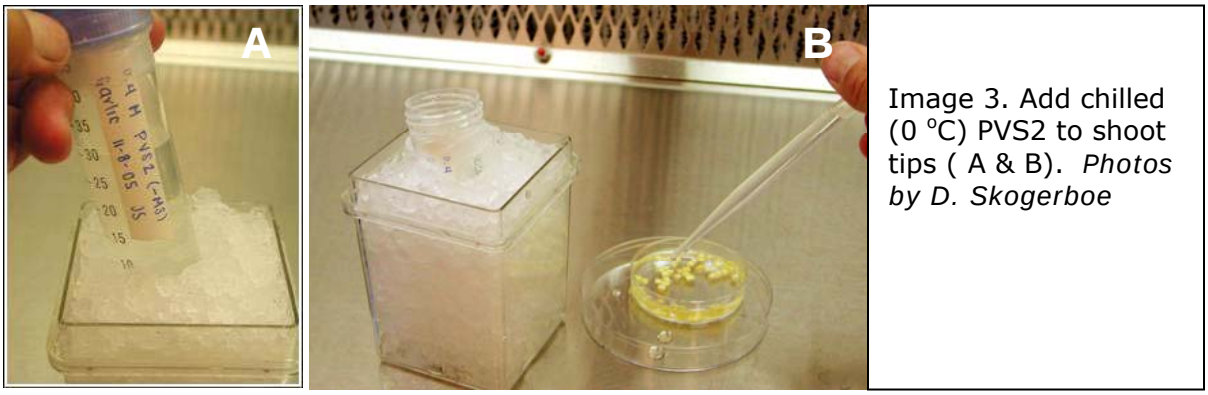


Image 3. Add chilled (0 °C) PVS2 to shoot tips (A & B). *Photos by D. Skogerboe*

3. Vitrification

A. After 15 minutes, use forceps to place 4-5 shoot tips onto a sterile foil strip (0.5x15 mm). Do not remove excess PVS2 from the foil. Immediately plunge the foil strip into a shallow vessel of liquid nitrogen (LN).

B. Using pre-cooled forceps, insert the frozen foil strip into a LN-cooled, 1.2 ml cryovial. Cap the cryovial making sure it contains enough LN to cover the enclosed foil strip.

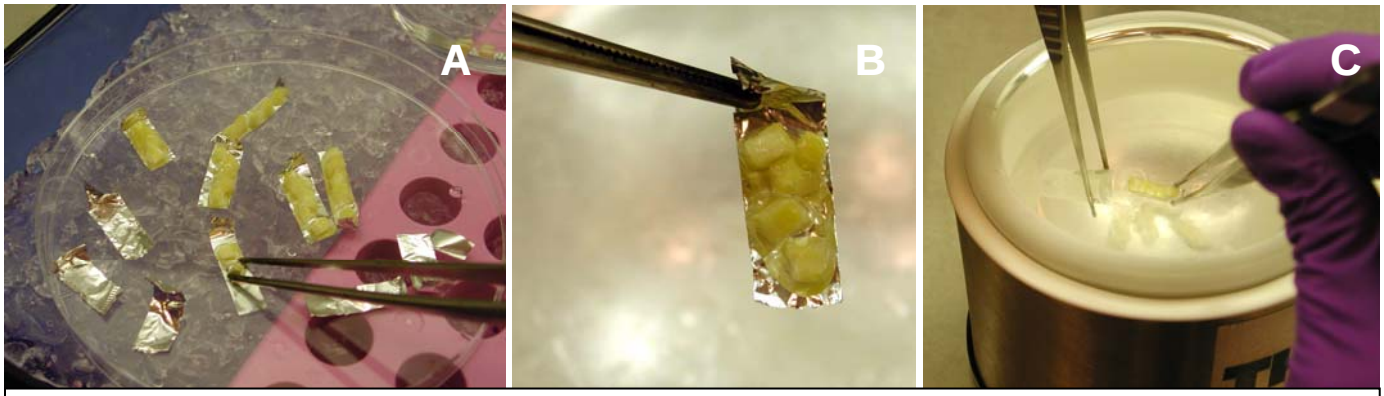


Image 4. Use forceps to place 4-5 shoot tips onto a sterile foil strip (A). Immediately plunge the foil strip into a shallow vessel of LN (B). Using pre-cooled forceps, insert the frozen foil strip into a LN-cooled, 1.2 ml cryovial (C). *Photos by D. Skogerboe*

II. Rewarming and recovery of PVS2-treated shoot tips
Keep cryopreserved shoot tips in liquid nitrogen for at least one hour prior to rewarming.

1. Rewarming

A. Remove the frozen foil strip from the cryovial as soon as the cap has warmed enough to open. Submerge the foil strip into **1.2 M sucrose+1/2 MS medium** for 20 minutes refreshing the solution once after 10 minutes. Remove the foil strip from the medium once the rewarmed shoot tips become detached.

B. After 20 minutes, transfer shoot tips to a Petri dish containing **Gamborg B5 basal medium+NAA+2iP**. Wrap the Petri dish with aluminum foil (to prevent light exposure) and place in growth room (see supplemental information) for 24 hours.

