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PRESERVATION AND MAINTENANCE OF VIRUS-FREE CLONAL CITRUS  
GERMPLASM AT THE NATIONAL CLONAL GERMPLASM REPOSITORY FOR  
CITRUS, RIVERSIDE, CALIFORNIA

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ABSTRACT

The United States Department of Agriculture's National Clonal Germplasm Repository for Citrus is currently establishing a virus-free clonal germplasm collection of 830 accessions of citrus and related Aurantioideae genera at its Riverside, California, facility with the objective of reducing genetic vulnerability of the crop and providing a source of virus-free clonal germplasm to researchers worldwide. Emphasis of the collection centers on the acquisition and ex-situ preservation, maintenance, evaluation and distribution of genera, species and cultivars representing the widest possible genetic diversity with the citrus subfamily. The Repository plan includes a comprehensive program of genetic characterization, pathogen detection and elimination, pest management, propagation in a controlled and protected environment, and documentation. All accessions are evaluated for trueness-to-type using leaf isozymes and characterized for 58 descriptors. Research is expanding into RFLPs for the development of an usable genomic map. The pathogen detection program evaluates accession for 9 virus and viroid and 2 bacterial diseases. Infected accessions receive shoot-tip micrografting and thermotherapy to eliminate pathogens. All accessions are maintained as potted trees in aphid-proof screen- or greenhouses. Citrus seed, pollen and buds are being investigated for extended preservation under low-temperature and cryostorage. Research investigations into in vitro culturing of explants have begun. A complete computer-based record of each accession is maintained on site and in the USDA-ARS Germplasm Resources Information Network (GRIN).

INTRODUCTION

Citrus is cultivated in nearly every tropical and subtropical country of the world, from the semi-arid climates of California, Australia and the Mediterranean countries to the intermediate tropical/subtropical climates of Florida and Brazil, and the tropical climates of Southeast Asia. Total cultivation worldwide exceeds  $3 \times 10^6$  ha (Foreign Agricultural Service, 1990). Most of the world production is concentrated in a relatively small number of cultivars representing "elite" germplasm selections (Giacometti et al., 1984). These cultivars, bud or limb sport selections, represent a very narrow genetic base within Citrus and the subfamily Aurantioideae. This has created an undesirable situation where pure acreages of common

commercial cultivars may be vulnerable to attack by new strains of pathogenic organisms, while other less widely distributed and "wild type" germplasm is being lost due to inadequate preservation or, through habitat destruction. Diseases affecting citrus have been, for a considerable time, a major limiting factor in nearly every citrus growing region of the world. In areas affected by endemic disease, the selection of naturally resistant genotypes suppressed damage from pathogens; however, as budwood is moved from infected areas into new locations these diseases become established in plantings lacking resistance, frequently with devastating effects (Bar-Joseph et al., 1989; Roistacher and Moreno, 1989). The spread of "tristeza" and "psorosis" virus strains into South America provides an excellent example of the severity of this problem (Tan Juan et al., 1984). It has been only recently in the history of citrus culture that increased emphasis has been placed on eradicating diseases and moving only tested, pathogen-free propagating material. This has been coupled with efforts in breeding programs to identify and incorporate specific resistances and desirable horticultural characteristics from citrus germplasm into hybrid plants. The value of accessibility to a widely diverse base of genetic resources for these programs is obvious.

In consideration to these factors and for the benefit of the United States and the international citrus community, the USDA-ARS through its National Plant Germplasm System, established in 1987 the National Clonal Germplasm Repository (NCGR) for Citrus at Riverside, California. The overall goal of the program is to reduce the genetic vulnerability of citrus by collecting and preserving the widest possible genetic diversity within the crop. More specifically, the objectives of the NCGR-Citrus are to collect, identify, preserve, evaluate and distribute clonal germplasm of Citrus and its related genera within the Aurantioideae subfamily, thereby providing the international citrus research community with a source of pathogen-free clonal germplasm.

#### Citrus and Related Aurantioideae Collections

The Citrus Germplasm Repository, in cooperation with the University of California- Riverside Citrus Variety Collection, has acquired 851 accessions of Citrus and related genera. These accessions, summarized in Table 1, represent the most genetically diverse collection of citrus germplasm in the world. Twenty-eight of the 33 genera in the subfamily Aurantioideae are represented. Acquisition priorities are based on obtaining species from non-represented genera as well as greater diversity within represented genera. Similar efforts are made toward obtaining "wild type" Citrus species. Acquisition of promising commercial cultivars is an ongoing priority. Efforts will include exploration of centers of origin of the world identified as promising for new Aurantioideae germplasm and other centers of domestication in danger of imminent habitat destruction.

