# SURVEYS AND CERTIFICATION

## The Citrus Variety Improvement Program in Spain in the Period 1975-2001

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ABSTRACT. The Citrus Variety Improvement Program in Spain (CVIPS) started in 1975. It has the following objectives: a) to recover pathogen-free plants of local cultivars by shoot-tip grafting *in vitro* (STG); b) to import foreign genotypes through a STG based quarantine procedure; c) to maintain healthy genotypes in a Germplasm Bank; and d) to release healthy budwood to citrus nurseries through a certification program. Plants recovered by STG are biologically indexed by inoculation to the following indicator plants: Mexican lime, Pineapple sweet orange, Dweet tangor, *Citrus excelsa*, Etrog citron, and Parson's Special mandarin. In addition, they are indexed by sPAGE or imprint-hybridization for viroids, by RT-PCR for Citrus leaf blotch virus, by dsRNA analysis for viruses that produce dsRNA during their replication cycle, and by tissue print-ELISA for *Citrus tristeza virus*. Only healthy genotypes are included in the Germplasm Bank, which has a field collection used for research and horticultural evaluation, a cryo-stored collection for long-term maintenance, and a screen-house collection that is used to release budwood to nurseries. It contains a total of 428 genotypes, 237 selected in Spain and 191 imported from other countries, representing 43 *Citrus* species and 32 species from 17 *Citrus*-related genera. Release of healthy budwood from this program to nurseries started in 1979. At that time, there were only 10 registered nurseries, but in the last few years the number has now increased to 39. For commercial propagation all nurseries are using budwood from the Germplasm Bank. Since the beginning of the program, about 85 million certified nursery trees from this origin have been produced. This represents more than 70% of the Spanish citrus industry. The CVIPS has had a very high impact on the citrus industry. Virus and virus-like diseases do not currently induce any significant damage in the new plantings, and a wide selection of healthy material from the best varieties is available for growers.

The tristeza outbreak in Spain in 1957 was the cause of an historical change in the Spanish citrus industry since, at that time, the highly susceptible sour orange was practically the only rootstock used for propagation. In 1962, and especially in 1968, the disease caused very severe damage in citrus plantings, and the danger of a potential disaster became evident. To prevent this situation, special regulations for the production of citrus plants were legislated in 1968. New plantings and replantings on sour orange rootstock were prohibited, and tristeza-tolerant rootstocks, such as Troyer citrange and Cleopatra mandarin, were introduced for sweet orange, mandarin and grapefruit scions. Several regulations were established for citrus nurseries: They had to be located in tristeza-free areas and they should have a minimum production capacity of 300,000 plants per year. Some new nurseries were established, and many of the small ones merged to form larger units. Only nine nurseries were initially authorized to legally propagate citrus plants.

Early surveys and indexing (14, 16, 36, 37) disclosed that other virus and virus-like diseases were widespread in all Spanish cultivars. Psorosis, impietratura, and concave gum were causing important damage. Exocortis was present in all varieties and consequently, Troyer citrange, which was the tristeza tolerant rootstock better adapted to the Spanish growing conditions, could not be used for propagation. Similarly, the presence of cachexia in lemons did not allow the use of alemow, the most suitable rootstock

for lemons. Tristeza alone has caused the death of more than 40 million trees grafted on sour orange rootstock (5), and other virus and virus-like diseases produced losses estimated to be 10-25% of the total production.

An additional problem of the Spanish citrus industry was the small number of cultivars available for propagation. Many of the best cultivars existing in other countries could not be used because importation of citrus budwood was forbidden to avoid the risk of introducing new diseases. This risk could be overcome by the importation through quarantine stations, but Spain did not have any in operation.

In the early 1970s, the Spanish citrus industry covered 225,000 ha, with a production of 2.5 million tons, and it was very important from the socio-economic point of view. However, the industry was seriously threatened by graft-transmissible pathogens that severely limited its development and maintenance.