2. Recovery

A. After 24 hours, transfer shoot tips to a new Petri dish containing **Gamborg B5 basal medium+ NAA+2iP** and wrap with foil. Place Petri dish in growth room for an additional 4 days, then remove foil but keep in dim light for another 5 days and after, transfer to full light. Leave shoot tips on the Petri dish until they outgrow the space and then move them to Magenta® GA7 culture vessels containing **Gamborg B5 basal medium+NAA+2iP**.

Method 2: Vitrification using PVS3 as a cryoprotectant (day 3)

I. Loading, dehydration and vitrification

1. Loading

A. Transfer preconditioned shoot tips to a Petri dish containing **2 M glycerol+0.4 M sucrose+1/2 MS loading medium**. Allow shoot tips to soak for 20 minutes.

2. Dehydration

A. After 20 minutes, remove the loading solution with a Pasteur pipette and replace with **PVS3**. Allow shoot tips to soak for 2 hours.

3. Vitrification

A. After 2 hours, move shoot tips to a 1.2 ml cryovial containing 0.5 ml **PVS3** and plunge into LN.

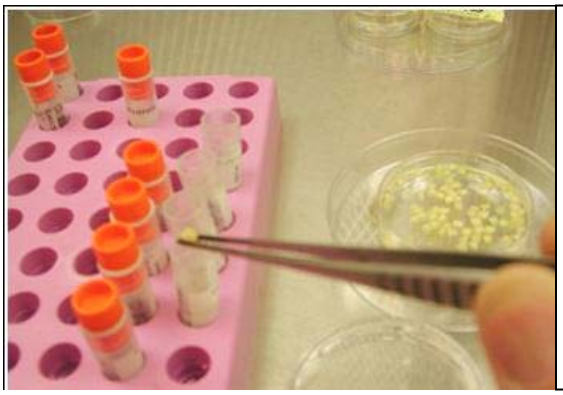


Image 5. Move shoot tips from PVS3, after soaking for 2 hours, to a 1.2 ml cryovial and plunge into LN.
Photo by D. Skogerboe

II. Rewarming and recovery of PVS3-treated shoot tips
Keep cryopreserved shoot tips in liquid nitrogen for at least one hour prior to rewarming.

1. Rewarming

   A. Submerge the LN-cooled cryovial in a 40 °C water bath for 2 minutes.

   B. Transfer shoot tips to a Petri dish containing **1.2 M sucrose+1/2 MS medium** for 20 minutes refreshing the solution once after 10 minutes.

2. Recovery

   A. After 20 minutes, transfer shoot tips to a Petri dish containing **Gamborg B5 basal medium+ NAA+2iP**. Wrap Petri dish with aluminum foil (to prevent light exposure) and place in growth room (see supplemental information) for 24 hours.

   B. After 24 hours, transfer shoot tips to a new Petri dish containing **Gamborg B5 basal medium+NAA+2iP** and wrap with foil. Place Petri dish in growth room for an additional 5 days, then remove foil but keep in dim light for another 5 days and after, transfer to full light. Leave shoot tips on the Petri dish until they outgrow the space and then move them to Magenta® GA7 culture vessels containing **Gamborg B5 basal medium+NAA+2iP**.

   C. Regrowth of LN-treated shoot tips is visible as early as three 3 days post-thaw. Final determination of survival can be confirmed after 14 days.

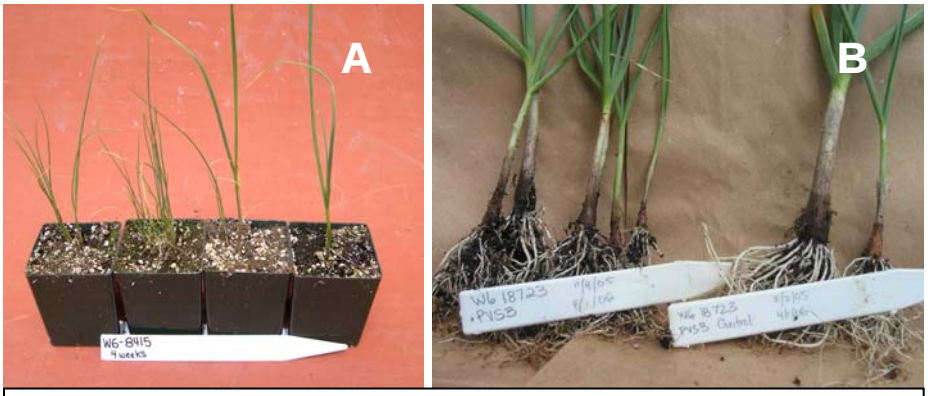


Image 6. LN-recovered shoot tips are moved from Magenta® GA7 culture vessels to a greenhouse once the root and shoot sytems are well-developed (2-4 months post-thaw). (A) Four weeks after being moved to a greenhouse and 20 weeks post-thaw. (B) Comparison of PVS3-treated plants (left) and controls (right) 12 weeks after being moved to a greenhouse and 24 weeks post-thaw. *Photos by D. Skogerboe*

Supplemental information

In vitro cultures of *Allium* 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}.

Garlic bulbs were kept dry and stored in brown paper bags in a cool (~17 °C), dark room for a maximum of 8 weeks until shoot tip excision.

Garlic plants were moved from Magenta® GA7 culture vessels to a greenhouse house once the root and shoot sytems were well-developed; 2-4 months post-thaw.

References and supplemental reading

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