STATUS REPORTS - BUDWOOD REGISTRATION PROGRAMS

Citrus Sanitation, Quarantine and Certification Programs

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ABSTRACT. Control of citrus virus and virus-like diseases in any citrus area requires the establishment of three related but separate programs: sanitation, quarantine and certification. Sanitation programs have the objective to recover healthy plants from local varieties. They are generally operated by research institutions. This program may not be necessary for countries that only grow international varieties available in germplasm banks maintaining healthy plants. Quarantine programs have the objective of importing foreign varieties avoiding introduction of new pest and diseases and usually are operated by Plant Protection Services. Quarantine could be bypassed in some cases, by importing material from germplasm banks that maintain healthy plants under screenhouses. Certification programs have the objective to guarantee that the sanitary status of the initial material is maintained during the process of commercial propagation at the nurseries. They are usually operated by Nursery and Seed Services and mainly consists of legal regulations for the different steps of nursery operations and requirements for periodical indexing of the trees in foundation and increase blocks used at the nurseries. Certification programs are always necessary to achieve an adequate control of diseases. These programs have to be used to protect citrus even from naturally spreading diseases existing in a given country. In this case, the main objective will be to avoid an early infection and/or to avoid introduction and spread of severe strains of pathogens with the nursery plants and the programs may have to be complemented with other control measures.

Index words. shoot-tip grafting, virus and virus-like diseases, indexing.

Citrus virus and virus-like diseases produce very important economic losses in most citrus growing areas. In general, they cause decline, loss of vigour and short commercial life of trees, low yields and poor fruit quality and they restrict the use of some rootstocks. In many areas they are the main limitation for the development of the citrus industry.

To control these diseases it is necessary to use healthy and high quality trees in the new plantings. The production of these trees requires the establishment of three different but related programs: sanitation, quarantine and certification (Fig. 1). Often concepts and organization of these programs are misleading in the literature. In this paper, the concepts and procedures of the three programs are reviewed.

SANITATION PROGRAMS

These have the objective to recover healthy plants from selected local cultivars. In general they are carried out by research institutions and they require the participation of specialists in horticulture, virology and tissue culture.

The recommended procedure for sanitation programs involves the following steps (Fig. 1): selection of mother trees of local cultivars, indexing of mother trees, recovery of pathogen-free plants by shoot-tip grafting *in vitro*, indexing of micrografted plants, horticultural evaluation of healthy plants and maintenance of healthy plants.

Selection of mother trees. Individual trees of the different cultivars included in the program have to be selec-

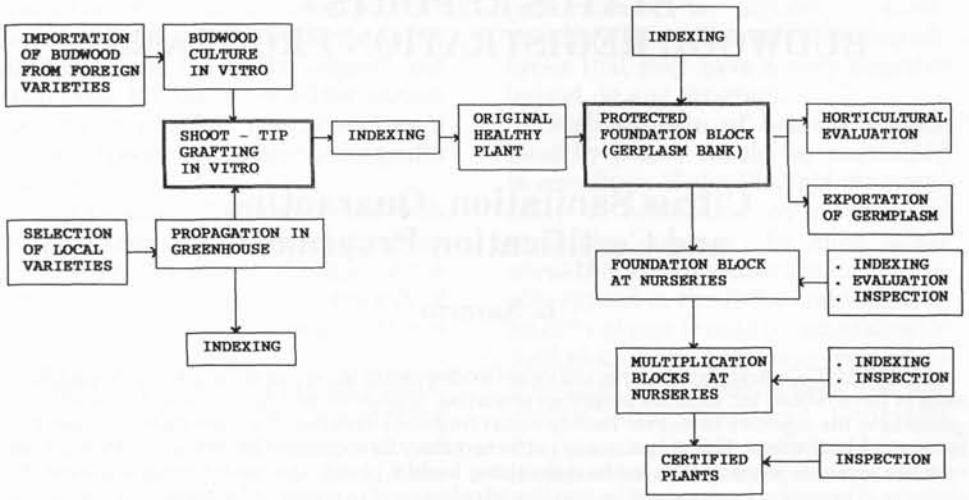

Fig. 1. Diagram of citrus sanitation, quarantine and certification programs.

ted according to horticultural criteria. Disease content should not be taken into consideration for tree selection, since there are several methods to recover healthy plants from infected ones. Discarding trees with disease symptoms may result in elimination of clones of superior horticultural value. Selected trees should be propagated on vigorous rootstocks (e.g. rough lemon) in the greenhouse to be use as a source of material for indexing and shoot tip grafting.