## THE CITRUS VARIETY IMPROVEMENT PROGRAM IN SPAIN (CVIPS)

**Objectives and outline**. It was evident that the control of virus and virus-like diseases required the use of pathogen-free varieties grafted on tristeza tolerant rootstocks. Since all Spanish cultivars were infected, the first step to achieve this goal was to recover healthy plants from infected ones. The techniques available in the early 1970's for this purpose were the selection of nucellar seedlings of polyembryonic varieties (38, 45) and thermotherapy (38). Both methods had important limitations to solve the problems of the Spanish citrus industry. Nucellar embryony is very efficient for elimination of pathogens, but nucellar plants are juvenile and require many years before they become commercially acceptable (38). This method was not appropriate to solve the urgent need of healthy varieties. Thermotherapy also allowed recovering pathogen-free citrus plants without juvenile characters, but it had been shown to be ineffective to eliminate viroids (38). Since all Spanish varieties were infected by viroids, the procedure was not practical.

The standard protocol of the new technique of shoot-tip grafting (STG) *in vitro* to recover pathogen-free citrus plants (19, 30) was developed through a joint project of our Institute with the University of California, Riverside. Plants recovered by STG do not have juvenile characters and all citrus pathogens, including viroids, can be eliminated.

The availability of STG allowed the establishment of the CVIPS in 1975 (18) with the following objectives: a) recovery of pathogen-free plants by STG from all varieties grown in Spain; b) establishment of a germplasm bank with pathogen-free plants; and c) releasing pathogen-free budwood to nurseries through a mandatory certification program.

Although citrus propagation in nurseries had been regulated since 1968 (35), the procedures were not adequate in some aspects, such as the periodical disease indexing of the budwood source trees or the procedure for budwood increase. In 1976, a new mandatory certification program was established (1), which regulated in detail all aspects of nursery tree production, including propagation through different blocks and their periodic indexing. The protected germplasm collection maintained at Instituto Valenciano Investigaciones Agrarias (IVIA) is the block of initial material for the certification program. The nurseries had to establish their own classical foundation, multiplication and nursery tree blocks (Fig. 1).

Once the recovery of pathogen-free plants of local cultivars was underway, the next problem to be addressed was the introduction of high quality foreign cultivars.

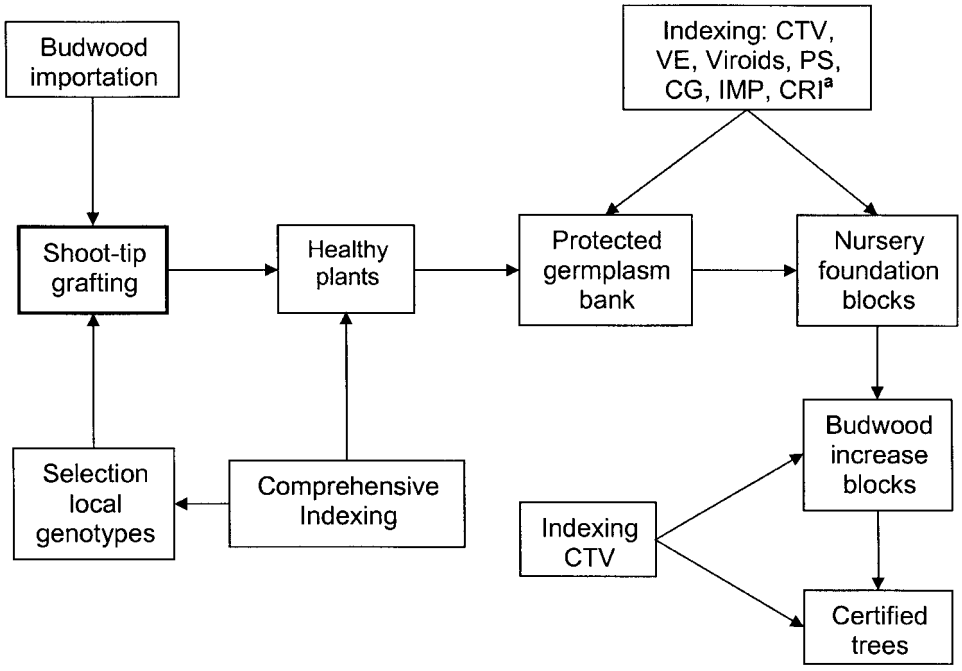


**Fig. 1. Diagram of the Citrus Variety Improvement Program in Spain. a) CTV, tristeza; VE, vein enation; PS, psorosis; CG, concave gum; IMP, impietratura; CRI, cristacortis**

Research was carried out to establish a new *in vitro* quarantine procedure for citrus as an alternative to the classical quarantine stations (26). The procedure consists of *in vitro* culturing the imported budwood at 32°C. This produces flushes in 10-14 days, from which shoot tips are excised and micrografted *in vitro*. With this procedure, only a small shoot tip, usually free of pests and diseases, is introduced into the country, and pathogens that may infect the original material are eliminated in the early steps of the introduction (24, 26). With the availability of this technology, the Spanish legislation was changed in 1982 to allow the importation of citrus through the *in vitro* procedure. Consequently, a new objective for importing foreign citrus genotypes was added to the CVIPS in 1983.

