6

Healthy Plant Material and Certification

Christian Vernière

Crop protection, in its widest approach, should take into account the cost of treatment for the producer, loss of homologation of some phytosanitary products, problems concerning environmental protection and the demand for quality products by the consumer. Furthermore, the development of a control strategy should take into consideration three kinds of factors directly or indirectly affecting the health of the trees and quality of the crops: abiotic factors (structure of the crop, cultural practices, environment), biotic factors (host resistance, nature of the rootstock, physiology, competition) and policies (sanitation programme and registration of the plant material, quarantine, eradication). The joint management of these factors should result in the development of the best possible control method. The policy component appears to be of prime importance for crop protection because it helps to propose strategies so that the grower is guaranteed the good quality of the plants before planting and to prevent the introduction of contaminated plants in a region.

INTERESTS AND OBJECTIVES OF A SANITATION AND CERTIFICATION PROGRAMME

Regulation methods are mainly powerful in two kinds of situations where they have proved to be an efficient barrier. In the first situation, when a disease is not yet present in a region, the first defence is to avoid its introduction and to get rid of the pathogen should it be introduced. Quarantine is a very effective means of controlling the passage of infected plants and detecting the presence of healthy carriers contaminated but not showing disease symptoms. Sanitation programmes and production of healthy plants therefore helps to satisfy the demand of professionals and thereby check the uncontrolled or illegal introduction of material whose sanitary status is not

known. The second situation is represented by the appearance of a new disease with a low incidence. Here control is through reduction of the inoculum or its elimination through an eradication programme and distribution of healthy plant material which prevents the diffusion of infected plants.

The objectives of sanitation and certification programmes are to provide propagation materials and plants which are free of the major degenerative diseases and whose true-to-typness is recognised.

Since 1994, all plant material has to be accompanied by a phytosanitary passport for free circulation among the countries of the European Union. This passport, which is issued following the approval of the plant protection service, guarantees that the plant is free of quarantine plant pathogens, which are organisms subjected to control in the European Union. This phytosanitary passport is given only for plants coming from a zone known to be free of these plant pathogens or produced under a certification programme.

For example, to satisfy this European directive, citrus plants produced in Corsica should be produced under such a programme because *Spiroplasma citri*, agent of stubborn disease and a quarantine organism, is present on the island.

To illustrate how a sanitation programme is conducted, examples will be given of different fruit crops, especially citrus plants for which such a programme has been built since the early 1960s in Corsica. A number of studies have been carried out on this crop, subjected to strong pathological constraints, and have conducted to formulate a national certification programme since 1996.

INTRODUCTION AND SAFE MOVEMENT OF PLANT MATERIAL

Exchange of plant material always carries a risk of accidental introduction of plant pathogens. Pathogens present on symptomless hosts acting as healthy carriers are therefore particularly risky. Measures should be taken while selecting and collecting material, and also when material is exported and imported. Plant material should be disinfected and despatch of rooted plants should be banned if another solution can be found. In the case of transport of vegetative material, quarantine measures have to be enforced (observation of plants in a protected place, requirement of indexing procedures). The measures described below for various species could be combined whenever possible for a better sanitary control of plant material.

Citrus

For research or for production, it is desirable to introduce new plant species or cultivars produced from hybridisation or mutations and potentially

interesting for certain pedoclimatic conditions. This tranfer can sometimes be done without any phytosanitary risk. Such is the case with polyembryonic citrus varieties for which the seeds can be exchanged with practically no risk of transmitting pathogenic organisms. However, very often introduction is through vegetative propagation, which may result in importing new pests and diseases because there are a large number of healthy carriers depending on the host-pathogen interaction. Establishment of a quarantine offers a sanitary guarantee for the introduction of vegetative material. It prevents contamination of neighbouring plants by managing potentially infected plant material during its passage from the introduction until its use by nurserymen and growers. This quarantine functions following three main principles: control and isolation of introduced material, regeneration and indexing (Navarro *et al.*, 1984).

As soon as the plant material is received, it should be examined to detect any anomalies, especially if it comes from a region where a serious degenerative disease is known to exist. This material is then disinfected; for example, the stems of citrus scions are immersed in a solution of sodium hypochlorite containing a wetting agent (Frison and Taher, 1991; Navarro *et al.*, 1984).

These rules should also be applied when distributing the plant material so that exchange of pathogens and pests is limited as much as possible.