Indexing of mother trees. Indexing of mother trees is necessary in order to know the diseases that have to be eliminated by shoot tip grafting. In addition, this will allow one to get an accurate knowledge of the pathogens present in the citrus area. As an example, in the citrus variety improvement program in Spain this step has allowed us to have very comprehensive information about the incidence of different diseases, the type of isolates of each disease, and to discover the presence of vein enation-woody gall (14), a severe isolate of tristeza introduced with an early satsuma illegally imported from Japan, and a graft-transmissible bud union abnormality of sweet orange on rough lemon (24). Indexing of mother trees has also been the basis for the establishment of the collection of virus and virus-like isolates which is actively being used for research on these pathogens.

Table 1 shows a recommended set of biological and laboratory methods that should be used for a comprehensive indexing of mother trees. A detailed description of indexing methods and facilities needed to apply them has been published (30).

Shoot tip grafting (STG) *in vitro*. Pathogen-free citrus plants can be recovered by nucellar embryony *in vivo* or *in vitro*, by thermotherapy and by STG.

Nucellar embryony is effective for elimination of virus and virus-like pathogens, but nucellar plants have juvenile characters and consequently they are excessively vigorous, thorny and late in bearing and they have to be grown for many years until these characters disappear and they become acceptable for commercial propagation (2, 7, 17, 19, 29, 31). In addition, nucellar plants are not always true-to-type (29) especially those obtained by nucellus culture *in vitro* (7, 22) and seed transmission of psorosis or related pathogens has been occasionally described (3, 4, 28).

Thermotherapy produces true-to-type plants without juvenile characters, but the technique is not effective

TABLE 1
RECOMMENDED INDEXING METHODS FOR CITRUS SANITATION AND QUARANTINE PROGRAMS

Biological Methods

Indicator Plant	Diseases Detected
Mexican lime	Tristeza, vein enation, leaf rugose, witches' broom.
Pineapple sweet orange	Psorosis, ringspot, concave, gum, cristacortis, impietratura, greening, kumquat disease.
Dweet tangor	Psorosis, concave gum, cristacortis, impietratura, mosaic.
Troyer citrange	Tatter leaf, kumquat disease.
Etrog citron	Exocortis, other viroids, infectious variegation, tristeza, kumquat disease, satsuma dwarf, others.
Parson's Special mandarin	Cachexia, ringspot

Laboratory methods

Method	Disease detected
sPage	Exocortis, cachexia, other viroids
ELISA	Tristeza
dsRNA	Tristeza, others.
EM, DNA hybridization	Greening

for elimination of exocortis, cachexia and other citrus viroids (6, 29).

STG is effective for elimination of all virus and virus-like citrus pathogens, including those not eliminated by thermotherapy, and produces true-to-type plants without juvenile characters (11, 12, 14, 25). Consequently STG is the best available and the recommended technique to recover pathogen-free citrus plants. This has been internationally recognized and STG is being successfully used in most citrus sanitation programs worldwide (13).

There are several factors that influence the recovery of healthy plants by STG (11). The pathogen itself plays an important role. Most pathogens are very easy to eliminate, whereas others like psorosis, concave gum, impietratura, cristacortis and tatter leaf are more difficult. The recovery incidence of plants free from these pathogens difficult to eliminate was increased by growing the shoot tip source plants under relatively warm conditions (8, 18). As a routine procedure in the citrus variety improvement program in Spain, the infected cultivars are propagated on rough lemon rootstocks and grown in pots in a relatively cool greenhouse at 18-25 C. Then they are defoliated by hand and placed in a growth chamber at constant 32 C, or 35 C day and 30 C night, and exposed 16hr daily to 350 μE/m2/sec illumination. After 8-12 days, new flushes are produced and used as a source of shoot tips for grafting . Following this procedure more than 90% of plants recovered by STG are free of virus and virus-like pathogens, including those that are difficult to eliminate (21).