The CVIPS is in fact the combination of three different but coordinated programs; sanitation, quarantine and certification (20). All programs are necessary to produce healthy nursery plants as the most important means to control virus and virus-like diseases (Fig. 1). Each of these programs is the responsibility of a different public institution, but the existing cooperation allows carrying out all technical operations related to disease elimination and indexing at IVIA under the same technical supervision. In addition, the excellent cooperation with nurserymen ensures quick and efficient distribution of budwood. The three institutions involved and the nurseries provide financial support for the CVIPS. Previous reports about the CVIPS have been published (22, 23, 27). In this paper, the general situation of the program and the methodologies used are reviewed and discussed.

**Selection of genotypes.** Initially the CVIPS placed emphasis on commercial cultivars. However, very soon the program evolved to a more general approach to include as much genetic variability of *Citrus* and *Citrus*-related genera as possible, with

the final goal of establishing a Germplasm Bank. This could then be used as starting material for commercial propagation and for research activities, particularly those focused on citrus genetic improvement.

Two hundred and thirty seven genotypes have been selected in Spain. Selection was based on horticultural criteria regardless their sanitary status. One hundred and ninety one genotypes were imported from other countries though the quarantine station.

Presently most new varieties are being registered worldwide. Therefore, the CVIPS has made provisions to include registered privately owned varieties. In these cases, the owners cover the cost of pathogen elimination (sanitation or quarantine) and maintenance at the Germplasm Bank.

**Shoot-tip grafting *in vitro*.** STG is done following the standard technique (19, 30), using Troyer citrange as rootstock for all species and grafting the shoot tips inside an inverted T-incision made at the top of the decapitated rootstock seedling. Micrografted plants are transplanted to the greenhouse 4-6 weeks after grafting either directly to pots (19, 30) or by regrafting on rough lemon rootstocks growing in the greenhouse (7). With both procedures, we usually obtain over 95% survival, but growth is faster with the latter method.

The most important factors that influence elimination of citrus pathogens by STG are the growing temperature of the shoot-tip source plants and the size of the shoot tip. It was found that incidence of recovery of plants free from diseases difficult to eliminate (psorosis, concave gum, impietratura and tatter leaf) increased by growing the shoot-tip source plants under warm conditions (15, 25). As a routine procedure in the CVIPS, the infected cultivars selected in Spain are propagated and grown in containers in a greenhouse at 18-25°C. They are then defoliated by hand, and placed in a growth chamber at constant 32°C and exposed to 350 $\mu E/m^2/s$ illumination 16 hr daily. After 8-12 days, new flushes are produced and used as source of shoot tips for micrografting. Similarly, budwood of imported genotypes is cultured *in vitro* at 32°C to induce production of new flushes in 8-16 days that are used as source of shoot tips for STG. This procedure is also being increasingly used for genotypes selected in Spain, since it is faster than propagation of plants in the greenhouse, and it gives a better grafting success.

The frequency of successful grafts increases with shoot tip size, but the incidence of recovery of healthy plants decreases (31). Consequently, it is necessary to choose a size that will give a realistic degree of grafting success with the highest possible number of pathogen-free plants, since indexing is more expensive and time consuming than STG. In the CVIPS we routinely use shoot tips composed of the apical meristem and three leaf primordia. The size, measured from the cut end to the tip of the largest leaf primordium, varies from 0.1 to 0.2 mm, depending on the citrus species.