New pathological problems which have appeared recently and the existence of severe strains with geographic limits make it necessary to adopt special control measures to restrict these threats (citrus variegated chlorosis, witches' broom, chlorotic dwarf, severe strains of citrus tristeza virus).

Coffee and cocoa

It is strongly recommended that exchanges of plant material among producing countries should first pass through a non-producing country to undergo a quarantine. With this objective, the CIRAD undertook measures to conserve, enrich and safeguard the biodiversity of coffee and cocoa by establishing a quarantine in Montpellier in order to ensure intercontinental exchanges of plant material by providing adequate sanitary guarantees.

Recommendations have been proposed to ensure the collection and transport of plant material with minimum risk.

Movement from a producing country

Before any despatch of plant material from a collecting area, it is advisable to avoid collecting from a region where the disease is present. If this is not possible, only healthy looking plant material should be taken—which, however, is not an absolute guarantee—ensuring very strict selection.

Various procedures are adopted depending on the plant parts to be exported.

If the material is taken in the form of ripening or ripe fruits or pods, the fruits or pods must have a healthy appearance. In the case of coffee, the seeds

are depulped, washed and dried if possible; if not they are preserved as they are. In the case of cocoa it is preferable to take the whole pod.

The seeds should be treated with a fungicide and an insecticide powders and then put into plastic bags. The pods should be sprayed or immersed in a fungicidal solution.

If the material is taken in the form of budwood or cuttings, the stems with or without leaves should be collected from healthy looking material. They should be immersed in an appropriate fungicidal and insecticidal solution and packed in newspaper moistened with this medicated solution.

For material taken in the form of rooted cuttings or plants, the good sanitary condition of the plant should be controlled, paying special attention to the roots. The roots should be washed and the plant debris and mud as well as the necrotic parts should be removed.

Rooted plants should be sprayed or immersed first in an insecticide solution and then in a fungicide solution and then packed in newspaper moistened with this medicated solution.

Reception of plant material in the quarantine laboratory

Sanitary control of the material received is carried out and all doubtful material is destroyed. The plant material is placed in an isolated compartment with limited visitors. Plant fragments remaining after taking the material, as well as the packing material, are destroyed by incinerating in an autoclave.

Under certain conditions, when a plant material is introduced in vitro and passes through a greenhouse, the presence of an eventual fungal or bacterial pathogen would be revealed in the culture medium and in such cases it should be eliminated as a contaminating agent. This elimination is done even without knowing the nature of the contaminant. Thus the introduction of a fungal or bacterial parasite is highly improbable.

The situation is different in the case of viruses (swollen shoot of cocoa) because the conditions for the survival of the virus are not well known. In this case, the only precaution that can be taken is to avoid any introduction of plant material coming from regions where the disease is present or to ensure the healthy status of the plant material by electrophoretic analysis (Amefia *et al.*, 1988).

Export of plant material

After passing the required time in the quarantine and sanitary control, the plant material can be re-exported to production areas where it will once again pass through a quarantine for a brief period under tropical conditions before it is ultimately planted in the nursery or field.

Important diseases

For coffee, the major diseases are orange rust—although rust-free countries

are now very few, the transport of more or less virulent strains has to be avoided—and coffee berry disease, at present limited to Central and East Africa. In many countries, the dispersal of nematodes belonging to the genera *Pratylenchus* and *Meloidogyne* should be taken into consideration. Strict treatments should be carried out in nurseries (disinfection of the substrate with methyl bromide and periodic treatment with nematicides such as aldicarb, carbofuran, terbufos, etc.) in order to distribute rigorously healthy plants to coffee planters.

For cocoa, several redoubtable diseases are still confined to a group of countries or a continent: swollen shoot disease in Ghana and Togo, moniliosis and witches' broom in a number of South American countries. Genus *Phytophthora*, which causes brown pod rot of cocoa, has a worldwide distribution, but in the chapter on Pathogens it was seen that species, or rather strains, can show varying degrees of aggressiveness.

Hevea

Production of healthy plant material mainly concerns *Microcyclus ulei* with a view to preventing the spread of this serious South American disease to other continents. A phytosanitary station, similar to the one set up for coffee and cocoa, was established in Guadeloupe where there is no hevea outside the station, and where the plant material comes from Guyana in the form of grafted rootstocks. Before despatch, the material is kept under observation for at least one year. In Guadeloupe *Microcyclus* has never been observed in a tree park containing 300 genotypes.

