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Recent Developments in Therapy and Virus-Detection Procedures for International Movement of Clonal Plant Germ Plasm

Domestic in-country and international movement of vegetatively propagated plant material plays a fundamental role in modern agriculture. In addition to commercial bulk shipments of plant material, there is considerable movement of smaller quantities of clonal plant germ plasm for various scientific purposes. The realization that reservoirs of plant genetic resources are rapidly vanishing has heightened interest in preserving endangered plant species. This has prompted the collection of germ plasm from natural habitats and its movement to ex situ collections in the same or other countries. Advances in breeding techniques have focused attention on the potential use of wild relatives of plants in crop improvement schemes. In recent years, wild relatives of potato, citrus, peanut, and strawberry have been extensively collected.

One of the greatest concerns regarding movement of vegetatively propagated plant material is the accidental introduction and distribution of plant quarantine pests into areas where they do not occur. The term "pests" is used to describe all harmful biotic agents ranging from viroids to weeds. Pathogens that are often symptomless, such as viruses, pose a special risk. For convenience, the term "viruses" will be used here to include viruslike agents and viroids, unless otherwise stated. In vegetatively propagated plants, virus infections may be symptomless or may induce symptoms that are easy to confuse with nutritional disorders or pesticide damage. Viruses present in one clonal generation are transferred by vegetative propagation to the next and therefore are rightly considered to be a quarantine risk. This concern is magnified when germ plasm that is likely to carry viruses with unknown or poorly understood etiology is collected in its natural habitat and moved internationally.

Safe movement of germ plasm depends on reliable virus detection methods and effective therapeutic procedures. Significant improvements have been made in both areas. Serological as well as nucleic acid hybridization methods using complementary DNA (cDNA) or RNA (cRNA) probes and, recently, nucleic acid amplification techniques involving the polymerase chain reaction (PCR) are available for the detection of many well-characterized plant viruses. The three main methods of virus elimination—thermotherapy, chemotherapy, and tissue culture—have been reviewed (6,8,9, 24,29). The recent trend is to combine elimination methods into a multistage operation to increase the probability of obtaining plants that are virus-free. Information about detection and elimination of a specific virus in a particular crop may be found in several continuing compilations (16,26) and disease compendia.

Plant quarantine systems in many countries have been effective in preventing the entry of viruses. For example, the United States has prevented the introduction of plum pox virus, known to severely damage the stone fruit production industry (Fig. 1) in several countries. In many cases, however, determining virus status and selecting therapeutic procedures for clonal plant germ plasm remain areas where decisions are made in the absence of reliable information.

In this article we give an overview of therapeutic and detection techniques for plant viruses in clonal plant germ plasm and suggest ways to minimize the risk inherent in collecting and moving this plant material internationally.

Virus Elimination Procedures

Thermotherapy, chemotherapy, and tissue culture techniques have been used, either alone or increasingly in combination, to eliminate viruses from plants or plant parts that will be regenerated subsequently into plants. Traditionally, these methods have been applied to potted plants. Current trends are to apply therapeutic procedures to plant material maintained in vitro.

Thermotherapy. Replication of many plant viruses is significantly reduced when the host plant is grown at elevated temperatures. The effects of prolonged high temperatures on virus and host are not clearly understood. Direct and immediate suppressive effects of exposure to 40 C on the synthesis of host and viral RNAs and on the activity of viral replicases were demonstrated in cowpea and tobacco plants infected by various viruses. However, when the plants were transferred after a few hours at 40 C to an optimal temperature, host RNA synthesis was resumed immediately, while virus synthesis was delayed (14).

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A basic thermotherapy procedure involves maintaining plants at a constant high temperature (usually 36–38 C), within the limits of their physiological tolerance, for periods of time ranging from several weeks to months. During this time, parts of or entire plants may become virus-free. In general, the higher the temperature and the longer the exposure time, the greater the reduction in virus titer. In practice, the selected temperature is a compromise between virus elimination and plant survival. Alternating high and low temperatures were found to be less stressful for plants than constant high temperature and effectively eliminated viruses in many cases. In procedures combining thermotherapy and tissue culture techniques, shoot tips (0.3–0.8 mm in length) may be excised from new growth produced during the heat treatment and established in vitro to regenerate new plants. Alternatively, shoot tips may be grafted to virus-free plants or seedlings grown in vitro or in pots. The resulting plants have a high probability of being virus-free, but this needs to be verified.

