IMPORTANCE OF CERTIFIED CITRUS PLANTS FOR ESTABLISHING A GROOVE WITH FREENESS FROM VIRUSES AND DISEASES

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Planting fruit trees is a long lasting asset but the results of this investment are not obvious until after many years from planting when the trees start bearing fruit. An unhealthy tree could be a carrier of diseases or have inherent genetic defects. The genetic defects arise due to excessive load of somatic mutations. The diseases are carried by nursery plants either because of the use of infected bud wood or may originate from nursery conditions and exaggerate by mismanagement. As a result, most orchards are short lived and produce only a fraction of potential yields. The losses due to poor quality of fruit from sick and variable plants reduce the return further.

Fruit production in Pakistan has had an imposing history of achievement and development. Fruits are grown on an area of 0.657 million hectare which is about 3% of the total cultivated area of 21.870 million hectares. Citrus fruits contribute about 40% of the total production of fruits. The area and production of fruits has been expanding since early 1960s' due to increasing demand in the domestic and foreign markets. The demand for fresh fruits in the neighboring countries offered profitable returns, which has helped the sustained growth of fruit crops. Unfortunately, the citrus/Kinnow plantations have had shorter than desired life span, low yields and deterioration in quality due to diseases and genetic defects. After the implementation of WTO it will not be possible for us to export citrus and other fruits with out ensuring its quality and phyto-sanitary status.

The world's best fruit growing countries have self sustaining nursery certification programs executed by government agencies and assisted by R&D institutions. A certification program ensures supply of healthy and true-to-type nursery plants to the orchardists. As a result, the orchards have long productive life, high yields and quality of fruits. Among mandarins Kinnow and Feutrell's Early and among Sweet Oranges Musambi and Blood red are the important cultivars grown in Punjab.

There are about 4000 virus and virus like diseases of plants now known. Only 1000 of them are identified. In citrus the first virus was Psoriasis or scaly bark. Up till now more than thirty disorders in citrus have been known to be caused by viruses. There are many others that are grouped as virus like on the basis of their infectivity, means of transmission (usually grafting) and apparent absence of other disease causing agents. It has been known that some of these are caused by Viroid (virus without protein coat or simply naked RNA), phytoplasmas and phloem limited bacterium.

During 1930's a disease, now called citrus tristeza virus wiped out millions of citrus trees in the major

citrus areas of the world; Brazil, Argentina and California. Efforts were being started to save citrus from viruses as a result researchers revealed that Tristeza and several other virus and virus like diseases can be checked if their vectors are controlled and by ensuring use of virus free bud wood. These viruses are not spread by seed; hence, rootstocks are virus free. To ensure use of virus-free bud wood the world's first bud wood certification program was launched in 1937 voluntarily by the Nursery Service of the California Department of Food and Agriculture in consultation with the University of California, USA scientists. It did much to free the new plantings that it was modified at different stages and continued for more then 30 years. As it was the first and successful virus sanitation program for citrus any where in the world, it was gradually copied by most of other citrus regions in various parts of the world.

Several methods are available to produce such virus free propagules. Use of shoot tip grafting or micro grafting, in combination with thermotherapy to produce virus free plants of citrus is a common method of bud wood sanitation. Resistance and/or prevention of infection are the only alternatives for control of plant viruses. Each viral disease has its own characteristics and need a specific control strategy. The preventive approach is especially effective for viruses without a living vector, or for viruses that have an insect vector. Such is the case with most viruses and virus-like diseases of citrus.

The sanitation program is of utmost importance as once the plant is infected with virus it remains infected until it dies. So plants used as bud wood source or mother stock must be carefully and continuously monitored to ensure that they remain virus free. Young orchards and new plantings must then be carefully managed to minimize the chances of viral infection. This involves monitoring insect vector and controlling it with best suited pesticides.

Therapy Methods for Clean-up

If the source is infected, it must be subjected to therapy procedures that can eliminate the disease or virus from it. Following are the methods used for clean-up of source bud line.

Thermal Therapy

The infected material is subjected to 32°C for at least 30 days. These triggers up plant growth and the new flushes dominate the disease and viruses. This technique is helpful to eliminate the chances of viruses and diseases from the bud line. After that the bud is grafted onto a healthy rootstock seedling.

Shoot-tip Grafting

Shoot-tip grafting (STG) is an exceptional technique to recover pathogen free plants from infected source. The technique consists of grafting a small shoot tip (0.20.3mm) from an infected plant onto a young rootstock seedling growing *in vitro.* The principle is based on the fact that the shoot tip or meristem of an infected plant is often free of pathogens, and a plant regenerated from young shoot tip is usually healthy.

The procedure of STG is carried out under sterile conditions and is comprised of five steps.