Oil palm and coconut

Exchange of plant material between countries or continents is done only in the form of pollen or seeds.

Disease transmission through seeds has never been observed, except of course external contamination which is always possible. As an additional precautionary measure, despatch of plant material from countries or regions contaminated by virus or viroid diseases is prohibited. FAO's (Food and Agricultural Organization) guidelines may be referred for additional information on these diseases.

The pollen is collected under very strict conditions for obvious reasons of legitimacy. Preparation is done in a sterile enclosure; the pollen is dried, kept in vacuum and then preserved at a low temperature.

Before despatch, the seeds are treated with a mixture of standard fungicides and insecticides to avoid eventual external contamination. Depending on the sanitary regulations of the receiving country, additional sanitary measures are undertaken on request.

In vitro material can also be exchanged but once received it must undergo adequate indexing procedures.

Besides all this, preventive measures can be taken by providing information to the public through posters about the risks of contamination while tranporting plants (Roistacher *et al.*, 1977). Technical guidelines indicating the rules to be followed for safe movement of plant genetic resources are regularly published by the FAO in collaboration with IPGRI (International Plant Genetic Resources Institute). These are available for species such as citrus, coconut and cocoa.

SANITARY IMPROVEMENT: THE CASE OF CITRUS

Availability of healthy varieties or free of the major degenerative diseases is a trump card in citriculture management. Although all virus-like diseases are transmitted vegetatively, a few are mechanically transmissible (Bové, 1995). Only one virus of citrus, the agent of psorosis, can be transmitted by the seed (Campiglia *et al.*, 1976) and some diseases may be transmitted by pollen (Vogel and Bové, 1976). The absence of seed transmission enables the production of rootstocks from seeds with a good sanitary guarantee. It has also enabled regeneration of polyembryonic varieties through nucellar selection. However, this procedure cannot be applied to monoembryonic varieties and a longer persistence of juvenile characters in nucellar plants has been observed.

Certain pathogens reduce the choice of rootstocks. For example, the tristeza virus can cause decline of varieties grafted on sour orange and the exocortis viroid can alter *Poncirus trifoliata* and its citrange type hybrids, thereby restricting the growth and production of the trees. Regeneration of citrus varieties allowed the use of these rootstocks by eliminating the major citrus pathogens (virus, viroids, phytoplasmas, spiroplasmas, endocellular bacteria).

Regeneration by shoot-tip grafting

The technique of shoot-tip grafting, developed by Navarro *et al.* (1975), has been used at the INRA-CIRAD agronomic research station in San Giuliano (Corsica) since 1978. Additionally to the elimination of the juvenile characters associated with the nucellar selection, it allowed to recover true-to-typness plants from monoembryonic varieties (Nicoli, 1985; Vogel *et al.*, 1988a). Shoot-tip grafting involves the aseptic isolation of meristems from infected plants and then grafting them on young seedlings of etiolated rootstocks that had been grown in vitro.

The shoot tips, composed of the apical meristem and two or three leaf primordia, are excised from the young shoots obtained from plants cultivated

in isolation cages or sticks which are forced in a phytotron at 32°C (Vogel *et al.*, 1988a). The temperature selected is a compromise between elimination of the virus and survival of the plant. This thermotherapy is applied depending on the origin of the plants and the pathogens that could be potentially present (psorosis virus, tatter leaf virus). The meristem is kept at the base of an inverted. T shaped incision done on the in vitro rootstock (Photo 107). The percentage of recovery at the end of the shoot-tip grafting experiment is about 36% and varies from 23 to more than 60% depending on the species (Vogel *et al.*, 1988a). When the grafted plantlet has a few expanded leaves, acclimatisation of the young plant is done by grafting on a vigorous well-rooted rootstock in a pot which is then put in a polythene bag (Photo 108). The plant is thus made up of three species: the rootstock in the pot, the sandwiched in vitro rootstock and the variety that is left to grow. Under these conditions, the transfer on a bearing plant has improved the results of acclimatisation by direct transplantation in a substate (Navarro *et al.*, 1975; Nicoli, 1985). Considering the rate of recovery during the second grafting, the percentage of shoot-tip grafted and acclimatised plants obtained is around 25% on an average. The plants are then cultivated in a greenhouse and indexed for the major graft-transmissible diseases.

