Production of virus free fruit plants in temperate regions

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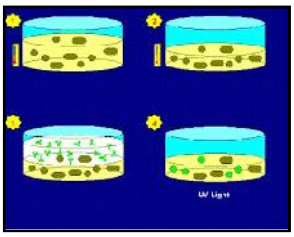
The diseases caused by viruses and virus like pathogens need special mention because of particular nature and habitat of these pathogens. Once a crop is infected the virus lives within the plant till its death and no chemical or other control measures are available against these pathogens and the losses caused by virus are huge in terms of quantity, quality and productive age of the plant. As most of our planting material today is not virus tested, these infections are definitely playing a major role in reducing productivity of fruits. Unlike fungal and bacterial diseases, attempts to control virus diseases in plants have met with limited success so far. This is due to the mechanism of infection of the host by the viruses. Majority of the plant viruses are RNA viruses. These RNA viruses having no independent metabolism, they depend upon their host for replication and synthesis of their virus particle. Virus infection in plants passes to their progenies owing to its being systemic in nature and is responsible for the degradation of the fruit plants in the years to come. Therefore, it becomes very important to manage the virus diseases in these crop including stone fruits by producing virus free propagative plant material. The process of producing virus free plants is linked with the indexing and detection method of virus in the tissues of the plant. The detection techniques are mainly of three types:

- Biological method of detection
- Serological method of detection
- Modern molecular techniques

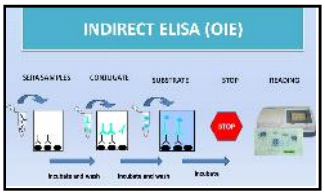
Biological method of detection: Biological method is more time and labour consuming assay than the laboratory assays but still very important in the detection and diagnosis of the plant virus and viriod, not least, biological method are the only means to propagate these pathogens. Biological techniques involve the use of certain indictor hosts both woody as well as herbaceous hosts for the detection of virus infection in the test plant material. These indicators are inoculated with in the plant material to be tested through grafting or /budding in case of woody indictors and also through mechanical sap inoculation on to the herbaceous hosts. After inoculation theses indicators are observed for the production of typical symptoms of viruses. As such the presence or absence of virus infection in the test plant material is ascertained.

A disadvantage of bioassay related to laboratory assay (Serological and molecular techniques) is the higher risk of inoculum escape into the environment. It is therefore essential that tests are performed in insect- proof growth facilities and that plant waste is handled correctly.

Serological method of detection: Serology is a traditional technique for virus detection, and based on the use of antibodies, proteins of the immunoglobulin type, raised in animals and capable of specific binding to antigens. Among the different serological methods of detection of viruses in plants, enzyme linked immunosorbent assay (ELISA) is most commonly used in the detection of viruses.



ELISA: ELISA is diagnostic techniques utilized for identifying plant viruses. The presence of viral specific antigens in infected sap is detected through a colorimetric reaction, that develops because of the reaction of an enzyme (alkaline phosphatase or horseradish peroxidase) conjugated to antibodies in the presence of an appropriate substrate (paranitrophenylphosphate or tetrametyl-benzidine, respectively). Among several variants of ELISA, DAS (double antibody sandwich) ELISA is a standard procedure. Schematically, viral antigens are first trapped by virus-specific antibodies coating the internal surfaces of polystyrene wells, and, then, covered by enzyme-conjugated virus antibodies. Finally, the addition of the substrate induces a colorimetric reaction in the presence of the antigen-enzyme antibody conjugate complex. ELISA has been applied to viruses of stone fruit trees since its first introduction into plant virology in 1976. The first approach was with arabis mosaic virus (ArMV) and plum pox virus (PPV), representatives of isometric and filamentous viruses, respectively. The technique was applied later to the majority of the viruses for which antisera were already available. Up to now, almost 30 viruses are reported as actual or putative agents of diseases affecting stone fruits.



Molecular technique: Molecular techniques of detection of plant viruses are generally based on nucleic acid (RNA or DNA) of plant viruses. The most commonly employed molecular techniques include nucleic acid hybridization and

polymerase chain reaction (PCR) with its variants reverse transcriptase PCR (RT-PCR), immunocapture-RT PCR (IC-RT PCR) which are able to detect the virus in a very low concentration.

Hybridization: Hybridization technique is used for the detection of RNA or DNA viruses in seeds, vegetative propagules and insect vector successfully. Basically, the method involves immobilization of spot or dot of sap extract of plant to be tested on to a solid matrix and detection of viral nucleic acid in the spot by hybridization with nucliec acid probes. The probe consists of radioactively labeled or biotin labeled nucleic acid sequences complementary to viral nucleic acid. If the test samples contain viral nucleic acid it is hybridized by homologous sequences in the probe under appropriate conditions. Non -hybridized probe is washed away and positive reaction assessed by autoradiography. This technique is called dot or spot hybridization assay. When the total nucleic acid extracted from the infected plant electrophoresed in agarose gels, the DNA forms in the gel can be transferred to solid matrix by capillary action, process is called Southern hybridization.

Some basic principles involved in nucleic acid hybridization techniques are:

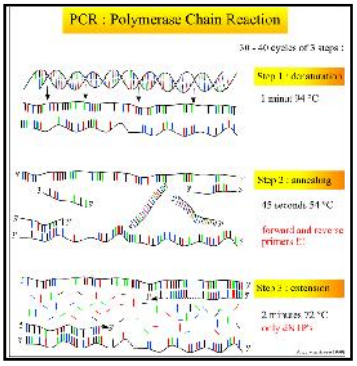
– When a double stranded nucleic acid is heated the bonds linking the strands to each other 'melt' (DNA denatures)

– If the two complementary strands are incubated at appropriate condition one strand hybridizes to the other and the double strand is reformed (DNA renatures)

– The temperature at which 50 per cent of the sequences are denatured is called the melting temperature (Tm) and is affected by the factors like compostion of the nucleic acid, salt concentration.