Table 1. Distribution of accessions (totals and virus-free)  
by genus and species at the USDA National Clonal  
Germplasm Repository for Citrus

| Genus/species               | Group  | Total number<br>of accessions | Number of<br>virus-free<br>accessions |
|-----------------------------|--|-------------------------------|---------------------------------------|
| <u>Citrus sinensis</u>      | sweet oranges                                  | 79                            | 19                                    |
|                             | navel oranges                                  | 39                            | 15                                    |
|                             | valencia oranges                               | 23                            | 5                                     |
|                             | blood oranges                                  | 16                            | 5                                     |
| <u>Citrus aurantium</u>     | sour oranges, including<br>bittersweet oranges | 50                            | 7                                     |
| <u>Citrus reticulata</u>    | mandarins, including<br>calamondins            | 119                           | 33                                    |
| <u>Citrus x tangelo</u>     | tangelos                                       | 27                            | 6                                     |
| <u>Citrus x tangor</u>      | tangors  | 15                            | 5                                     |
| <u>Citrus paradisi</u>      | grapefruit                                     | 49                            | 13                                    |
| <u>Citrus maxima</u>        | pummelos                                       | 77                            | 25                                    |
| <u>Citrus medica</u>        | citrons  | 41                            | 3                                     |
| <u>Citrus limon</u>         | lemons   | 91                            | 11                                    |
| <u>Citrus jambhiri</u>      | rough lemons                                   | 14                            | 1                                     |
| <u>Citrus aurantiifolia</u> | limes  | 31                            | 2                                     |
| <u>Citrus</u> sp.           | papedas  | 22                            | 1                                     |
| <u>Poncirus trifoliata</u>  | trifoliates                                    | 49                            | 7                                     |
|                             | trifoliolate hybrids                           | 44                            | 17                                    |
| <u>Fortunella</u> sp.       | kumquats                                       | 24                            | 3                                     |
| <u>Microcitrus</u> sp.      | Microcitrus                                    | 20                            | 0                                     |
| Other related genera        |  | <u>44</u><br>874              | <u>24</u><br>183                      |

## Evaluation, Preservation and Maintenance

Ex-situ preservation and maintenance of citrus genotypes requires a carefully monitored program of genetic characterization, pathogen detection and elimination, pest management, propagation and culture in a controlled and protected environment, and documentation. Guidelines developed by the USDA-ARS Citrus Crop Advisory Committee specifically address each of these areas.

### 1. Evaluation

- A. Genetic characterization. As an indicator of trueness to type and as a genetic tool, all accessions in the collection undergo leaf isozyme analysis using starch gel electrophoresis (Torres et al., 1978; Roose, 1988). Accessions are analyzed for eleven loci from seven enzymes (GOT, PGI, PGM, MDH, IDH, LAP, ME). Analysis of the isozyme patterns indicated accessions of hybrid origins and aids in grouping into representative species types (Soost et al., 1980). Limitations of isozymes for cultivar identification have led to initiation of cooperative research on RFLPs (restriction fragment length polymorphisms) in pursuit of a usable genomic map for citrus and possible cultivar-specific probes (Roose, 1988). Investigations utilizing PCR (polymerase chain reaction) rapid screening are in development.
- B. Morphological characterization. All accessions in the collection are evaluated for 66 specific descriptor categories covering tree, flower, fruit and crop data as well as environmental responses to temperature and salinity-related factors. This list, developed by the USDA-ARS Citrus Crop Advisory Committee, generally follows the International Board of Plant Genetic Resources citrus descriptor list. Evaluations are made primarily on field trees, although greenhouse growing trees may be used in the cases of cold-sensitive cultivars.
- C. Pathogen detection and elimination. As an integral part of the Repository program, a comprehensive pathogen detection program has been implemented from protocols conceived and developed by the American Phytopathological Society (Whiteside et al., 1988), the International Organization of Citrus Virologists (Navarro, 1990) and the California Citrus Clonal Protection Program (Roistacher, 1977a), and compiled by the International Board of Plant Genetic Resources (Frison and Taher, 1990). All accessions, prior to entry into the protected Repository collection, are evaluated for nine virus and viroid diseases (tristeza and seedling yellows, psorosis A and B, exocortis, cachexia, concave gum group, tatterleaf, infectious variegation, and vein enation/woody gall) and one mycoplasma (stubborn).

