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Storage Technique Preserves Citrus, Kills Pathogens



Left: Technician Remi Bonnart (foreground) flash-freezes plant shoot tips while plant physiologist Christina Walters places cryopreserved materials into cryovats for long-term storage.

Above: Plant shoot tips are frozen within droplets of cryoprotection solutions on foil strips. The strips are plunged into liquid nitrogen slush that is formed by placing liquid nitrogen in a vacuum.

human encroachment on the natural habitat of potentially valuable wild citrus genetic resources is also a concern.

Ensuring Citrus's Future

Fort Collins, Colorado, is the home of the “Fort Knox” of plant and animal germplasm preservation, the National Center for Genetic Resources Preservation (NCGRP). There, Agricultural Research Service plant physiologist Gayle Volk is spearheading a program to develop methods to back up U.S. citrus cultivars in the form of shoot-tip cuttings that have been cryopreserved—kept in frozen storage via immersion in liquid nitrogen.

The Fort Knox comparison reflects the value of the center’s germplasm collections, which are crucial to research and to food security of current and future generations. The analogy also reflects the structural strength of its large storage vaults, which can withstand flooding, tornado-strength winds, and the impact from a 2,500-pound object hurtled at 125 miles an hour.

In addition to state-of-the-art equipment, NCGRP has a staff of scientific personnel renowned for expertise in the storage and

Americans consume about 84 pounds of fresh and processed citrus per person each year, with oranges topping the list at 61 pounds annually. The availability of these favored fruits in grocery stores, fresh markets, and other consumer outlets is a testament to growers’ success in managing the ever-present threat of pests and diseases to the nation’s \$3.4 billion citrus crop (2011-12), most of which is produced in Florida and California.

A major threat is citrus greening disease, also known as “Huanglongbing.” First

detected in Florida in August 2005, citrus greening today is the target of a multi-faceted effort by federal, state, university, and industry partners to control this costly bacterial disease and the insect responsible for spreading it, the Asian citrus psyllid. The most visible symptoms include stunted growth of citrus trees; poor flowering; blotchy, mottled leaves; and off-flavored, misshapen fruit. A diseased tree cannot be cured and starts to decline in 5 to 12 years.

Other hazards, such as droughts, freezes, hurricanes, and earthquakes, can be just as devastating to the citrus industry. Globally,

evaluation of germplasm acquired from around the world—whether it be seed, pollen, and vegetative tissues (buds and stems) from endangered plants and cultivars; or blood, semen, and embryos from prized livestock breeds.

For example, Volk's laboratory, part of the center's Plant Germplasm Preservation Research Unit, is the only one in the United States that's adapted cryopreservation methods to safeguard citrus cultivars. "Once cryopreserved, citrus genetic resources are safe from biological or physical threats, and their associated maintenance costs are very low," she says. Cryopreserving shoot tips also offers a way to ensure pathogen-free germplasm for restoring lost or imperiled cultivars.

To date, Volk and her colleagues have cryopreserved shoot tips for 30 cultivars, including mandarin and navel oranges, from collections maintained by the ARS National Clonal Germplasm Repository for Citrus and Dates, located at the University of California-Riverside. The collections, among others there, contain nearly 900 accessions (specimens) and represent 132 taxonomic groups of *Citrus*, *Fortunella* (kumquat), and wild citrus species, whose rich genetic diversity can be tapped to improve economically important traits like yield, nutrient content, and pest and disease resistance.

"The citrus collection at Riverside is our current source of material, and we have been strategically selecting high-priority materials for the California industry from that collection as some of the first to be cryopreserved here at NCGRP," says Volk. "Riverside's citrus collection doesn't contain some of the key cultivars grown in Florida or Texas, and we will be working with people from those states to obtain a greater diversity of germplasm."

The cryopreservation process begins with removing a 1-millimeter-long shoot-tip segment from new citrus growth, called "flush," and immersing the tissue in a solution containing glycerol, sugar, and other cryoprotectants. This preparatory step is critical because it displaces water in shoot tip cells and prevents lethal ice

crystals from forming during the next phase—immersion in liquid nitrogen at -196°C (-321°F). As they are needed, the cryopreserved tips can be removed from the vials in which they are stored and carefully thawed for rootstock grafting procedures that lead to whole, young citrus plants.

Initial results showed that, on average, 53 percent of shoot tips survive cryopreservation, and they have now refined the technique to achieve average regrowth levels of 83 percent. A paper reporting the detailed methods has been published in the journal *CryoLetters*. Long-term experiments are under way to confirm the viability of the regenerated plants and make sure that they are identical, or true-to-type, to the plants from which they were originally derived.

Putting Pathogens on Ice

Cryopreservation has an added benefit of eliminating bacteria, viruses, and viroids to ensure disease-free germplasm for citrus research, breeding, and commercial operations. Volk's team has used the approach—dubbed "cryotherapy" by the Chinese researcher who first investigated it—to eliminate pathogens from citrus shoot tips, which are composed of meristem cells.

These cells are important to cryotherapy because they are especially small, densely packed, and often not infected with pathogens that reside in the more mature, vascular tissues. Most importantly, meristem cells survive being frozen in liquid nitrogen, unlike larger cells in the shoot-tips, especially those cells harboring pathogens. Additionally, the procedure leaves much of the shoot-tip intact, which expedites regeneration of whole plants.

"If cryotherapy proves to be an effective way to clean up diseased material, then it will facilitate movement of breed-

ing lines and cultivars among the states where quarantines are in effect to restrict the spread of citrus greening," says Volk.—
By **Jan Suszkiw**, ARS.

This research is part of Plant Genetic Resources, Genomics, and Genetic Improvement, an ARS national program (#301) described at www.nps.ars.usda.gov.

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Citrus shoot tips were cryopreserved and then micrografted onto seedling rootstocks. This recovered plant is about 2 months old.



RICHARD LEE MARCH (D2881-1)



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At ARS's National Clonal Germplasm Repository for Citrus and Dates in Riverside, California, technician Brittany Moreland selects citrus shoot tips for cryopreservation at the ARS germplasm repository in Fort Collins, Colorado.