When thermotherapy is applied to potted plants, preconditioning greatly improves their survival. This is achieved by growing plants under optimal conditions in large pots to allow a large root system to develop. In addition, the temperature in the heat chamber can be raised gradually over several days to the desired treatment temperature. In spite of proper preconditioning, the exposure of several cultivated woody species (e.g., *Prunus, Vaccinium*, and *Vitis*) to elevated temperatures results in very poor plant survival. This sensitivity can be overcome by either increasing CO_2 concentration in the heat chamber to approximately 1,000 ppm and/or reducing O_2 to approximately 5% (11).

Whereas viruses generally can be eliminated from plants by heat therapy, several viroids have been eliminated from plants by prolonged growth at low temperatures. Potato spindle tuber viroid was the first to be eliminated from infected potato plants by low-temperature therapy followed by shoot-tip excision and regeneration (13).

Selecting an effective thermotherapy method for a given plant material requires an empirical approach if no information is available regarding heat tolerance of the host plant.

Chemotherapy. Producing virus-free plants from infected stock by applying chemicals that inhibit or interfere with virus replication has been defined as plant virus chemotherapy. Among the three main techniques available for eradication of plant viruses, chemotherapy is the newest and the least utilized. A variety of natural and synthetic compounds, many of which are of interest in animal and human virology, have been tested for their potential to eliminate both RNA and DNA plant viruses. Research has been concentrated on two synthetic nucleotide analogues, ribavirin (Virazole) and 5-dihydroazauracil (DHT), and, recently, on a DHT derivative, diacetyl-dihydro-5-azauracil (DA-DHT) (21). The chemicals may be sprayed on or injected into infected plants or incorporated into a nutrient medium on which plants are grown in vitro. Shoot tips are subsequently excised and regenerated (8,21).

Viruses of various groups and in different hosts have been successfully eradicated in young shoots treated with ribavirin, DHT, and DA-DHT. With ribavirin, the best examples are in potato and apple. In some cases, chemotherapeutants are reducing virus titers only at phytotoxic levels, e.g., tobacco mosaic virus in systemically infected tobacco plants (21). Combinations of different methods of therapy are also discussed in other sections of this article.

The biochemical events leading to plant virus eradication by chemicals are not understood. Ribavirin may be active in its triphosphate form, which inhibits the capping of viral RNAs (4,12). Recently, Schuster and Huber (22) concluded that both ribavirin and DHT inhibit potato virus X replication at the early stage by impairing synthesis of RNA-dependent RNA polymerase and at a later stage by impairing synthesis of the coat protein.

Tissue culture techniques. Shoot-tip culture is the most commonly used tissue culture technique. It involves the excision of a shoot tip (0.3–0.8 mm in length), consisting of the meristematic dome and at least one set of leaf primordia from plants. The required minimum size of the excised tips varies with different plant species and viruses. The shoot tips are regenerated into plants on a nutrient medium in vitro and subsequently grown out in soil. Information is available in the literature for many plant species.

Although shoot-tip culture is the method of choice for obtaining virus-free plants wherever possible, it has not been very useful for many virus-infected woody plants. The reasons for this include failure to eliminate viruses in explants, lack of differentiation during tissue culture, and failure to induce rooting of plantlets in vitro. To overcome these problems, variations on the basic method have been developed, two of which will be briefly discussed here.

Shoot-tip grafting is a technique in which shoot tips, isolated aseptically from infected plants, are grafted onto decapitated, young, etiolated rootstock seedlings that have been grown in vitro (Fig. 2). Growing plants at an elevated temperature prior to excising shoot tips improved graft survival and recovery of virus-free plants, i.e., the success rate. The method has been adopted for various woody species (17). However, success rates vary greatly with different viruses and plant species and among various research groups.

Shoot tips of grapevine, fragmented in vitro, formed new adventitious buds from leaf primordia. Plants regenerated from these buds were found to be free from many economically significant viruses (1). To our knowledge, this method has been reported for grapevine only, but it may have potential for other "difficult" plant species.

