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OCCURRENCE AND DISTRIBUTION OF VIRUS AND VIRUS-LIKE DISEASES OF CITRUS IN NORTH-WEST FRONTIER PROVINCE OF PAKISTAN

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Abstract

In order to assess the occurrence and distribution of citrus virus and virus-like diseases, extensive surveys were conducted in citrus growing area of NWFP during 2001-03. Based on characteristic symptoms expression and serological indexing, the major virus, viroid and prokaryotic diseases commonly observed in citrus orchards and nurseries were citrus tristeza, citrus variegation, citrus exocortis, citrus cachexia (-xyloprosis), citrus greening and stubborn. Average incidence of citrus tristeza closterovirus (CTV) was 27%, citrus variegation ilarvirus (CVV) 31%, citrus exocortis viroid (CEVd) 16%, citrus cachexia viroid (CCVd) 4%, citrus greening (*Liberobacter* sp) 4% and stubborn (*Spiroplasma citri*) 2%, respectively. High incidence of these devastating pathogens has caused the severe citrus decline syndrome and drastic yield and quality losses in citrus fruits in the region. The field isolates of CTV and CVV were reproduced on diagnostic hosts through graft and mechnical transmission. CTV produced vein clearing and chlorosis on young leaves *of Citrus aurantium, C. lemon* cv. Eureka, *C. sinensis.* Field isolates of CVV was readily sap transmitted on young seedlings of *C. sinensis, C. aurantium* and *C. lemon* cv. Eureka and produced severe variegation and crinkling symptoms on *C. sinensis* and leaf cupping, variegation and crinkling symptoms on *C. aurantium.* CVV also produced local chlorotic lesions on *Vigna ungiculata* and *V. sinensis* and systemic chlorosis, mottling, vein banding and distortion on *Phaseolus vulgaris* cv. Red Kidney and Bountiful. The virus produced leaf thickening, vein banding and mottling in *Nicotiana tabacum* cv. White Burley and Samson NN and *Petunia hybrida*. The status of virus and viruslike diseases have been reported in 10 major citrus growing districts of NWFP. Futhermore, recommendations have been made to manage the citrus fruits through integrated disease management approaches.

Introduction

Citrus fruits represent approximately 40% of all fruit crops growing in Pakistan and mainly concentrated in Punjab and North-west Frontier Province (NWFP). The range of cultivated citrus cultivars in Pakistan is limited. Kinnow is major citrus cultivar in Punjab, whereas sweet orange (blood-red) is mainly grown in NWFP. Many cases of citrus decline have been reported from Punjab and NWFP, Pakistan in 1970s and citrus tristeza was considered as major cause of the decline (Bove, 1995). Preliminary survey conducted by a group of Italian and Pakistani experts in 1988 with the co-operation of Ministry of Foreign Affairs and Pakistan Agricultural Research Council (PARC), citrus was reported to be infected by a number of virus and virus-like diseases in NWFP and Punjab, Pakistan (Catara *et al*., 1988) and only CTV was confirmed by enzyme-linked immuno-sorbent assay (ELISA) and electron microscopy in a few locations in the country (Catara *et al*., 1988). After a period of about 12 years, the situation has deteriorated to the extent that most of the citrus orchards are about to collapse in NWFP as well as in Punjab. Almost 100% of citrus trees in Pakistan are infected with one or more virus and

virus-like diseases that resulted in high economic losses. The major virus and virus-like diseases of citrus trees reported in Pakistan are tristeza, infectious variegation, exocortis, cachexia-xyloporosis, greening and stubborn (Catara, 1987; Catara *et al*., 1988). Most of these diseases are wide spread in new plantations due to un-certified infected bud wood as scion. Unfortunately, no deliberate attempts have been made so far to produce pathogen-free bud-wood. As a result, citrus industry that could earn foreign exchange in billion is on decline and nearly to collapse. New plantation would also be un-successful until pathogen-free bud wood is provided to the citrus growers.

Tristeza caused by citrus tristeza closterovirus (CTV) is economically destructive disease of citrus fruits. The virus is restricted to phloem tissues of the infected plants. It occurs in most citrus producing areas of the world, especially in the areas where CTV-sensitive sour orange (*Citrus aurantium* L.) is used as rootstock (Bar-Joseph *et al*., 1989; Rocha-Pena *et al*., 1995). CTV was originated in the Orient from where it was spread world wide through infected bud-wood and plants in the quest for new citrus varieties (Bar-Joseph *et al*., 1979; Roistacher *et al*., 1991; Roistacher & Moreno, 1991; Rocha-Pena *et al*., 1995). Several epidemics of CTV have been reported from Argentina and Brazil during the 1930s, when over 30 million citrus trees were killed (Costa , 1956; Bar-Joseph *et al*., 1989) and in Spain during 1960s and Venezuela during 1980s, when 10.0 and 6.6 million trees were killed, respectively (Cambra *et al*., 1988; Rocha-Pena *et al*., 1995). Less severe epidemics of the virus have also been reported from USA (Rocha-Pena *et al*., 1995) and Israel (Bar-Joseph *et al*., 1989). Many CTV strains have been reported to be associated with CTV syndrome on a variety of citrus hosts (Garnsey *et al*., 1987; 1991). Maxican lime (*Citrus aurantifolia)* is the most sensitive indicator host for CTV and sweet orange (*C. sinensis*) grafted on to sour orange seedlings serves as the indicator host for decline-inducing (DI) strains of CTV (Rocha-Pena *et al*., 1995).