The above-described procedures have been routinely used in the CVIPS since 1985. They have been used with many genotypes of at least 43 C*itrus* species, and with *Poncirus* and *Fortunella* species. The overall percentage of grafting success has been 38%, but differences were found between species and shoot tip sources (Table 1). Comparative studies using the same genotype have not been done, but shoot tips excised from budwood cultured *in vitro* gave a slightly higher incidence of grafting success than shoot tips from plants growing in a growth chamber, albeit this is species dependant. In the case of satsumas the grafting success has been much higher with shoot tips isolated from budwood cultured *in vitro* (Table 1). Overall, more than 40%

TABLE 1
AVERAGE GRAFTING SUCCESS (%) OF SHOOT-TIP GRAFTING *IN VITRO* CARRIED OUT IN THE CITRUS VARIETY IMPROVEMENT PROGRAM IN SPAIN 1985-2001

| Group of genotypes | Shoot tips excised from plants growing in growth chambers | Shoot tips excised from budwood cultured *in vitro* |
|---|---|---|
| All genotypes | 33.6 | 43.5 |
| Sweet oranges | 40 | 40 |
| Clementines | 46 | 48.2 |
| Satsumas | 18.1 | 39.8 |
| Other mandarins | 39.3 | 45.8 |
| Lemons | 19.4 | 21.2 |
| Grapefruit | — | 58.3 |

grafting success was usually obtained with most commercially grown species using budwood cultured *in vitro* as the source of shoot tips, with the exception of lemons that gave only about 20% success.

Citrus viroids, mild strains of *Citrus tristeza virus* (CTV), vein enation, and citrus concave gum were very easy to eliminate with the standard procedure of STG. In the entire history of the CVIPS, none of the micrografted plants has been found infected with mild CTV strains or concave gum. Only one micrografted plant was found infected with a non-cachexia isolate of the *Hop stunt viroid* (HSVd), and another one with vein enation. *Citrus psorosis virus* (CPsV) and severe CTV strains were more difficult to eliminate, yet more than 85% of the micrografted plants were free of these pathogens. Some mother plants were infected with pathogens that induced psorosis-like young leaf symptoms on indicator plants, but they did not protect against challenge inoculation with psorosis B or concave gum. These pathogens were eliminated in 65% of the micrografted plants. Citrus leaf blotch virus (CLBV) was found in five mandarin plants from two genotypes imported from Japan. This pathogen has been recently characterized (13, 45) and although its distribution has not yet been assessed, it seems that it is not widely distributed. Therefore, we do not have enough data to evaluate the rate of elimination by STG. However, it seems to be difficult to eliminate from some genotypes. Experiments are under way to obtain this information.

The above data indicate that the standard procedure of STG used in the CVIPS gives satisfactory results. Grafting success is acceptable and most of the micrografted plants are free of the pathogens that infected the original plants. Reduction of the shoot tip size to include only two leaf primordia would probably increase the incidence of pathogen elimination, but then the grafting success will decrease to levels that are not practical for routine operations (30, 31).

In the CVIPS, several micrografted plants are recovered from each genotype, but only one or two are initially indexed, since this is usually enough to get a healthy plant. The total time required to recover a healthy plant is usually 14 to 16 mo, including STG and indexing.

## INDEXING

**Procedures.** Pathogen detection procedures used in the CVIPS includes biological indexing using the indicator plants listed in Table 2 and several laboratory methods. Biological indexing is done according to standard protocols (39). Pineapple sweet orange and Dweet tangor plants are always challenge inoculated with psorosis-B lesion bark inoculum and with severe concave gum, respectively, as an addi-

TABLE 2
BIOLOGICAL INDEXING SPECIES ROUTINELY USED IN THE CITRUS VARIETY IMPROVEMENT PROGRAM IN SPAIN

| Indicator plant | Incubation Temperature | Diseases/pathogens detected |
|---|---|---|
| Etrog citron | 27-32°C | Exocortis and other viroids |
| Parson's special mandarin | 27-32°C | Cachexia |
| Pineapple Sweet Orange | 18-25°C | Psorosis, Cristacortis, Concave gum, Impietratura, Greening (HLB) |
| Dweet tangor | 18-25°C | Psorosis, Concave gum, Mosaic, Cristacortis, Impietratura, Dweet mottle, Leaf blotch. |
| Mexican Lime | 18-25°C | Tristeza, Vein enation, Leaf rugose, Witches' broom. |
| *Citrus excelsa* | 18-25°C | Tatter leaf, Tristeza, Psorosis |
| Etrog citron | 18-25°C | Infectious variegation, Crinkly leaf, Leaf blotch, Satsuma dwarf. |

tional safeguard and to determine if the original sources were infected with these pathogens (40). Citron indicators plants incubated at warm temperatures are kept without pruning for at least 7 mo to be able to detect the non-cachexia isolates of HSVd (10).

