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Authors
Navarro, L.
Juarez, J.
Pina, J. A.
et al.

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The Citrus Quarantine Station in Spain

L. Navarro, J. Juarez, J. A. Pina, and J. F. Ballester

ABSTRACT. A Citrus Quarantine Station has been recently established in Spain. It is based in a tissue culture procedure developed for safe budwood introduction, consisting of the following steps: a) preliminary budwood inspection; b) budwood disinfestation; c) budwood culture *in vitro* at 32 C for flushing; d) disinfestation of flushes obtained; e) isolation of 0.1-0.2 mm long shoot tips; f) grafting shoot tips *in vitro*; g) transplanting micrografted plants to a quarantine greenhouse; h) indexing of micrografted plants; i) release of budwood. Any abnormal or contaminated tissue or plant found during the process and all introduced plant material except the shoot tips are destroyed. Pests, fungi, bacteria and even virus and virus-like diseases introduced with the original material can be easily eliminated by this procedure. The estimated time from budwood introduction until budwood release is 24-30 months, including 18-24 months for xyloporosis indexing.

Index words. tissue culture, shoot-tip grafting, *in vitro*, virus-free, citrus diseases.

Transfer of citrus species and cultivars from one country or growing area to another is very often desirable. Cultivars existing in some areas may be suitable for commercial use in others where they have not been previously grown. New high quality cultivars are being obtained by artificial hybridization or as a result of natural bud mutation in the field. In addition, it is sometimes desirable to introduce new species and cultivars for research purposes. Polyembryonic varieties can be safely introduced as seed although nucellar seedlings have undesirable juvenile characters (11). Monoembryonic varieties do not reproduce true-to-type plants when propagated by seed. The exchange of vegetative plant material may lead to the introduction of new pests and diseases which, in some instances may be devastating for the citrus industry.

One approach to this problem is the total embargo of citrus importation, but this may lead to illegal introduction of budwood. Presently, it is recognized that importation of vegetative plant material should be allowed through plant quarantines, which can prevent the introduction and spread of dangerous, or potentially dangerous, pest and diseases (4).

This situation also applies to the Spanish citrus industry. Although many citrus selections are available (7, 8), it is desirable to introduce new cultivars to cover the production throughout the season and to complete the citrus germplasm bank. Imports of citrus budwood without adequate control measures have a high risk. Over one hundred important pests have been reported in different citrus growing areas that have not yet been detected in Spain. Severe citrus diseases caused by fungi (e.g. mal secco, citrus scab, black spot), bacteria (e.g. cancrosis, greening), viruses (e.g., severe tristeza strains, tatter leaf and diseases of unknown cause (e.g., blight and similar declines) are not yet present in Spain.

The establishment of a citrus quarantine to allow safe introduction of citrus vegetative material in Spain was decided. Use of traditional quarantine procedures, which require the introduction of material into an isolated area where citrus is not grown, has presented many problems, mainly due to lack of facilities and trained personnel in these areas. Then, the study of alternative procedures based on tissue culture techniques was initiated. These techniques have only been used to a limited

extent in plant quarantine procedures, but it is expected that will be extensively used in the future (2).

In a previous paper (12), a tissue culture citrus quarantine procedure was proposed. It consisted of the culture of axillary buds *in vitro* to produce flushes that could then be used as source of scion material for shoot-tip grafting *in vitro*. This preliminary work was done using buds from plants grown in glasshouses (10). However, when later buds were isolated from budwood of field trees nearly one hundred per cent of the cultures usually became contaminated. A different method was then developed and as a result, a citrus quarantine station was established in Spain in September 1982. In this paper we describe the procedures used in this quarantine station.

PROCEDURE OF THE SPANISH CITRUS QUARANTINE STATION

Figure 1 shows a diagram of the procedure. It involves the following steps:

Budwood introduction. A history and description of the budwood source tree is recorded. It includes the location where it is grown, age, rootstock, observed abnormalties in trunk, branches or leaves, pests and results of any previous indexing for virus and virus-like diseases. The presence of diseases caused by fungi, bacteria or diseases of unknown origin in the area is also noted. Budsticks are washed with detergent and water and whenever possible treated with a suitable pesticide. Preferably they should be packaged in transparent polyethylene bags.

Preliminary inspection. Upon arrival at the laboratory, located at the Citrus Research Center in Moncada, Valencia, budwood is inspecteded without opening the bags. If it is found with abnormalities, heavily contaminated or infested with living pests, the whole package is destroyed by autoclaving.