Indexing and sanitary control

Indexing methods that are reliable and easy to implement are necessary and have been developed for sensitive detection and reliable identification of pathogens. Serological and molecular detection techniques have been recently proposed for routine use and combine speed with specificity. However, for viruses that are poorly described and for unknown pathogens, these detection methods are not available or are not sensitive enough. In such cases, biological indexing through inoculation or mechanical transmission is the only detection method possible (Roistacher, 1991; Spiegel *et al.*, 1993).

Biological indexing

Indicative symptoms of a viral infection may appear on plants from which the material for regeneration had been taken and can thus direct a diagnosis. Nevertheless, the existence of symptomless tolerant species, which are healthy carriers of certain pathogens, makes it necessary to use susceptible indicator plants for detecting a certain number of pathogens (Bové, 1995; Roistacher, 1991).

Expression of symptoms of various diseases may require the application of an appropriate temperature. For example, in citrus plants the symptoms of psorosis and related diseases (concave-gum, *cristacortis, impietratura*) express at temperatures of around 20-25°C, whereas symptoms of stubborn and viroid diseases (exocortis and cachexia) are better expressed at temperatures of 32-34°C. In practice, implementation of such an indexing programme

requires two independent compartments, each devoted to the detection of these different diseases (Frison and Taher, 1991; Roistacher, 1991).

For every indexing series, a positive and a negative control should be incorporated; they would serve as proof of the good application of environmental conditions and of effects other than those induced by the pathogen (Roistacher, 1991). This requires the constitution of a pathogen bank which will attempt to conserve mild strains.

Serological techniques

These immunological methods are based on the specificity of the reaction between antibody and antigen. A given antibody is produced against an antigenic determinant or a specific epitope. A population of antibodies, called polyclonal antibodies, is synthesised in response to the presence of several epitopes. Under a certification programme, if the pathogen exhibits wide antigenic variability, an antibody or a mixture of antibodies will be selected to enable detection of all the strains of the pathogen.

Enzyme labelling of antibodies has led to the development of two techniques which are widely used in diagnosis: the ELISA test and the dot-blot immunoassay. These techniques are used for detecting pathogens because they are easy to perform in routine manipulations. In the case of citrus pathogens, they enable the detection of the tristeza virus, *Spiroplasma citri* (agent of stubborn disease) *Xanthomonas axonopodis* pv. *citri* (agent of citrus canker) and the satsuma dwarf virus (Civerolo and Fan, 1982; Garnsey *et al.*, 1993; Roistacher, 1991).

Molecular techniques

For serological detection, the antigen protein must always be expressed in the infected plant, which could be a limiting factor. Molecular techniques which can directly detect the presence of nucleotide sequences help us to overcome this constraint besides being highly specific. Two methods are mainly used to detect and identify plant pathogens: molecular hybridisation and polymerase chain reaction (PCR). Molecular hybridisation is based on the capacity of two complementary chains to separate and reassociate depending on salinity and temperature. Labelled monocatenary sequences, or probes, can then hybridise with targeted sequences and their presence can then be detected. In routine operations, cold probes are preferred to radioactive probes, which are more sensitive but have a short half-life period and are more difficult to use. In polymerase chain reaction, a DNA sequence is amplified with the help of specific primers of targeted strands and a thermostable polymerase DNA. Repetition of a denaturing cycle, annealing of the primers and DNA synthesis results in amplification of the targeted sequence which can be observed on agar gel.

Polymerase chain reaction improves the sensitivity of molecular hybridisation, whose degree of response is found to be limited by the number

of targeted sequences present in the sample. Although these methods are still not routinely applied in sanitary indexing programmes for citrus, their ongoing development for some pathogens should help to considerably improve their detection (*Liberobacter* for huanglongbin or greening disease, *Spiroplasma citri* for stubborn disease).

To enable the detection of a RNA virus requiring a retrotranscription phase or to limit inhibitory effects from plant extracts, a preliminary purification may be necessary. Immunocapture is a solution which helps to quickly treat a large number of samples and can be routinely used.