In 1989, conventional biological indexing using Etrog citron and Parson's Special mandarin as indicators of exocortis and cachexia was complemented with sPAGE analysis of inoculated citrons (10). This detection procedure combining properties of Etrog citron and nucleic acid analysis was reliable, faster and cheaper than conventional biological indexing for routine indexing of all citrus viroids. When cDNA clones of citrus viroids were available, protocols for detection by molecular hybridization using cold labeled probes were also developed (33). A method based on the hybridization of imprinted membranes (41), which avoids the need for nucleic acid extract preparation, was developed for the routine testing of large numbers of inoculated citron plants (34). This method is been used in the CVIPS since 1998.

CLBV is being indexed by RT-PCR. Total RNA extracts from the candidate plants are obtained from fresh or freeze-dried young leaf tissue, using TRIzol$^{R}$ reagent (Invitrogene), which contains isothyocyanate, following the manufacturer's instructions for samples with high sugar content. cDNA is synthesized by one-step reverse transcription and PCR amplification (RT-PCR) in the conditions described previously (11, 46) using primers based on the CBLV genomic RNA sequence (45).

Serological DAS-ELISA tests for CTV detection (12) have been used in the CVIPS from 1978, initially using polyclonal antibodies, and later specific single monoclonal antibodies (MAbs) (44) or a mixture of two MAbs (3DF1 and 3CA5) that recognize all CTV isolates tested (4). The development of tissue print-ELISA for CTV detection in imprinted sections of plant material in nitrocellulose membranes (13) allowed a sensitive and reliable diagnosis of samples without the need to prepare extracts (6). This procedure has been extensively used in the CVIPS since 1995, using the mixture of antibodies 3DF1 and 3CA5 (5).

Potential infection with other unknown RNA viruses is indexed by double-stranded RNA (dsRNA) analysis of candidate plants. While healthy plants do not have detectable amounts of dsRNA molecules, plants infected with RNA viruses usually contain one or more types of dsRNA which correspond to replicative intermediates of viral genomic, subgenomic or defective RNAs (2, 9).

These dsRNA molecules sometimes allow identification of the infecting virus, at least to the genus level, or differentiation of virus isolates (8, 17, 42, 43). Analysis of dsRNA is routinely used in the CVIPS. Extracts enriched in dsRNA are obtained by phenol extraction of total nucleic acids and dsRNA purification by non-ionic cellulose column chromatography in the presence of 16-18% ethanol, and then they are characterized by polyacrylamide gel electrophoresis (8, 17).

**Indexing of trees selected in Spain.** Field candidate trees selected in Spain are indexed with the whole set of indicators listed in Table 2 and by sPAGE. This identifies the pathogens that need to be eliminated by STG, and also provides detailed information on the incidence of different diseases and the type of isolates of each pathogen present in Spain, and the identification of new diseases. Vein enation-woody gall (21), a severe isolate of tristeza introduced with an illegal importation of satsuma from Japan (3), a graft transmissible bud union abnormality of sweet orange on Rough lemon (29), and a kumquat disease (28) later found associated with CLBV (13, 45), were first detected in Spain in the context of the CVIPS. Indexing of mother trees has also been the basis for the establishment of the IVIA collection of graft transmissible citrus pathogens that has been very important for research purposes.

All Spanish varieties included in the CVIPS were initially infected at least with one pathogen and more than 50% of the genotypes were affected by three or more pathogens (27). This illustrates the consequences of propagating budwood without adequate sanitary control, and provides additional evidence of the damage caused by graft transmissible pathogens.

**Indexing of micrografted plants.** Micrografted plants, either from genotypes selected in Spain or imported through the quarantine station, are also indexed using the whole set of indicators listed in Table 2, and with all the above described laboratory procedures. In the case of viroids, imprint hybridization is usually used for genotypes selected in Spain, whereas sPAGE is used for imported material. The reason is that hybridization procedures are based on the sensitivity of the probes to recognize only known homologous viroid sequences, whereas sPAGE analysis is less specific and may detect unknown viroids. The viroid content of genotypes selected in Spain is found in the previous step, whereas the viroid content of imported genotypes is unknown as the budwood sources are directly micrografted without previous indexing.

We have not found discrepancies among biological and laboratory methods. Duplication of diagnosis of the same pathogen by two different procedures gives an additional safeguard to the CVIPS. In addition, analysis of dsRNA may allow the detection of unknown pathogens.