Shoot tip size also plays an important role on the incidence of healthy plants recovered by STG. It has been shown that increasing shoot tip size resulted in a higher incidence of successful grafts, but also in a reduced incidence of healthy plants recovered (26). The use of a shoot tip composed of the

apical meristem and three leaf primordia, measuring 0.1-0.2 mm form the cut surface to the tip of the largest leaf primordia, is recommended for routine application of STG. This size gives a realistic frequency of successful grafts and healthy plants.

The relatively low numbers of healthy plants recovered in some laboratories is probably due to the use of too large shoot tips and/or the growth of shoot tip source plants at relatively cool temperatures.

Indexing of micrografted plants. Plants recovered by STG, or any other method, should be carefully indexed for the diseases found in the mother trees to ascertain that they are healthy. It should never be assumed that a plant is healthy because it has been submitted to a sanitation treatment, even in the cases where diseases present in the mother plant are very easily eliminated. As an example, in Spain cachexia has been eliminated by STG always in 100% of the micrografted plants. However, in the citrus variety improvement program, plants recovered by STG are routinely indexed for this disease, despite the relative long time required to carry out the standard biological test on Parson's Special mandarin indicator (30), that delays the release of healthy budwood.

Horticultural evaluation of healthy plants. Plants recovered by STG have been, so far, true-to-type and to our knowledge there is no report on abnormal plants obtained through this procedure. This was expected, since STG is just a grafting technique that does not involve the neoformation of buds *in vitro* or the use of growth regulators. However, it is well known that natural mutations often occur in citrus and there is always the possibility to graft *in vitro* meristems carrying one of this mutations. In addition, the whole process requires a lot of labeling in the laboratory and the greenhouse and there is also the possibility of errors in this process. Consequently, horticultural evaluation of healthy plants to ascertain that they are true-to-type and to know their horticultural performance is strongly recommended. This will avoid releasing off-types that may have a very negative impact on any program.

Maintenance of healthy plants. Healthy plants should be maintained in conditions that will avoid the possibility of reinfection with virus and virus-like diseases. In most citrus areas there are diseases that are naturally spread in the field, and therefore healthy plants should be maintained in field plots located far away from citrus plantings or in screenhouses that precludes vector infestation. Generally, the latter method is preferred because it allows location of the screenhouses at citrus experiment stations and this facilitates the studies and control of the plants.

The collection of healthy plants should be simultaneously the protected foundation block source of budwood for the certification program and the germplasm bank for research studies and exchange of cultivars with other countries (Fig. 1).

QUARANTINE PROGRAM

Quarantine programs have the objective of importing foreign varieties avoiding the introduction of new pest and diseases that may be carried on the original material. These programs are usually operated by Plant Protection Services.

Movement of citrus species and vaieties between different citrus areas for commercial and scientific purposes is often desirable. Particularly in citrus industries oriented to commercialization of fresh fruits in international markets, as is the case of all citrus producing countries in the Mediterranean area, the availability of the best cultivars of the different species is a high priority. However, uncontrolled importation of budwood has the risk of introducing new pest and pathogens, that in some instances, may be devastating or may produce very important economic damage.

This risk may be overcome by the introduction of varieties through quarantine stations (9). In citrus there

are two different quarantine procedures that can be safely used for importation of plant material (5). The classical method consists in propagating the imported budwood in greenhouses located far away from citrus growing areas. Then, the newly propagated plants can be indexed or submitted directly to shoot tip grafting, followed by indexing. This procedure requires the availability of facilities and trained personnel on citrus pest, diseases and cultural practices. It is used in countries with a long tradition in quarantine (e.g. USA, Australia), having central facilities and personnel for importation of plant material of several crops, but it is very difficult to establish just for citrus in most countries.

An alternative citrus tissue culture procedure was developed for the safe introduction of citrus varieties (20) which has been proven to be very efficient to exclude citrus pest and diseases (16,20,21). It consists of culturing *in vitro* the imported budsticks at a constant 32 C or 35 C day and 30 C night, and exposed 16 h daily to 45 μE/m2/sec illumination to induce the sprouting of lateral buds and formation of flushes from which shoot tips can be isolated and micrografted *in vitro*.

