THERAPY AND VARIETY IMPROVEMENT

The Citrus Variety Improvement Program in Spain after Eleven Years

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ABSTRACT. The Citrus Variety Improvement Program in Spain (CVIPS) started in 1975 with the following objectives: a) to recover virus-free plants by shoot-tip grafting *in vitro* from cultivars selected in Spain; b) to release virus-free budwood to citrus nurseries and c) to establish a germplasm bank of healthy plants. In 1983 these objectives were expanded with the importation of citrus budwood from other areas through a quarantine station based on tissue culture *in vitro* techniques. Sixty-three species and varieties have been introduced through this quarantine station so far.

Presently the CVIPS has 267 accessions and 112 have been freed of virus and virus-like diseases and are available to citrus nurseries. So far, over 12 million healthy trees from the CVIPS have been planted in commercial orchards.

Index words. shoot-tip grafting *in vitro*, quarantine, viruses, indexing.

Virus and virus-like diseases are widespread in older Spanish citrus plantings producing very important economic losses to the citrus industry. Tristeza, exocortis, cachexia, concave gum, impietratura, ringspot, cristacortis, vein enation-woody gall, crinkly leaf, and stubborn have been detected in commercial orchards (9), where most trees are infected with more than one disease.

These diseases posed important limitations to new plantations, because virus-free budwood of Spanish varieties was not available and virus and virus-like diseases restricted the use of many rootstocks (5).

To solve these problems the Citrus Variety Improvement Program in Spain (CVIPS) was established in 1975 (4, 8, 9) with the general objective of producing virus-free, true-to-type plants of a wide range of species and varieties for commercial propagation and research purposes.

Presently, the CVIPS has the following activities:

1) Recovery of healthy plants selected in Spain.
2) Introduction of foreign species and varieties through the citrus quarantine station.
3) Release of healthy budwood to growers.
4) Establishment of a citrus germplasm bank.

RECOVERY OF HEALTHY PLANTS SELECTED IN SPAIN

This part of the program is based on shoot-tip grafting *in vitro* (6, 12, 14), and it includes the following steps (8, 9).

Selection of mother trees. Selection of mother trees of each cultivar is based on their horticultural performance regardless of virus content. Commercial varieties were selected in private orchards, whereas other varieties were selected in citrus variety collections. They include varieties presently grown in Spain, old varieties not currently grown commercially, new varieties obtained by spontaneous bud mutations in the field, and foreign varieties introduced in to Spain before the establishment of the CVIPS (often without adequate sanitary control). Presently, 204 accessions have been included in the program (table 1).

Indexing of mother trees. Indexing is performed by graft inoculation to the following indicator plants:

TABLE 1
ACCESSIONS OF THE CITRUS GERMPLASM BANK OF INSTITUTO VALENCIANO DE INVESTIGACIONES AGRARIAS (IVIA)

Genus	Species	No. of accessions		
		Selected in Spain	Introduced by quarantine	Total
Citrus	*amblycarpa*	—	1[y]	1
	aurantifolia	2	—	2
	aurantium	19	1[z]	20
	bergamia	3	—	3
	clementina	39	3[y]	42
	deliciosa	1	2[y]	3
	depressa	—	1[y]	1
	excelsa	1	—	1
	grandis	—	2[y]	2
	halimii	—	1[y]	1
	hystrix	—	1[z]	1
	ichangiensis	—	1[y]	1
	karna	—	1[y]	1
	latifolia	2	—	2
	limettioides	1	—	1
	limon	25	4[y]	29
	macroptera	—	1[y]	1
	madurensis	4	—	4
	medica	4	—	4
	meyeri	1	—	1
	myrtifolia	2	—	2
	nobilis	—	1[y]	1
	paradisi	3	4[y]	7
	shunkokan	—	1[y]	1
	sinensis	67	6[y]	73
	sunki	—	1[y]	1
	tachibana	—	1[y]	1
	tangerina	1	—	1
	temple	1	—	1
	unshiu	9	6[y]	15
	webberi	—	1[z]	1
	hybrids	17	10	27
Afraegle	*paniculata*	—	1[z]	1
Atalantia	*bilocularis*	—	1[z]	1
	citroides	—	1[z]	1
	ceylanica	—	1[z]	1
Eremocitrus	*glauca*	1	—	1
Fortunella	*crassifolia*	—	1[z]	1
	hindsii	—	1[z]	1
	margarita	—	1[y]	1
Glycosmis	*pentaphylla*	1	—	1
Microcitrus	*australasica*	1	—	1
Murraya	*paniculata*	—	2[z]	2
Pamburus	*misionis*	—	1[z]	1
Poncirus	*trifoliata*	—	2[y]	2
Severinia	*buxifolia*	1	—	1
Triphasia	*trifolia*	—	1[z]	1
	TOTAL	204	63	267