Budwood disinfestation. Budwood is taken out of the package, disinfested by immersion for 20 minutes in a 2% sodium hypochlorite solution containing 0.1%

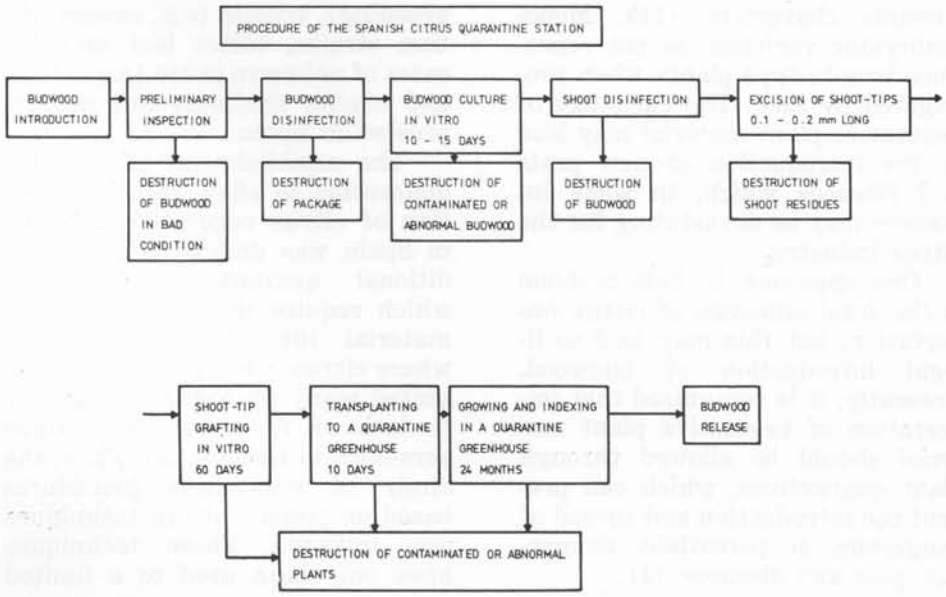

Fig. 1. Diagram of steps of the Spanish Citrus Quarantine Station.

Tween 20 wetting agent and rinsed three times with sterile distilled water. Package and disinfesting solutions are discarded after autoclaving. This severe disinfestation treatment eliminates most superficial contaminants and any possible living pests that could remain on the budwood.

Budwood culture *in vitro*. Culture medium is composed of the Murashige and Skoog salt solution (5) and the medium is distributed in 20-ml aliquots in 38x200-mm culture tubes containing fine sand as a substrate (fig. 2). Tubes are capped with polypropylene caps and sterilized by autoclaving at 121 C for 15 minutes.

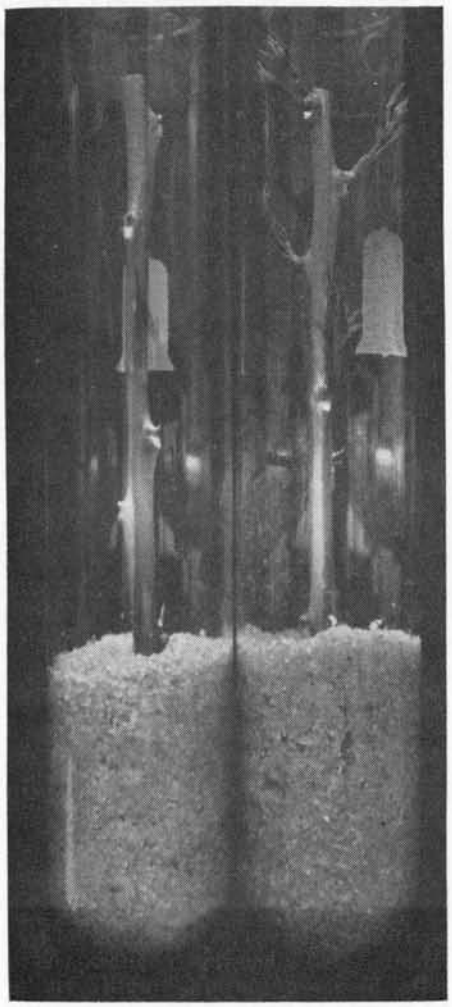

Fig. 2. Culture *in vitro* of Salustiana sweet orange budwood. Left: Initial culture. Right: Flushes produced after 12 days in culture at 32 C.

Budsticks about 15 cm long and 4-6 mm diameter containing 4-6 buds are used for culture. One budstick is placed upwards in each tube, with its base introduced in the sand. Cultures are kept in a tissue culture incubator at 32 C constant temperature and exposed 16 hours daily to 10,000 lux illumination. After 10-15 days, over 80% of the buds produce flushes (figs. 2 and 3).

Several experiments were done to study the optimal conditions for the production of flushes from the budsticks. Thickness of budwood between 4 and 6 mm in diameter did not influence sprouting of buds. Triangular budwood from vigorous twigs also gave similar results. No differences were found among budwood collected in different seasons of the year or collected in winter and stored at 8-10 C up to 4 months. After this storage period, budwood of several culti-

Fig. 3. Detail of flushes produced by budwood of Washington Navel orange cultured *in vitro* for 14 days.

vars was sent by regular air mail to the Canary Islands and then back to Valencia. After a total transportation time of 12 days, budwood behaved like the controls kept in Valencia at 5 C. The percentage of sprouting buds and growth of new flushes was not influenced by culture temperatures between 25 and 32 C. At 35 C the percentage of sprouting buds was the same, but growth of flushes was slower and they had an abnormal appearance. At 38 C none of the buds sprouted. The temperature of 32 C has been selected for the routine procedure of the Spanish citrus quarantine station because recovery of virus-free plants by shoot-tip grafting *in vitro* is enhanced using flushes produced at warm temperatures (9).