This tissue culture method has several advantages over the traditional quarantine method. Pests and diseases that might be in the original material are eliminated at the early stages of introduction, and this shortens the quarantine period. For example, Rio Red grapefruit had a high demand in Spain. One budstick was introduced from Texas, the tissue culture procedure was carried out in January 1988 and in May 1989 10,300 healthy buds were released to citrus nurseries (23).

Another advantage is that the quarantine stations may be located at citrus research stations, because test tubes are the substitute of the greenhouses located in isolated areas. At many of these stations STG is being used for sanitation of local cultivars and the needed facilities and personnel are usually available. Consequently the tissue culture procedure can be easily established in many countries to safely import citrus vegetative material.

As a consequence of the above advantages this method is recommended for importation of citrus budwood (5). In Spain, it has been successfully used to import over 80 varieties from different citrus areas. In California they have recently changed their legal regulations for quarantine and are also now using this procedure (D. Gumpf, personal communication).

After STG the quarantine programs have exactly the same steps as the sanitation programs previously described (Fig. 1). Special emphasis has to be placed on the indexing of micrografted plants. In addition to the indexing methods indicated in Table 1, it may be necessary to use additional methods for diagnosis of specific diseases existing in the country of origin of the plant material.

The main problem related to quarantine is the introduction of material from areas with diseases for which indexing methods do not exist, like blight. In this cases importation should be restricted as much as possible and introduced material should undergo additional cycles of therapy. The primary micrografted plant should be subjected to thermotherapy and then to shoot-tip grafting again (5).

CERTIFICATION PROGRAMS

These have the objective of producing certified nursery trees that will guarantee that the sanitary status and trueness-to-type on the initial material is maintained during the process of commercial propagation at the nurseries. In addition, they also control the horticultural quality of nursery plants. These programs consist of legal regulations for the different steps of nursery operations and requirement for periodic indexing and inspection of trees of the different blocks used at the nurseries. Usually they are operated by Nursery and Seed Services or other state agencies with legal authority to impose restrictions and to inspect nurseries and all steps of the propagation takes place at private nurseries.

Certification programs have to be adapted to the specific situation of each country. However, a recommendation on the general outline can be established. Propagation should be based on four blocks of trees; protected foundation block, foundation blocks in the field, budwood increase blocks and blocks of nursery trees (Fig. 1).

Protected foundation blocks should be maintained by research or other state agencies. They are composed of pathogen-free plants recovered through sanitation and quarantine programs, and grown in containers in insect-proof screenhouses to avoid recontamination with vector transmitted diseases. These blocks are the prime source of budwood to establish the foundation blocks. Plants should be indexed periodically to ascertain their health status and inspected to detect any possible growth or fruit abnormality. In Spain we maintain two plants per accession, propagated on Troyer or Carrizo citranges. Plants are pruned every year and they produce fruits regularly. Some plants are already 13-yr-old and they are growing normally.

Foundation blocks usually belong to private nurseries, although they also may be maintained by state agencies. They are field plantings propagated with budwood from the protected foundation blocks. Trees have to be reindexed periodically. In general, it can be recommended that trees will be indexed annually for vector transmissible diseases, every 3 yr for mechanically transmitted diseases and every 6-10 yr for other diseases. Any infected tree should be removed immediately. Trees also have to be inspected annually during the fruit production season by horticultural specialists for identification of possible off-type branches or trees with abnormal growth, that should be removed. Budwood should be collected from these trees only when they have produced enough fruit to give acceptable evidence that they are true-to-type. This requires the observation of at least three normal crops. The amount of budwood collected from each tree should be limited to allow a consistent fruit production. Usually two to six trees are maintained per each accession. The number should not be increased because then it is very costly to carry out the indexing controls. As an example, in the certification program in Spain, that produce 3 to 5 million certified trees per year, only four trees per accession are maintained in the foundation block.

Budwood increase blocks are nursery plants propagated directly from foundation trees to increase the number of buds for propagation of certified plants. The establishment of these blocks allows a reduction in the number of foundation trees which can be then more easily inspected and indexed. Increase blocks may be established under normal field conditions or in plastic greenhouses where a faster and better growth can be obtained. Buds should only be collected from these blocks during a maximum period of 2-3 yr, to avoid the propagation of possible undetected mutations. Consequently, new blocks have to be periodically established with buds from foundation trees. Increase blocks should be inspected to detect any possible growth abnormality and indexed at random for serious vector transmitted diseases.