Successful thermotherapy and chemotherapy applied to plant material grown in vitro have been reported for various virus-host combinations. Constant high-temperature regimes followed by excision of shoot tips, which are then regenerated, were used to produce banana plants free from banana bunchy top virus (30) and potato plants free from potato virus S (2). Alternating high and low temperatures applied to several *Prunus* spp. grown in vitro were used to overcome the species sensitivity to heat and facilitated production of clones free from Prunus necrotic ringspot virus (25). In vitro chemotherapy was achieved for a few host-virus combinations by adding ribavirin or DHT to the medium on which plant material was grown. Recently, both strawberry mottle virus and strawberry crinkle virus were eliminated from shoot tips of strawberry plantlets grown on a nutrient medium with DHT (10). Combined thermotherapy, chemotherapy, and tissue culture applied to plant material grown in vitro successfully eliminated Prunus necrotic ringspot, prune dwarf, and apple chlorotic leaf spot viruses from shoot cultures of three sweet cherry cultivars, each infected with one of these viruses (5). Recently, peanut mottle virus was eliminated from peanut grown in vitro by the addition of ribavirin to the medium used to regenerate shoot-tip explants after thermotherapy. The virus was not eliminated unless thermotherapy, tissue culture, and chemotherapy were used in conjunction (3).

In vitro therapy permits treatment of large numbers of plants in a relatively small space, easy application of tissue culture procedures that increase survival (25), and application of chemicals to plantlets in a contained medium, minimizing their possible environmental effects. As is the case with all therapeutic procedures, the genetic integrity of the resulting clones should be evaluated.

Virus Detection Methods

Reliable virus detection as well as established therapy protocols are prerequisites for safe movement of germ plasm. For some viruses, especially poorly characterized, uncharacterized, or unknown viruses, detection methods either are not available or are not sensitive enough to detect virus. Diagnostic methods for plant viruses were recently

Fig. 1. Symptoms caused by plum pox virus on (A) ripe peach fruit and (B) leaves and ripe fruit of apricot. (Courtesy F. Dosba, France)

Fig. 2. Successful graft of a shoot tip from *Citrus sinensis* on a decapitated rootstock seedling of Troyer citrange. (Courtesy L. Navarro, Spain)

Fig. 3. Approximately 50 micropropagated banana plantlets packaged for international shipping in a tissue-culture vessel. (Courtesy Rahan Meristem Propagation Nurseries, Israel)

summarized (15). Following a therapy step, virus titers may be very low and plants may need to be grown for a prolonged period of time (often through a natural dormancy period) to allow virus titers to reach detection thresholds. The time required to reach reliable virus detection may vary with host plant, virus, and geographic location. Extensive retesting is necessary for plants presumably free from poorly characterized viruses or viruses that reach detectable titers very slowly after therapy. This unavoidable step often causes considerable delays in the release of plant material maintained in quarantine.

Detection of poorly characterized, uncharacterized, or unknown viruses. Bioassays using sap- and graft-inoculation to a range of sensitive indicators are still the only methods available to detect viruses that are not well characterized. In some cases, obvious symptoms in the original germ plasm are indicative of a virus infection. However, when the original host is symptomless, bioassays should be used. Both sap- and graft-transmission assays may be inaccurate, time-consuming, and season-dependent. Therefore, conclusions based on negative results are reliable only when testing is repetitive and includes alternative methods utilizing different virus properties for detection.

Double-stranded RNA (dsRNA) analysis is based on the isolation of disease-specific dsRNAs from virus-infected tissue and their electrophoretic separation on a gel, which is then stained and viewed. This assay can be used to detect both uncharacterized and characterized viruses. For plant species with no information regarding virus status and/or with uncharacterized viruses, dsRNA analysis is a rapid tool that can add to the information obtained from bioassays. However, the presence of nonviral dsRNAs in healthy plants, the apparent absence of a dsRNA profile in some viruses, the low concentration of some viral dsRNAs, and the presence of host plant components that may interfere with RNA extraction may result in false negatives or false positives (27). Negative dsRNA tests should be confirmed by other methods before plant material is regarded as virus-free.

The application of broad-spectrum tests such as those developed recently for potyviruses (23) will be very useful for routine testing of germ plasm and, in particular, for testing wild relatives of crops.

Detection of well-characterized viruses. Reliable detection tests are available for many well-characterized viruses. Published data describe variables related to sampling methods and the influence of seasonal factors on test reliability. The three most commonly used detection methods are briefly discussed here.