- Root stock preparation
- Scion preparation
- Grafting
- Culture of grafted plants
- Transfer of soil

Briefly, young growing flushes from infected plants are collected, surface sterilized, and used for shoot tip excision. A shoot tip, composed of the apical meristem and two to three leaves primordial and measuring 0.2-0.3 mm in height, is cut and places in an inverted-T incision made at the top of the decapitated rootstock with the aid of a microscope. The micro grafted plant is placed in a tube with nutrient medium and maintained in a culture room at 26°C and exposed at 16 hrs daily to 4 mol m-2s-1 illumination. When the graft takes and 2-3 leaves form the scion develops, the micro grafted plant is transplanted to soil or grafted onto a healthy vigorous root stock in the green house to encourage rapid growth. Using this procedure 90% of the recovered plants by STG is free of virus and virus like pathogens, including those that are difficult to eliminate by ordinary practices.

Indexing Methods

Biological Indexing

This term refers to the preferred method of testing STG or other citrus source trees to confirm the absence or presence of viruses and other pathogens. In biological indexing, tissue from the source tree is inoculated or grafted onto a battery of indicator plants. These indicator plants show symptoms for specific pathogens under specific temperature regimes. After successful inoculation, indicator plants are cut back and laced in a' chamber or room of a green house that has the specific temperature range for symptom expression of the pathogen being tested. The plants are checked for disease symptoms over a period of months. If none are seen, the plants are declared free of pathogens being tested for, and mass multiplication can begin.

Laboratory Indexing

ELISA is a sensitive serological test based on an antigen-antibody reaction that is routinely used for the detection of both plant and animal pathogen. There are numerous modifications of ELISA technique; however, the one we generally perform is DASELISA to trap potential virus particles in plant sap. This is done with the use of two antibodies, trapping the virus in it which is referred to as the double antibody sandwich method.

In contrast to biological indexing, ELISA is a quick way to test the presence or absence of a virus, as hundreds of samples can be tested in a very short period of time.

The Citrus Quarantine Program at UAF

Commercial fruit production demands better control of fruit trees in growth and production. The process begins in the nursery. The performance of future tree is based on genetics and health of rootstocks seeds and scion bud wood. In Pakistan the condition of nurseries is unhealthy and phytosanitary rules are hardly followed. The propagating materials for raising stock as well as scion do not pass through a screening program to ensure uniformity in quality and freedom from diseases. It has been reported that almost 20% of the citrus trees grown in Punjab are infected with Tristeza virus. It is the most destructive citrus virus that actively refers to several disease symptoms. Luckily we mostly have Rough lemon rootstock that is tolerant to CTV otherwise the situation could be different. Anyhow to tackle the increasing infection (from infected to

non-infected by the vector; brown citrus aphid) the Institute of Horticulture Sciences, University of Agriculture Faisalabad took lead and lunched a citrus bud wood certification program with the sponsorship of Ministry of Science and Technology. The project is located at Faisalabad that falls in the middle of citrus belt covering districts of Khushab, Sargodha, Jhang, Toba Tek Singh, Sahiwal, Okara, Khanewal, Multan etc. Moreover, the University of Agriculture, Faisalabad is the best-equipped institution to conduct research and development work proposed in this project proposal.

The Program is stated to achieve the following objectives

1. To initiate a base for bud wood certification program.
2. To establish rootstock tree
3. Selection and propagation for citrus and mango.
4. To initiate container grown citrus nursery plant production.
5. To provide basis for the sale of certified fruit plants.

The program consists of following stages.

1. Selection of Trees

Initially healthy and well-shaped fruits are collected form healthy, vigorous & true to type trees of rough lemon from PARS, UAF. Fruits were washed, seeds extracted and rootstock seedlings rose both in soil mixtures and *in vitro* on tissue culture media.

For bud wood source, healthy looking vigorous, high yielding scion tree are Selected from the University citrus groove located at Experimental Fruit Garden.

2. Indexing of Scion Trees

The selected candidate trees are indexed for CTV. For quick and reliable indexing Serological tests as ELISA (Enzyme Linked Immunosorbent Assay) is used. Only bud wood from virus free trees is used to produces new Micro budded/STG (Shoot Tip Grafted) plants.

3. Microbudding/STG

Micro budding is a new technique used successfully as an alternate of shoot tip Grafted plants rose through tissue culture. In shoot tip grafting a bud of 0.2 mm is inserted on *in vitro* grown rootstock seedlings. This technique eliminates virus even if the scion is from infected plants. However, it is a laborious and tiresome method and if the Scion is taken from a plant that has been confirmed virus free after indexing, then Micro budding is equally efficient in the production of virus free citrus budded/grafted plants. Micro budding involves taking a wedge shaped small bud of from a scion and inserting it onto a rootstock plant cut in the form of a cleft.

4. Re-indexing of Micro budded/ STG plants

All the plants produced are re-indexed after six month using ELISA. The interval of 6 month for re-indexing is meant that during this period if there is any virus present would start multiplying and can be indexed.