[z]Introduced by seed
[y]Introduced by budwood

Arizona 861-S-1 citron for detection of citrus exocortis viroid (CEV); Parson's special mandarin for citrus cachexia viroid (CCaV); Mexican lime for citrus tristeza virus (CTV) and vein enation; Pineapple sweet Orange and Dweet tangor for psorosis, concave gum, impietratura and cristacor-

tis; Eureka lemon or Arizona 861-S-1 citron for crinkly leaf, and Madam Vinous sweet orange for stubborn. Inoculated plants of Pineapple sweet orange and Dweet tangor are challenge-inoculated with psorosis lesion bark inoculum (13) to determine the presence of psorosis A.

Indicator plants for detection of CEV, CCaV and stubborn are incubated in a warm greenhouse at 27-32 C, whereas indicator plants for detection of the other diseases are incubated in a cooler greenhouse at 18-25 C.

Table 2 shows a summary of indexing results for virus and virus-like diseases of mother trees of sweet oranges, mandarins and lemons of Spanish origin. The exocortis viroids (2) are the most common pathogens. Only two sweet orange mother trees were found to be free of them. One was a large and productive old Washington navel tree, that probably is a very early introduction of the Washington Parent navel from California. The other one is of Foia Menuda, an old variety located on the island of Majorca. All plants found infected with cachexia also were infected with exocortis, except a Cajel sour orange which is the only plant found so far in Spain infected only with citrus cachexia viroid. Cachexia is also quite widespread, particularly in mandarins. The diseases producing psorosis-like young leaf symptoms on indicator plants (psorosis, concave gum, impietratura and cristacortis) are very common on mandarins and sweet oranges, but are rare in lemons. Vein enation is widespread in sweet oranges, and also found in lemons and mandarins. Citrus tristeza virus was found only in mandarins; although this disease is widespread in Spain. Declining trees were not selected for the Program.

None of the Spanish varieties of the CVIPS were virus-free. Mandarins had the highest rate of infection, followed by sweet oranges and lemons. All the mandarins of the CVIPS had at least two diseases, and 87% has three or more diseases. Sixty-three per cent of the sweet oranges were also infected by three or more diseases. Lemons are usually infected by one or two diseases. These high rates of infection have been found in selected trees that, in general, did not show severe disease symptoms in the field. Most trees in commercial orchards are in worse condition, particularly due to tristeza and psorosis. These data clearly suggest the very important economic losses produced by virus and virus-like diseases to the Spanish citrus industry.

Shoot-tip grafting (STG) "in vitro". The technique applied is the standard procedure for STG (6, 12). The infected varieties are propagated on vigorous rootstocks in the greenhouse. When the resulting plants are

TABLE 2
INCIDENCE OF VIRUS AND VIRUS-LIKE DISEASES IN THE VARIETIES OF SPANISH ORIGIN INCLUDED IN THE CITRUS VARIETY IMPROVEMENT PROGRAM IN SPAIN (CVIPS)

Disease	Group of varieties[z]		
	Sweet orange	Mandarins	Lemons
Exocortis	95	100	100
Cachexia	32	67	38
Psorosis	27	55	0
Concave gum Impietratura and/or Cristacortis[y]	57	83	7
Vein Enation	75	16	21
Tristeza	0	22	0