Serological assays for virus frequently involve trapping virus particles on a supporting surface to which a specific antiserum has been attached. Then, either an enzyme-linked immunosorbent assay (ELISA) in a 96-well microplate or dot blots or squash blots are used to produce a color reaction dependent on virus concentration, or the trapped, negatively stained virions are viewed in the electron microscope, i.e., immunosorbent electron microscopy (ISEM). The use of ELISA has increased dramatically in the last decade with the proliferation and refinement of ELISA systems and the increasing availability of polyclonal and monoclonal antibodies. Serological detection methods for plant viruses were recently summarized (7). Various modifications have been introduced to reduce host antigen background reactions in ELISA and to enhance trapping efficiency and minimize nonspecific trapping of virus particles in ISEM. At present, for viruses for which antisera are available, ELISA is the most widely used detection assay, irrespective of sample number. It can be automated and is sensitive in the nanogram range. ISEM can be used when an available antiserum contains nonspecific antigens that reduce ELISA specificity. ISEM is mostly used on a small scale because it requires ex-

pensive, specialized equipment and highly skilled operators.

Nucleic acid hybridization methods using ^{32}P have been successfully employed for detection of nanogram amounts of plant viral genomes. The availability of cDNA probes and the short half-life, cost, and hazard of radioisotopes present drawbacks to large-scale use of this diagnostic tool. Nonradioactive probes such as biotin and photobiotin-labeled probes and, more recently, digoxigenin-labeled cDNA or cRNA probes have been developed and used for diagnosis of several viruses in plant extracts after overcoming problems with background and nonspecific color reactions.

The recently developed PCR method involves rapid and highly specific in vitro amplification of selected DNA sequences, for which specific primers are synthesized (19). The product obtained from this cyclic amplification process is separated by gel electrophoresis and identified by specific cDNA probes. The PCR method was used for detecting picogram quantities of several plant viral nucleic acids in infected tissues, including corms in which virus concentration is very low (28). The sensitivity of this assay could be further increased by transcribing the PCR products (18). With its relative simplicity and high sensitivity, the PCR method has high potential for detecting minute quantities of virus in plant tissue. However, the prerequisite of having known viral sequences to allow synthesis of suitable primers limits its application to well-characterized viruses.

Quarantine Regulatory Aspects

The ultimate task of quarantine is to prevent the entry of pests into areas where they do not occur or are not widely distributed. The modus operandi is based on inspection, treatment, and prohibition. The most common regulation is the exclusion of plant material originating from an area where pests of quarantine concern, which cannot be easily eliminated, are known to occur. When clonal germ plasm is being moved internationally, the risk of a possible introduction of unknown pests requires extra measures to be taken. For endangered germ plasm, a possible irreversible loss should be weighed against the risk of pest introduction through relaxation of phytopathological standards. Quarantine decisions regarding import of clonal plant germ plasm, usually moved in small quantities and of a high genetic value, can differ from those regarding bulk shipment of commercial material.

Various measures can be taken to minimize the risk involved in movement of plant germ plasm:

- Plant material should be chosen carefully. In general, there is a gradient of increasing risk from embryos to seeds, to fruits, to budwood or stem cuttings, to corms, roots, or tubers, to whole plants.
- A number of different treatments are possible, and therapeutic measures should be applied before plant material is shipped.
- Plant material should be moved in containment when possible (Fig. 3). In vitro cultures exclude insects and many pathogens except for viruses and MLOs.
- Testing methods should be applied to confirm the absence of pathogens of quarantine concern.

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The use of in vitro methods for international movement of plant material has increased significantly over the years both by commercial operations and by scientific organizations. For example, the in vitro exchange of cassava by the International Center for Tropical Agriculture (CIAT) in Colombia has increased from 206 samples in 1980 to 1,507 samples in 1990. It should be emphasized that regardless of the sophistication of the containment facility in which plant material is maintained, testing and elimination of viruses and MLOs have to follow the guidelines proposed for untested plant material.

Systematic application of appropriate therapeutic techniques plays an important role in minimizing the risk of transferring any plant germ plasm, especially wild relatives. It should be emphasized, however, that therapy alone cannot replace indexing for diseases. Sanchez et al (20), in their work on potato introductions, present an overall scheme for minimizing the risk posed by possible viruses in new clonal accessions of a crop.

There are no internationally accepted general protocols for the transfer of germ plasm. An ongoing series of crop-specific guidelines that provide relevant information on disease indexing and other procedures that will help to ensure phytosanitary safety when germ plasm is moved internationally is being published by the Food and Agricultural Organization of the United Nations and the International Board for Plant Genetic Resources (26). Since 1989, guidelines have been produced and published for banana and plantain, cassava, citrus, edible aroids, grapevine, legumes, sweet potato, vanilla, and yam. In cases where no guidelines are yet available, the maximum data possible should be gathered to allow informed decisions to be made.

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