5. Establishment of the foundation and bud wood increase blocks

The Release Program is based on the indexing of virus tested plants buds from STG/Micro budded plants. After mother plants have fruited and the fruit is found true to type then bud and/or plants will be released to nursery men for increased propagation. The certified buds will be propagated for distribution to nurserymen. The virus tested foundation

trees developed will be grown in containers and held in an insect proof screen house to prevent re-infection from insect transmitted diseases. These trees will be used as

Primary source material for propagation of clean bud wood until field grown foundation block trees are large enough for bud wood release.

6. Propagation & increase of healthy nursery stock

To meet the needs of new grafted/budded citrus plants container grown rootstocks will be produced in a large quantity. After six to eight months these were then micro budded with buds from virus indexed scion plants from the foundation block.

CONCLUSIONS

Nursery Problems

Nursery business has become an unregistered, a common profession with no scientific/technical background. No phytosanitary measures are taken in the selection of seeds, rootstock and bud wood. No measures like disinfection of tools which is the main Cause of spread of viral/varied diseases, gumming, bud union crease etc. No treatment against soil-borne diseases or nematodes is done. Presence of citrus canker and other diseases in the nursery stock. Budding/grafting on rootstock is practiced at very low height.

Field Problems

When farmers transplant budded/grafted plants in the field, the bud upon IS almost buried in soil which promotes *Phytophthora* root rot and gummosis. Mounds are made around the trunks that enhance instance of diseases and production of offshoots and suckers. Inter-cropping especially with crops like rice, barseem etc is harmful. Unbalanced fertilizers are applied that lack micro-nutrients which cause deficiency

Table 1: Status of Citrus Pathogens in Pakistan

Pathogens having independent metabolism; host-parasite interaction breakable	**Pathogens having dependent metabolism systematic, graft transmissible**
Fungi	Viruses (Mollicutes)
Bacteria	Viroids BLO's
Nematodes	*Spiroplasma
Phanerogames	Phytoplasma

Table 2: Insect Vectors of Citrus Viruses

Insect	**Scientific Name**	**Disease**
Aphids	*Aphis gossypii* *A. Citricola* *Toxoptera aurantii*	CTV
Psyllid	*Diphorina citri*	Greening
Leaf hoppers	*Neolitorus tenellus* *N. haematoceps*	CSD CIVV

symptoms and confused with that of viral diseases. Very less or no protective sprays are applied. Heavy hoeing is practiced that destroys feeder roots in upper zone and it is main reason of die back.

There is a great need to sustain the country's citrus industry through resolving problems relating to it. The functional significance of certified citrus nurseries is a subject we are currently addressing.

Table 3: Distribution of Virus and Virus-Like Diseases of Citrus in Pakistan

Disease	Host	Pathogens	Acronyms	Climate
Cachexia Xyloporsis	Viroid	CX	Warm	Mandarin Malta Mosabmi
Exocortis	Viroid	EX	Warm	Sweet oranges Sweet lime
Greening	BLO	CGD	Cool-Warm	Mosambi Malta, Lemon, RL
Stubborn	Spiroplasma	CSD	Warm	Mosambi Mandarin
Tristeza	Virus	CTV	Cool-Warm	SO, SWO Mand. Lime Mosambi
Infectious Variegation Ring spot	Virus	IVV	Cool-Warm	Lemon, RL, Kinnow SWO
Yellow vein	Virus	CYVCV	Cool-Warm	Lemon Sour orange

Table 4: Distribution of CTV in Punjab (ELISA-Based)

Location	%infection	Citrus variety	Infected Observed Trees	%Infection
Sargodha	13.2	Lemon	0/24	0
Faisalabad	13.0	Mosambi	15/66	22.7
Sahiwal	18.2	Kinnow	2/43	4.65
Sheikhupura	7.4	Sweet orange	5/28	17.85

(Anwar and Mirza, 1992)

Table 5: Distribution of Greening (Colour Test)
(Akhtar and Iftikha, 1999)

Locations (Province)	Infected/tested	Orchards
Punjab	89/142	81/161
NWFP	37/41	37/43
Total	126/183	118/204

Percent incidence

	Variety	Infection (%)
Sahiwal and vicinity 0.5-1.0	Kinnow Malta	22 40
Faisalabad and vicinity2.3-4.5	Musambi Grapefruit	25 15
Peshawar 5.3	Sweet lime Lemon	10 02

Table 6: Citrus Decline-Symptoms and possible causes

Citrus Decline

1. Leaves show mottling, vein clearing, vein banding, but general appearance still normal.
2. Leaves drop more fruits on some branches.
3. Profuse flowering, heavy fruiting, die back symptoms, secondary pests.
4. Trees collapse

Problems Involved

Faulty nurseries, defective management and agronomic practices, *Phytophthora,* CTV.

Table 7: Virus Detection Methods

Method	Detectable Organism
Biological Indexing	YVC, CTV, CX
PAGE	CEV, CX, RNA's
ELISA	CTV
EM	YVC,CTV,BLO,CIV

Flow Chart of Citrus Budwood Certification Program at IHS, UAF