[z]Data expressed as percentage of infected trees.
[y]These diseases are placed together because they produce similar symptoms on indicator plants.

used for STG, they are completely defoliated by hand and placed in a growth chamber at a constant 32 C. Flushes produced after 10 to 15 days are used as source of shoot tips. These are composed of the apical meristem and three leaf primordia, and measure 0.1-0.2 mm. Troyer citrange is used as the rootstock for all species except lemons, which are grafted on Etrog citron. Routinely, 60-70% grafting success is achieved and over 90% survival is usually obtained upon transplanting.

Indexing of micrografted plants. Plants obtained by STG are indexed by the same methods as described above. Tests for pathogens producing psosoris-like young leaf symptoms are repeated, because sometimes they produce very mild symptoms which are difficult to detect. These tests are usually conducted in spring, when symptom expression is best.

Following the pretreatment at 32 C previously indicated, more than 90% of the plants recovered by STG were free of virus and virus-like pathogens, including psorosis, concave gum, cristacortis and impietratura. These diseases were more difficult to eliminate when flushes were produced in field trees or in plants growing in greenhouses at 18-25 C or 27-32 C (10, 14).

Ninety accessions have been freed of virus and virus-like pathogens. They include all the cultivars presently grown in Spain, and the most promising new cultivars for commercial propagation, except for a few which have been very recently introduced. The other accessions are in different stages of the program.

Horticultural studies. Horticultural evaluation of virus-free plants is carried out to ascertain their fruit and growth characteristics. Three types of observations are made: a) leaf and fruit morphology on plants grown in greenhouses and screenhouses; b) fruit characterization on healthy plants topworked on adult trees; c) comparison of growth and fruit characters between virus-free plants and the original virus-infected plants of each cultivar. So far, the available data indicate that the plants freed of disease-agents are true-to-type.

INTRODUCTION OF FOREIGN VARIETIES

Although many citrus cultivars are currently grown in Spain, introduction of new species and cultivars from other areas is necessary to span the production period. In addition, it is also desirable to introduce many citrus species and varieties and citrus relatives, to increase the germplasm bank necessary for research work. However, the introduction of vegetative material may lead to the introduction of new pests and diseases.

To solve this problem, a Citrus Quarantine Station was legally established in Spain in 1982 and a new tissue culture procedure was developed for citrus introduction (11). This procedure basically consists of culturing the imported budwood *in vitro* at 32 C to induce formation of flushes, from which shoot-tips are isolated and micrografted *in vitro* to obtain plants that are grown and indexed in a quarantine greenhouse.

This STG method has not been adapted yet for many citrus relatives, and these are introduced as seed. To enhance seed germination, and to exclude possible soilborne pathogens, seed are germinated *in vitro*, according to the procedure used to grow seedling rootstock for STG (6, 12). The resulting plants are also grown and indexed in the quarantine greenhouse.

Sixty-three species and varieties have been introduced from California, Argentina, Italy, France, Morocco, Cuba and Japan (Table 1). Both *in vitro* procedures have been successfully applied to a wide range of citrus species and citrus relatives (Table 1).

Indexing of introduced plants is done by the same procedure as described above. In addition, indicator plants of Troyer citrange, *C. excelsa*

and Arizona 861-S-1 citron are inoculated and incubated in the greenhouse at 18-25 C for possible detection of other pathogens not present in Spain. All indicators are observed for leaf symptoms and stem pitting.

For additional safety, we have recently included enzyme-linked immunosorbant assay (ELISA) testing for tristeza (3, 16), electrophoresis of purified RNA extracts for detection of viroids (2, 15) and ds-RNA assays for unknown viruses (1).

Indexing has been finished for 22 introductions and it is underway for the others. In all cases the resulting plants were free of virus and virus-like pathogens.