This method has been used with several cultivars of sweet oranges, mandarins, lemons, grapefruit and Nagami kumquat. In all cases flushes produced *in vitro* were similar to the ones produced in field or glasshouse trees (fig. 3).

Culture medium may become contaminated, but this does not affect the growth of flushes. Lack of sucrose in the medium avoids fast growth of contaminating microorganisms.

In some cases fungi grow on wounds left after leaf removal. These cultures, as well as any showing abnormalities are destroyed. Symptoms of some diseases like citrus canker, may develop in plants growing *in vitro* (3). Any budwood culture showing these symptoms will also be destroyed.

Shoot disinfestation. Flushes produced *in vitro* are excised from budwood under aseptic conditions with the aid of a long forceps. They are immediately disinfested by immersion for 5 minutes in a 0.5% sodium hypochlorite solution containing 0.1% Tween 20 wetting agent and rinsed three times with sterile distilled water.

The original budwood cultures are destroyed by autoclaving.

Shoot tip excision. Shoot tips containing the apical meristem and three leaf primordia, measuring 0.1-0.2 mm are isolated from the terminal growing point, following to the standard procedure of shoot-tip grafting *in vitro* (STG) (10). This is the smallest size of shoot tip that can guarantee a reasonable number of successful grafts, but if large amounts of material are available, smaller shoot tips can be used. The axillary buds of the flushes can be used as source of shoot tips when necessary. Flush tissues remaining after shoot tip excision are destroyed.

Shoot tip grafting *in vitro* **(STG).** Excised shoot tips are grafted *in vitro* following the standard procedure described by Navarro, *et al.* (10), using the most appropriate rootstock for each species (6). Grafted plants are kept at least 60 days in culture before transplanting to soil to allow the development of possible microbial contamination or disease symptoms (3). Any culture showing abnormalities is destroyed.

Transplanting to soil. Successfully grafted plants are transplanted according to the standard procedure of STG (10) to pots containing a steam sterilized artificial soil mix (1) that are enclosed in plastic bags and placed in a quarantine greenhouse. The bags are kept closed for a minimum of ten days. Plants showing abnormalities are destroyed before opening the bag.

The quarantine greenhouse is divided in small compartments, equipped with heating and cooling systems, and windows are protected with an insect-proof screen.

Indexing. Indexing of established plants is done in the quarantine greenhouse following standard procedures (8, 13) for all virus and virus-like diseases present in the

country from which budwood was imported. Micrografted plants will be maintained in the quarantine greenhouse until indexing is finished. This requires a two-year period after transplanting, allowing for xyloporosis indexing. If during this time micrografted plants show any abnormality they will be destroyed together with all inoculated indicator plants.

If a plant is found infected by any common virus or virus-like it will be destroyed or submitted again to STG according to the importance of the cultivar.

Experiments done following this procedure have shown recovery of plants free of tristeza, exocortis, psorosis and concave gum which infected the original budwood. The incidence of virus-free plants was similar to the standard STG technique (6). The procedure was also effective to obtain clean plants from budwood heavily infested by several arthropods and sooty molds.

Budwood release. Budwood from introduced material can be released about 27 months after importation. This budwood can then be distributed to legal citrus nurseries for commercial propagation or used for experimental purposes.

DISCUSSION

The procedure described here is considered very safe for importation of citrus vegetative material. Pests and epiphytic fungi and bacteria should be eliminated in the disinfestation steps; any remaining pest or microorganism would contaminate the culture medium during STG and the plant would be destroyed. Internal fungi and bacteria will be easily eliminated by STG because they do not infect meristematic tissues of the shoot tip. During the process, plants are grown in test tubes or in pots enclosed in plastic bags, with almost 100% relative humidity and adequate temperature for symptom expression of diseases.

Also virus and virus-like diseases that could infect the introduced budwood can be eliminated through the process. The only material really introduced in the country is a 0.1-0.2 mm long shoot tip that is free of pests and fungal and bacterial diseases and in most cases also free of virus and virus-like diseases.

One of the major advantages of this procedure is that pests and diseases that could be carried by the original budwood are eliminated at the initial stages of introduction. Another advantage, is that the quarantine station can be located at citrus research stations having tissue culture facilities. Test tubes are the substitute of greenhouses located in areas where citrus is not grown. In many countries, central isolated quarantine facilities are not available and it is very difficult to establish them only for citrus. At the same time, STG is being used in many citrus research stations (6). Thus, this quarantine procedure based on tissue culture may allow an ease and safe importation of citrus vegetative material in many areas whereas now it is being introduced without sufficient precautions.

The Spanish Citrus Quarantine Station located at Moncada (Valencia) has already introduced four sweet orange cultivars from the Canary Islands and four mandarin, one tangelo and two lemon cultivars from Italy.

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