CTV is transmitted by several aphid species in a semi-persistant manner (Raccah *et al*., 1989; Yokomi *et al*., 1994). However, each aphid species vary in transmission efficiency. The most efficient aphid vector is *Toxopetra citricida* (Roistacher & Bar-Joseph, 1987; Rocha-Pena *et al*., 1995). *Aphis gossypii* Glover is also an efficient vector of severe strains of CTV in most areas of the world (Roistacher & Bar-Joseph, 1987; Yokomi & Garnsey, 1987). *A. spiraecola* Patch (Yokomi & Garnsey, 1987), *Toxoptera aurantii* Boyer de Fonscobmbe (Norman & Grant, 1958), *A. craccivora* Koch and *Dactynotus jacae* (Roistacher & Bar-Joseph, 1987; Bar-Joseph & Lee, 1989) have also been reported vectors of CTV. *A. gossypii* and *A. spiraecola* are the major vectors of CTV in Pakistan (Catara, 1987). The virus is transmitted not through seed (Wallace, 1978).

CTV is a species of Closterovirus genus. Virus particles are flexuous, approximately 2000 x 11nm in size (Gonsalves *et al*., 1978; Bar-Joseph & Lee, 1989), having single stranded positive-sense RNA of about 20Kb (Gonsalves *et al*., 1978; Bar-Joseph *et al*., 1985; Bar-Joseph & Lee, 1989) and encapsidated by a coat protein (Bar-Joseph & Lee, 1989; Sekiya *et al*., 1991). The complete genome of the virus has been sequenced (Karasev *et al*., 1995) and the open reading frame (ORF) coding for the coat protein of several severe isolates have been characterized (Pappu *et al*., 1993a,b; Sekiya *et al*., 1991).

Infectious variegation is another disease caused by citrus variegation ilarvirus (CVV) and widely distributed through out the world and also widely prevalent in all citrus growng areas of Pakistan (Catara, 1987). In some citrus growing areas of NWFP, 100% CVV infection was observed in citrus where sour orange was used as root-stock (M. Arif, un-published). About 63% losses due to CVV have been reported (DesJardin & Bove,

1975). The virus is transmitted through seed (Wallace, 1978), mechanically and through grafting, but not known to be vector transmitted (DesJardan & Bove, 1975).

Exocortis is a bark-scaling and tree-stunting disease induced by citrus exocortis viroid (CEVd), widely prevalent in citrus growing areas of the world (Timmer *et al*., 2000) and also in Pakistan (Catara, 1987; Catara *et al.*, 1988). In Pakistan, symptoms of exocortis have been observed on sweet orange trees grafted on citrange or sweet lime (Catara, 1987). Polyacrylamide gel electrophoresis (PAGE) analysis confirmed the association of viroid in symptomless tree (Baksh *et al.*, 1984; Catara *et al*., 1988). It is readily graft-transmitted, and through infected bud-wood (Duran-Vila *et al*., 1988).

The cachexia-xyloprosis disease of citrus is found in most citrus growing countries of the world (Roistacher, 1983). The disease is caused by cachexia-xyloprosis viroid (CXVd), containing 300 nucleotides (Roistacher & Garnsey, 1988) and is a major pathogen of mandarin and tangerine species in Pakistan (Catara *et al.*, 1988) causing tremendous losses in yield and quality of the fruits. Symptoms observed in susceptible citrus hosts were discoloration and gum impregnation of the bark. The inner bark surface became bumpy, with numerous rounded bumps or pegs and depression in the wood. Cachexia has been associated with a viroid that contains approximately 300 nucleotides RNA

Greening is highly destructive and widely prevalent disease of citrus, previously thought to be a virus disease but now confirmed to be caused by a fastidious, phloem-limited endocellular bacterium (Garnsey, 1988). The disease is a potential threat to citrus cultivation all over the world (Garnsey, 1988) but is causing tremendous yield and quality losses in citrus fruits in Pakistan (Coehran, 1976). In Pakistan, the disease has been reported on sweet orange trees grafted on sour oranges (Catara *et al*., 1988). ELISA and electron microscopy of infected trees confirmed the identity of the disease in Pakistan (Coehran, 1976; Catara, 1987; Catara *et al*., 1988). The causal organism of the disease is transmitted by *Diaphorina citri*, a psyllid vector (Catling, 1970). *D. citri* has been reported on citrus in Pakistan and it transmits the greening organism (Catling, 1970).