RELEASE OF HEALTHY BUDWOOD

Virus-free cultivars recovered in Spain or introduced through the Quarantine Station are available to citrus nurseries for commercial propagation through a Certification Program (7, 8).

Budwood is released in small amounts to establish the foundation and the initial multiplication blocks for budwood increase. Only 16 legal citrus nurseries are operating in Spain, and they are associated in two groups, one composed of 14 nurseries and the other of two. Each group has a foundation block. Inspection and control of sanitary status, trueness-to-type and plant quality are easily achieved because of the small numbers of nurseries and foundation blocks.

Table 3 shows the number of plants of different varieties recovered through the CVIPS that have been planted in commercial orchards since 1982. The total number of plants is over 12.7 million and represent almost 12% of the Spanish citrus industry.

All varieties propagated by legal citrus nurseries in Spain are free of known virus and virus-like diseases. This is a great change, because before 1982 all the Spanish varieties commercially propagated were infected with one or more pathogens.

The CVIPS has made available to growers many new varieties that

TABLE 3
NUMBER OF VIRUS-FREE PLANTS, ORIGINALLY RECOVERED THROUGH THE CITRUS VARIETY IMPROVEMENT PROGRAM IN SPAIN, PLANTED IN COMMERCIAL ORCHARDS[z]

Variety	Year					
	1982	1983	1984	1985	1986	TOTAL
Foyos Washington navel	—	—	—	50,000[y]	254,923	304,923
Navelina navel	275,000[y]	560,352	734,422	879,747	885,772	3,335,293
Newhall navel	300,000[y]	681,000[y]	615,686	802,638	1,037,281	3,436,605
Navelate navel	100,000[y]	223,645	208,578	229,779	286,984	1,048,986
Nules clementine	125,000[y]	409,735	581,240	644,349	534,923	2,295,247
Oroval clementine	100.000[y]	174,732	132,018	50,012	25,089	481,851
Fina clementine	20,000[y]	24,517	8,139	23,403	4,585	80,644
Hernandina clementine	—	—	3,161	30,153	42,918	76,232
Arrufatina clementine	—	—	—	18,938	31,416	50,354
Esbal clementine	—	—	—	8,662	12,490	21,152
Fortune mandarin	—	—	—	8	71,464	71,472
Clausellina satsuma	50,000[y]	134,261	214,200	231,971	162,720	793,152
Fino lemon	50,000[y]	90,743	100,499	35,299[x]	162,096	438,637
Verna lemon	50,000[y]	54,154	50,052	14,123[x]	78,663	246,992
Others	—	—	14,634	10,074	34,367	59,075
TOTAL	1,070,000	2,353,139	2,662,629	3,029,156	3,625,691	12,740,615

[z]Source: Instituto Nacional de Semillas y Plantas de Vivero
[y]Estimated amounts
[x]This year there was a severe freeze that affected nursery plants, especially lemons.

were not previously propagated, particularly in the mandarin group. The program is having a very important impact on the Spanish citrus industry and most of the problems posed by virus and virus-like diseases to new plantations are being solved.

CITRUS GERMPLASM BANK

The species and varieties selected in Spain and introduced through the quarantine station are included in the citrus germplasm bank. Presently it includes 207 accessions (table 1). The recovered virus-free plants are grown in a field block and in a protected block.

The field block is used for horticultural evaluation and characterization. It is composed of three trees of each accession, propagated whenever possible on Troyer citrange. For commercially important varieties, three additional trees propagated on Cleopatra mandarin are included.

The protected block is composed of two plants of each accession propagated also on Troyer citrange, grown in containers in an insect-proof screenhouses. The principal objective of this block is to prevent reinfection of healthy material. It is the primary source of material for commercial propagation and for international exchange of citrus germplasm.

CONCLUSION

The CVIPS has made it possible for all the plants propagated in commercial citrus nurseries in Spain to be virus-free. A wide range of Spanish and foreign varieties are available to growers, who can select the most appropriate according to the orchard characteristics and the international market demand. This situation will produce very important economic benefits for the Spanish citrus industry.

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