**Indexing of the propagation blocks in the Certification program.** The Spanish Certification Program includes indexing for the following pathogens or diseases: tristeza, vein enation, psorosis, concave gum, impietratura, cristacortis, exocortis and cachexia. The regulations for periodical indexing depend on the degree of isolation of the different blocks. Presently, the initial block at IVIA and the 11 foundation blocks at the nurseries (Fig. 1) are grown in screen or greenhouses. In this situation, all trees have to be indexed every 3 yr for tristeza, vein enation, and exocortis, every 6 yr for cachexia and every 10 yr for the other diseases. Indexing is done by inoculation on Mexican lime and tissue print-ELISA, Pineapple sweet orange, Dweet tangor and Etrog citron followed by imprint hybridization with probes for all viroids. Nearly 2000 trees have to be

periodically indexed, as a consequence of the recent increase in the number of foundation blocks.

Multiplication blocks are also grown in plastic houses and it is estimated that there are about 250,000 trees in the existing 39 nurseries. Ten per cent of these trees are randomly selected each year and indexed for CTV by tissue print-ELISA or DAS-ELISA by the Plant Protection Services; however, several nurseries are doing their own indexing of all trees in their multiplication blocks using the commercial kits available.

In recent years, citrus nurseries are producing 6-8 million plants annually which are indexed at random. Random samples from these plants are also indexed for CTV by the Plant Protection Services. Between 0.1 to 1% are indexed, depending on location and CTV incidence in the area.

**Germplasm bank**. In August 2001 the program had 428 genotypes representing 43 *Citrus* species and 32 species from 17 related genera. The highest number of genotypes correspond to the three species most widely used in commercial plantings: Sweet orange (Spain is a secondary center of diversification of this species) with 114 accessions, Clementine with 58 accessions, and lemon with 35 accessions. A list of all accessions of the Germplasm Bank is available on the website http://www.ivia.es/deps/biot/germop.htm.

The Germplasm Bank is composed of three different collections: a) a protected collection grown in containers in screenhouses, that is used as the block of initial material for the certification program; b) a field collection, used for genotype characterization following IPGRI (International Plant Genetic Resources Institute) and UPOV (International Union for the Protection of New Varieties of Plants) descriptors and c) a cryo-preserved collection of embryogenic callus used for long term conservation and also as source of material for protoplast fusion and genetic transformation (32).

Field characterization of genotypes is critical to ascertain their trueness-to-type and to avoid duplications. Characterization data are stored in a data base developed at IVIA named GERMO, which allows a quick and efficient comparison of genotypes.

**Release of budwood.** All genotypes of the germplasm bank are available to citrus nurseries. The first budwood release was done in 1979 and up to now 157 genotypes have been released to nurseries for their foundation blocks. They include commercial and ornamental scion varieties, and rootstocks. The choice of varieties to be grown varies according to consumer demands, and new improved varieties are often replacing older ones. Presently over 40 varieties are being propagated in the nurseries for commercial plantings. Nurseries started to sell certified plants originated in the CVIPS in 1982, and by 2000 they had sold 85 million plants, representing about 70% of the total trees in the Spanish citrus industry.

Initially there were only nine nurseries associated with only two foundation blocks. The number of nurseries increased slowly to 16 in the period 1975-1984, but they were all still grouped around the two foundation blocks. However, in recent years, the number of nurseries has increased quickly to 39, and the number of foundation blocks to 11. All nurseries and foundation blocks are using only budwood from the CVIPS to produce certified plants since the beginning of their operation. The increased number of nurseries, and especially of foundation blocks, forced several adjustments in the financial management of the CVIPS in order to be able to meet the additional needs of budwood and indexing. Nurserymen are now providing an important part of the budget.

## CONCLUSIONS

The Spanish citrus industry now comprises approximately 285,000 ha with a total production close to 6 million tons. It is the fourth-producing country in the word and the largest exporter of fresh fruit. The CVIPS has played a very important role for Spain to reach this leading position. About 70% of the citrus area is now planted with healthy high quality varieties from the program. In addition, there is now a much larger number of varieties available for the growers to choose from considering what is most appropriate for their orchards and consumer preferences. Diseases produced by graft-transmissible diseases that were the main limitation of the Spanish citrus industry in the past, do not now cause damage in new plantings.

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