Certified nursery trees are propagated with budwood from the increase blocks. They may be produced under regular field conditions or in different types of greenhouses, according to the specific needs and technology available at each nursery. These trees are inspected mainly to guarantee that they meet the horticultural quality required in the certification regulations.

In addition to these blocks, it is also necessary to establish trees for seed production. These trees have to be free of psorosis and other diseases producing similar young leaf symptoms, that are occasionally transmitted through seed (3, 4, 28). It is also desirable that they should also be free from other diseases.

There are several aspects that have to be included in certification programs, such as cultural practices, pest control, labelling and planting location.

Plants should be grown with the best available cultural practices. Special precautions should be taken to control pests and fungal diseases. All pruning and grafting tools should be adequately disinfested prior to any fruit picking, grafting or cutting on any tree or nursery plant to avoid spread of mechanically transmitted diseases. Minimum requirements for certified plants could be established to meet the needs of different growing areas. They can include plant size, height of budding, etc.

A very important aspect of certification programs is careful labelling of plants during the whole process of propagation. This should avoid mixing of rootstocks or cultivars and allow the tracing back any abnormality or infection found at any stage of propagation. Continuous inspection should be done throughout all nursery operations. Special attention should be paid to collection, treatment and packing of seeds and budwood, and to grafting increase blocks and certified plants.

Planting locations should be approved by the legal institution in charge of the program. They should be in areas with the minimum risk of infection and suitable for growing citrus plants of good quality. Foundation blocks, increase block and the nursery should be located at a certain distance from any established citrus orchard. The distance will depend on the particular conditions of each area. In countries with non-endemic vector transmitted diseases, the location should be in disease-free areas, to avoid contamination of certified plants that later could disseminate the disease. Presently several nurseries are using greenhouses for establishing multiplication blocks and producing certified trees. In this case the location may be closer to areas infected with vector-transmitted diseases.

Certification programs are necessary even in areas with endemic vector-transmitted diseases, although additional control measures may be necessary. In areas with an endemic infection of tristeza several measures and modification of certification programs could be adopted. Nursery plants should, obviously, be propagated on tristeza tolerant rootstocks. In addition, nursery plants could be certified free of severe strains of tristeza, to avoid their dissemination as has recently happened in Florida (27). As a further step, plants could be certified as being infected with a local protective mild strain, for those countries that use preimmunization to control tristeza. Obviously quick and specific methods for strain diagnosis have to be developed. For these cases plants infected with specific strains, rather than healthy plants should be maintained in the foundation blocks.

In areas infected with greening and stubborn, it is essential that nursery plants are free of these diseases. In addition specific cultural practices have to be adopted to control the vectors and delay as much as possible the reinfection of new plantings. These situations may force the production system to be in greenhouses. In this case the protected foundation block may have a larger number of trees that could be used directly as source of budwood for the multiplication blocks.

In some countries citrus certifications are voluntary, whereas in others they are mandatory. This aspect should be decided according to the situation in each particular country, but mandatory programs are recommended.

DISCUSSION

Sanitation, quarantine and certification are three separate programs that are necessary to control citrus virus and virus-like diseases. In general, these programs are carried out by different institutions, but in order to be effective they should be very well coordinated. As an example the programs presently in operation in Spain (10, 15, 21) follow the general outline previously presented and each program is the responsibility of a different institution. However, there is very close cooperation that allows to carry out all technical operations related to disease elimination and indexing at the IVIA research institute under the same technical supervision. This has resulted in a

very successful operation, that has produced over 40 million certified trees during the last 12 yr.

There are some countries with small citrus industries mainly growing international varieties, where it may not be necessary to establish sanitation and quarantine programs. These countries may introduce healthy budwood from the few germplasm banks that maintain healthy plants under screenhouses. The imported material should be kept in a greenhouse or isolation for at least three growth flushes before being released (5).

However, a certification program has to be established in every country which wishes to produce high quality and healthy nursery plants. In practice, this program is the more difficult to operate, especially in countries with large numbers of small nurseries lacking appropriate technology. There are many countries where STG is used to produce healthy plants and the basic technology for indexing is available, but very few where the growers could widely use certified nursery plants for their plantings. This gap can only be overcome with a good organization to establish a certification program adapted to the specific local conditions of each area.

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