Additional tests are performed when the origin of the source germplasm warrants further examination. These include tests for greening, canker, mal seco and Phytophthora. Indexing protocols used conform to those accepted by the Animal and Plant Health Inspection Service (APHIS) of the USDA. These include bioassay inoculation tests on pathogen-sensitive indicators, rapid serological screening (ELIZA) and electrophoretic (PAGE) techniques, and culturing. Accessions found to be infected are shoot-tip micrografted (STG) in culture to eliminate the disease organisms (Navarro, 1981). They may also receive thermotherapy as specific conditions warrant (Roistacher, 1977b). Methodologies are used in accordance with established protocols. Shoot-tip micrografted and thermotherapy treated accessions are reindexed to confirm the absence of pathogens. Research is continuing on the development of additional rapid screening techniques utilizing PCR technology.

## 2. Preservation/Maintenance and Distribution

The presence in the Riverside area of the insect Aphis gossypii, an important vector of the tristeza virus, precludes the maintenance of pathogen-free trees outdoors. Source germplasm accessions, backup trees for the Repository collection, are maintained in a field planting at the University of California Citrus Variety Collection. These trees are subject to tristeza infection. The pathogen-free accessions in the Repository collection, depending upon cold tolerance, are maintained in aphid-proof screenhouse enclosures or greenhouses. Temperatures at Riverside during winter months can drop below 0°C; therefore, many non-citrus accessions within the Aurantioideae subfamily, being tropical in origin, are protected in heated greenhouses. Backup trees for cold-sensitiveness are maintained at alternate field site locations in Florida, Malaysia, Hawaii and the coastal area of California. These accessions provide seed sources for species and cultivars which due to environmental factors do not fruit at Riverside. Physical facilities at the Repository include, 336 m<sup>2</sup> of temperature controlled greenhouses, 755 m<sup>2</sup> of screenhouse space, headhouse work areas, laboratory and offices. Most accessions of Citrus, Fortunella, Poncirus, Eremocitrus and Microcitrus are maintained as potted trees budded onto appropriate rootstocks. These rootstocks may be genotypes more adapted to pot culture than to field growing conditions. The other genera represented are most often propagated from cuttings or seeds. Except for the genus Citrus, trueness to type from seed is a generally uniform characteristic among other genera of the Aurantioideae subfamily. Two or more trees per accession are kept at all times, depending on the demand for budwood. The two trees are usually three years apart in age, with individual trees kept a maximum of seven to eight years for Citrus, and longer for other genera. This allows a constant replacement of

trees by age, insuring maximum budwood production in spite of the space constraint of a screenhouse and the limitation of tree growth in pots. Heavy pruning of dormant trees is necessary in January of each year to control tree size. Pathogen-free trees are reindexed periodically to insure their disease-free status. The source of budwood for distribution is the screenhouse protected trees. Seed requests are provided from field collections. Seed of citrus generally does not transmit virus and viroid disease organisms and therefore can be collected from field trees when certain precautions are observed. Prior to shipment, the seed is treated with hot water and surface-sterilized with a fungicide. The budwood is surface-sterilized with a 0.5% hypochlorite solution. All material distributed receives a phytosanitary certification. Distributions are limited to qualified researchers and research/breeding programs.

Research on the long-term preservation of citrus pollen, seed and buds is under way in cooperation with the USDA-ARS National Seed Storage Laboratory (Towill, 1988). Citrus pollen samples have been stored at low temperatures in liquid nitrogen ( $-196^{\circ}\text{C}$ ) and freezer ( $-20^{\circ}\text{C}$ ) for one year, after which they are retrieved, checked for germination and used to pollinate field citrus trees. Early tests using monoembryonic seed parents and isozyme analysis of progeny seedlings have indicated a successful hybridization with the stored pollen. Similar low temperature studies are being initiated with seed and buds using cryoprotectants.

A complete computer-based information record is maintained for each accession in the Repository collection. This record includes passport information on acquisition data, inventories, evaluations (descriptor data), pathogen testing records and orders/distributions. These records are also maintained in the USDA-ARS's GRIN system (Germplasm Resources Information Network), the government's database for plant germplasm resources (Perry et al., 1988). The GRIN database is accessible to public users by obtaining a password code from the GRIN system. Orders for germplasm can be made and processed through this system.

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