This technique is used for indexing with a view to produce healthy material, free of *Xanthomonas axonopodis* pv. *citri*, in the island of Réunion where—and this is true in many other tropical regions where citrus is cultivated—the primary inoculum is often brought to the plantation by nursery plants. The sanitary status of the plants in the nursery is therefore a factor that has to be vastly improved. In Réunion it was shown that the present way of managing the nurseries—in open air with overhead irrigation system—is highly favourable for the development of bacterial canker. Moreover, heavy rainfall of high intensity but short duration is most propitious for the dispersal of the pathogen through projection. The efficiency of modified cultural techniques in the nursery to restrict the development of bacterial canker lies in the evaluation phase. The production programme retained is as follows: conservation of mother trees serving as the source for scions and rootstock seedlings in insect proof greenhouse and raising grafted plants in a plastic tunnel with drip irrigation.

It is also very important that the sanitary state of the mother trees serving as the source for scions be regularly reevaluated. For this, the technique to detect *Xanthomonas axonopodis* pv. *citri*, based on immunocapture—nested-polymerase chain reaction assay (Ic-n-pcr)—developed by Hartung *et al.* (1996), has proved to be very sensitive. It enables the detection of about 100 bacterial cells per gram of tissue.

A few experimental orchards were set up with grapefruits which are extremely susceptible to bacterial canker and produced under conditions where *Xanthomonas axonopodis* pv. *citri* was not detected by Ic-n-pcr. These orchards help to evaluate the durability of a strategy based on the production of plants free of symptoms, and carriers of population levels that are lower to the detection level of the Ic-n-pcr, compared to plants obtained following a standard integrated control strategy. Preliminary results show that because of the almost systematic presence of infected citrus plants within a boundary that enables dissemination by wind and rain, the plants in experimental orchards get infected during cyclones (climatic conditions favourable for the long distance dispersal of *Xanthomonas axonopodis* pv. *citri* prevail). Hence under the conditions prevailing in Reunion, it is very difficult to grow citrus crops which are very susceptible to bacterial canker (grapefruit, lime, combava).

Choice of a technique

Three criteria should guide the choice of an indexing technique in a sanitation or certification programme: its sensitivity, specificity and easy application. The cost of a test is also an important factor to be taken into account, but it is relative to the value of the indexed plants (mother trees, producing plants).

An ideal sensitivity level is one which enables the detection of pathogens in the latent phase, in small samples or trees where the pathogen is irregularly distributed. The highly sensitive molecular tests offer these qualities and they should be developed despite the difficulties in their handling.

The specificity of a test is primordial for excluding a given quarantine organism. Nevertheless polyvalent techniques are useful in indexing programmes for reducing the cost of a test. For example, a single procedure associating amplification on a susceptible host and electrophoresis under denaturing conditions helped to detect the eleven viroids of citrus (Duran-Vila *et al.*, 1988). On the other hand, varietal management of an orchard may require the identification of races or pathotypes within the same pathogen species and hence a wider specificity.

Lastly, detection techniques should be simple and rapid. They should enable the treatment of a large number of samples (automation) at minimum cost. The choice of a test from an array of existing techniques should take into account these advantages and constraints.

CERTIFICATION PROGRAMMES

The success of an orchard depends on the quality of the plants used while planting. For this the grower should be able to obtain plants with the best sanitary and pomological qualities from nurseries, whether it is for rootstock or for variety.

Sanitary and pomological guarantee

Sanitation programmes are available for obtaining healthy plants (Vogel *et al.*, 1988b). They can be developed for specific pathogens depending on the constraints. With certification, the true-to-typness of the healthy lines of the foundation block, guaranteeing the physico-chemical characters of the fruits, is confirmed (Vogel *et al.*, 1988a). An initial plant is selected to serve as the base for vegetative multiplication. Sanitary and pomological selection of citrus conducted in the INRA-CIRAD research station at San Giuliano served as the starting point for a certification programme set up for citrus fruit plants (Vernière, 1995; Fig. 1). After regeneration and sanitary certification, the plants are registered and maintained in insect-proof cages. They are multiplied and planted in an orchard for the authenticity of the variety.