Stubborn is widely prevalent and destructive disease of citrus in hot and arid citrus areas (Bove *et al*., 1988). In Pakistan, the symptoms of the disease as aborted seeds and fruit malformation in Mosambi sweet orange were reported (Catara *et al*., 1988). The disease is caused by culturable phytoplasma (*Spiroplasma citri*) (Fudl-Allah *et al*., 1972; Tully *et al.*, 1973; Garnsey & Gumpf, 1988). This present report gives an up-to-date information on the incidence and distribution of virus, viroid and prokaryotic diseases of citrus fruits in major citrus growing area of the NWFP, Pakistan.

Materials and Methods

Field surveys-assessment, sampling and indexing of mother citrus trees for virus, viroid and prokaryotic diseases: Field surveys were conducted from March to September 2001-03 in 10 major citrus growing districts (Peshawar, Nowshera, Charsadda, Mardan, Swabi, Haripur, Malakand, Swat, Dir, D. I. Khan) of the NWFP. Two citrus orchards were selected in each district. Total number of trees in each orchard were counted, assessed and trees showing symptoms of virus, viroid and prokaroyotic diseases were recorded. In some cases where total number of trees were over many hundred, then a scheme of random assessment was made. Known virus, viroid and virus-like diseases of citrus were assessed in orchards/ fields either on the basis of diagnostic

symptoms or ELISA-based surveys or both. A key for the field diagnosis of virus, viroid and virus-like diseases was developed on the basis of previous information and from elsewhere in the world and prevalence of these diseases in Pakistan (Table 1). Trees showing characteristic symptoms of virus, viroid and virus-like diseases were tagged for future reference. For serological indexing of mother trees for CTV, leaf samples were collected from infected and healthy trees from all 20 orchards in 10 districts surveyed. Samples were collected in plastic bags, kept in ice bags and transported to the laboratory for serological detection. The specimen of insects associated with citrus plantation were collected in Petri dishes containing moistened blotting paper and kept for identification.

Table 1. Key for field diagnosis of virus, viroid and prokaryotic diseases of citrus

Disease	Pathogen	Diagnostic symptoms
A. Virus diseases		
Tristeza	CTV	Phloem necrosis, innerface tiny projections (trees grafted on sour orange) vein clearing and stem pitting (sweet orange/ Maxican lime/ grapefruit)
Infectious variegation	CVV	Leaf variegation, distortion, vein clearing, rugose fruits
B. Viroid diseases		
Exocortis	CEVd	Bark cracking, exofoliation stunting, epinasty, bark scaling
Cachexia	CXVd	Guming inside the bark, wood pitting, pegs, stunting
C. Prokaryotic diseases		
Greening	FPEB[1]	Late ripening fruit, mis-shapen, small, bitter, poor in juice
Stubborn *Spiroplasma citri*		Bunchy growth, off-season flowering, fruit drop, aborted seed

[1]Fastidious, phloem-limited, endocellular bacterium (*Liberobacter* sp).

Vector population and their culture: Comprehensive surveys were conducted from March to September 2001-03 to assess the population, identity and association of insects and transmission of virus and virus-like diseases of citrus. Two to five samples of each type of insect were collected from selected trees in each location surveyed. The population of these insects was estimated. The insect specimens were taken in moistened Petri plates at each location and identified as reported by Blackman & Eastop (1984). *Aphis gossypii, Myzus persicae, Aphis fabae, Toxoptera aurantii* and *Diaphorina citri* were reared on *Brassica* species. A group of 10 aphids was tested on indicator plant species to ensure the aviruliferous nature of the insects. Before using the aphid culture in transmission experiments, pool sample of 10 aphids per lot were also tested by DAS-ELISA using antibodies of CTV to ensure that they are virus-free.

Transmission of CTV by insect vector: Young CTV-infected leaves were kept on moistened filter paper in a plastic Petri dish. Aviruliferous insects (*A. gossypii M. persicae, A. fabae, T. aurantii)* were starved in a Petri dish (10-15 insects/petri dish and replicated three times) for 24 h. Insects were transferred with fine camel hair brush to

Petri dish containing source and caged for about 24-48 h. After required acquisition period, the insects were transferred to three test plants (5-10 aphids/ plant) while in case of citrus psylla 10–15 psylla / plant with clean camel hairbrush. Insects were caged on test plants by inverting six inches high plant tumblers with base removed and replaced with muslin cloth for ventilation over them. Insects were given an inoculation feeding period of maximum 24 h each for CTV and greening. At the end of inoculation period, insects were killed with Tamaran EC600 sprays. The plants were kept in insect-free screen house for 30-60 days for symptom development.