Thus for the hybridization temperature and salt concentration are the most important factors to be considered.

Polymerase chain reaction (PCR): Detection of viruses in a given sample by PCR is not only dependent on the performance on the PCR assay itself, but also on the efficiency of the procedure employed to extract the nucleic acids from the plant material. The sensitivity of detection can be reduced by inhibitors that may be present in the extract of nucleic acids. To check for substances that may interfere with the amplification process, internal controls can be designed for each pair of primers, or real-time PCR can be employed. The enrichment step can be performed in solid or liquid medium, and even in planta and has to be optimized for each pathogen and amplification protocol. PCR efficiency is controlled by many parameters, such as polymerase type, buffer composition and stability, purity and concentration of dNTPs, cycling parameters as well as the characteristics of the starting template. In addition, the quality of the DNA to be amplified is critical. The very long, complicated and time consuming protocols developed for DNA extraction in the 1990s have often been replaced by rapid, simple DNA extraction protocols or by commercially available DNA-extraction kits. Among them, the RNeasy and DNeasy Plant System (Qiagen, Hilden, Germany) and the Easy-DNA-Extraction kit (Invitrogen, Carlsbad, California, USA) have been used successfully for different types of plant material. Several expensive commercial integrated systems allow for the automated extraction and analysis of nucleic acids from microorganisms, but they are not efficient with all types of plant material and need to be evaluated before they can be adopted for routine detection. Due to its high sensitivity, reverse transcription coupled to PCR in a single step (RT-PCR) is the molecular method most frequently used for the detection of plant viruses. Different RT-PCR variants have been developed, including immunocapture RT-PCR, which has been used with plant extracts or with immobilised targets on paper print/squash-capture (PC/SC) RT-PCR allowing the detection of minimal quantities of RNA targets from plant material or insect vectors without extract preparation.



Besides PCR, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) is the other recent molecular technique for the detection of viruses in the infected plant

Production of virus free propagating material: Virus free propagating material can be obtained in three ways:

Selection supplemented by testing or indexing: Indexing or testing is used both in selecting fruit clones (scion and rootstock) free of the virus. Indexing is carried out by using the indicator plants or serological techniques. Indexing woody indicator is carried out either in the field under isolated condition or in the glasshouse. Field indexing however, proved to be very uneconomical because of the expenses incurred. Glasshouse testing can considerably increase accuracy, efficiency and economy as the average

time for symptom development reduced from years to weeks or months.

Heat therapy: Heat therapy is not applicable to cure the trees in the commercial orchards; however, virus free progeny from the infected varieties would be obtained by thermotherapy alone or in combination with meristem tissue culture. Two types of thermotherapy are known in the treatment of fruit trees. In the short treatment, the scions are exposed for a short time to high temperatures whereas in the long treatment potted plants are kept for a longer time at 37 or 38^0 C. The mode of treatment, the temperature and exposure period applied depend upon many factors, primarily on the pathogen itself. Phytoplasmas are more easily eliminated by heat therapy than viruses. The ability of viruses to withstand *in vivo* high temperature is different. The length of heat treatment may show differences even for the same virus, according to host species and variety. Among fruit trees the pomaceous plant tolerate heat better than stone fruits; especially sweet and sour cherry are sensitive to high temperature.

Meristem culture: Meristem culture is the way of producing virus- free propagating material. The method is based on the fact that because of the low intensity of the virus multiplication in the apical meristem of plants a considerable number of cells are free of virus. Single detached meristem could be cultured, divided into several bits and each bit sub cultured. By repeating this several times over, an incredibly large number of plantings could be obtained from a single protocorn in one year. Meristems are dissected out with the help of a sharp razor-blade , the operation being carried out under a microscope. It should be 500 in length and about the same in diameter . These tiny bits of meristems are then sterilised with 2 % calcium hypochlorite solution and placed in a liquid nutrient medium in a flask under sterile conditions. The flask is then placed on a rotary shaker and rotated at a given speed. This is to prevent formation of any polarity to the developing meristem. Light equivalent to 200 foot candles and a temperature of 22^0 C are maintained throughout. Within a week the meristems will turn green. It starts developing several lumbs of tissue. At this stage the protocorn is taken out, washed and cut up into as many pieces as there are balls of tissue. Each bit is dipped in a sterilant and put in a tube containing fresh liquid medium and agitated. The procedure is repeated until as many plantings as required are produced. The tiny balls of tissue are then transferred to normal nutrient agar medium and kept static as in seedling culture. Each ball of tissue, after reaching 4 mm in length differentiates into an individual plant. The technique of propagation by meristem culture provided a great boost to the orchid industry. Rare plants of special qualities which could not be propagated by seeds due to genetic factors could be multiplied easily and that too at a fantastic rate. This naturally brought down the prices of these orchids which were otherwise too expensive for most people to possess. The only disadvantage with propagation by meristem culture is that removal of meristem causes temporary set-back in the growth of the mother plant. To avoid this, parts of the plant other than the apical meristem, have been tried in tissue culture experiments.

Maintenance of virus free clones: Virus free clones or progeny of virus tested plant material is maintained in the foundation greenhouse or foundation blocks in the field. The selection of suitable places for plantations one of the most important requirements in preventing reinfection. Foundation blocks or mother tree orchards shall be established only in an area where no orchards or any other plants occurred that are hosts of fruit tree viruses.

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