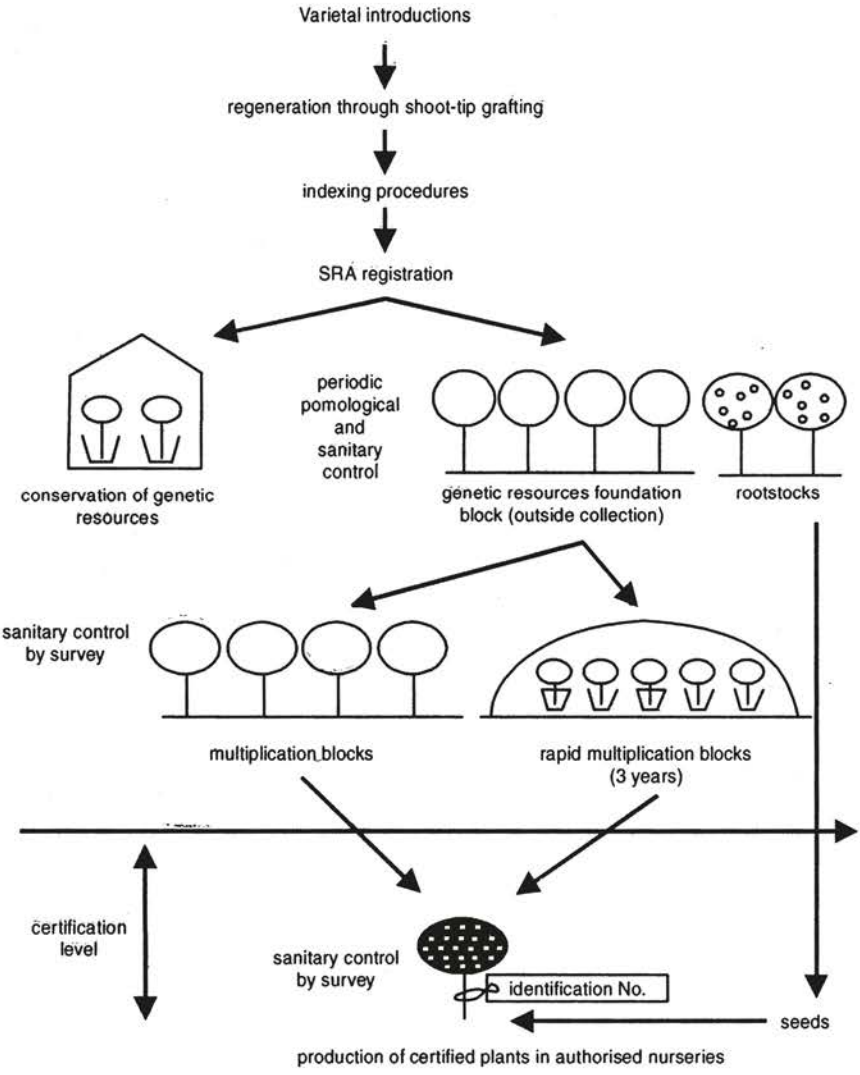

Fig. 1. Procedure for a phytosanitary certification programme: the case of citrus (from C. Vernière, 1995).

The exceptional phytosanitary situation in Corsica with respect to the major degenerative diseases facilitates observation of these genetic resources in open air. In order to conserve this status as much possible, the tests are performed by the plant protection service for certain quarantine agents for quick eradication in case of an introduction. Multiplication blocks are planted according to the requirements of professionals. Rapid multiplication blocks can be established to fulfil an order as quickly as possible and thus enable a significant increase in the production of budwood (Roistacher, 1992).

Plantation guarantee

A certification programme should be conducted under the supervision of an official organization. Plants are produced from scions and seeds of certified rootstocks following certain specifications. An agreement is granted to nurserymen who undertake to respect it. After testing the sanitary state and varietal authenticity, the plants are individually labelled with the information necessary for their identification.

CONCLUSION

The first condition to guarantee the success of a plantation is to have perfectly healthy plant material. Sanitary and certification programmes take on a particularly important character for citrus plants affected by a large number of diseases, especially the viral type in a broader sense, which are often difficult to detect.

The creation of such a sanitary and pomological improvement programme in Corsica in the early 1960s, and now integrated into an official certification programme, has helped to eliminate most of the graft-transmissible diseases in Corsican orchards and to protect the latter from the major degenerating diseases. However, to succeed, the whole citrus sector should be taken into account. In the case of citrus, ornamental plants do not come under the current certification programme, whereas pathogens do not have any barrier for fruit plants. The establishment of quarantine and certification programmes is nevertheless the main barrier for eliminating undesirable pathogens and for reducing the inoculum potential. However, for such a programme to succeed, it should be strictly and fully followed by professional nurserymen and cultivators.

Moreover, with increasingly frequent exchanges of plant material between continents, the risks of spreading serious diseases, which are often still localised, are high. Lastly, even for a disease with a global distribution, all the pathogen strains do not have the same aggressiveness. For these reasons, quarantine departments have a very important role to play.

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