Plant culture and growth conditions: Young plants of sweet orange grafted on sour orange root-tock were transplanted in 36 cm diameter clay pots using sterile potting mixture which consisted of field soil, peat, sand and farm yard manure (1:1:1:1 v/v). Sour orange germinated in big tray containing above potting mixture and 1-2 month seedlings were transplanted in 26 cm diameter clay pots. Seeds of indicator plants were germinated in soil mixture as mentioned above as and when needed. Plants were kept in insect-free screen house. Plant-lets of Eureka lemon were generated from cutting in above soil mixture.

CTV and CVV isolates: collection, preservation and inoculation: Leaf samples from CTV and CVV infected citrus trees from different locations in the NWFP were collected in polythene bags. For CTV ELISA-positive, selected samples and severely symptomatic in case of CVV were homogenised in pestle and mortar with 1:5 ratio of 0.1 M Potassium Phosphate buffer, pH 7.0 separately. Sufficient amount of carborundum powder was added during crushing to make a fine homogenate, passed through double layer of muslin cloth and immediately used for mechanical inoculation as reported by Garnsey *et al*. (1977). Infected sap was applied on carborundum (600 mesh) dusted young leaves of diagnostic hosts (Table 4). The plants were marked with date of inoculation and source, etc. Inoculated plants were kept in insect-free screen house for further studies. Characterization of the CTV isolates was not made at this stage but continued in second phase of the study. Control plants were inoculated with sap from healthy plant or even with buffer. The inoculated plants were kept in insect free screen house for symptom development.

Detection of CTV through DAS-ELISA: DAS-ELISA was used for the detection of CTV. The tests were performed in polystyrene micro-plates containing 96 wells (NUNC). ELISA-plates were coated with 100 µl aliquots of CTV-specific antibody (Agdia) with coating buffer, pH 9.6. Plates were kept at RT in a humid box for 3-4 h. Leaf samples were extracted by crushing through pestle and mortar in extraction buffer, pH 7.4. Leaf tissues were extracted in extraction buffer at 1:10 ratio (w/v), 100µl of prepared sample was dispensed in each well after washing. After first incubation was completed the plates were washed with 1 X PBST buffer pH 7.4. Control wells were filled using that same amount of sap from known CTV infected and known healthy citrus plants as positive and negative controls. ELISA-plates were incubated inside a humid box for 2 h at RT or overnight in refrigerator (4°C). After washing, 100 µl of enzyme conjugate (Agdia) was dispensed in each well of the plate and incubated in a humid box for 2 h at RT. The plates were washed 4 times with 1 X PBST. 100 µl of OPD solution (100 ml of OPD solution, pH 5.0 was prepared by dissolving hydrogen peroxide (30%) 0.4 ml, citric acid

(anhydrous) 5.1 g, sodium phosphate, dibasic (anhydrous) 7.33g in 900 ml of distilled water and volume was adjusted to 1000 ml by adding more distilled water) was added per well and the plates were incubated 15-30 min in humid box at RT or overnight (i.e. 16 h) at 4°C. The reaction was stopped by adding 50µl 3M sulphuric acid to each well. The reaction was assessed visually or measured at A_{405} nm times at with Titertek Multiskan, Model MC (Flow Laboratories Inc.) The samples were considered to be positive when the A_{405} nm values exceeded the mean of the virus-free samples by at least a factor of three.

Results

Disease incidence

Citrus Tristeza Closterovirus: CTV was present in all citrus growing areas of the NWFP surveyed (Table 2). The characteristic symptoms of CTV were commonly observed on sweet oranges grafted on sour orange. The infected trees developed phloem necrosis and inner face tiny projections. The affected wood produced tiny projections going in to small holes in the inner face of the bark. Vein clearing and stem pitting were also observed on Maxican lime and sour orange trees. Incidence of CTV ranged from 18 to 40%, with an average incidence of 27%. Lowest incidence of 18% was recorded in district Haripur, where rough lemon was used as rootstock, whereas highest incidence (40%) was recorded in Swabi district where sour orange was used as rootstock (Table 2).

Citrus Variegation Ilarvirus (CVV): Symptoms of CVV were observed in almost all ten districts surveyed (Table 2). Mild to severe leaf variegation, distortion, vein clearing was generally observed in sour oranges and sweet orange grafted on sour orange root stock. At NWFP Agricultural University Campus and ARI, Tarnab, where sour orange is being used for ornamental purposes, (hedges, etc.) 100% trees were found infected with CVV. Infected trees produced small, rugosed and mis-shapened fruits. Incidence of CVV ranged as mild to severe infection between 15 to 65 % with an average of 31% (Table 2). The identity of the virus was confirmed through biological tests. The virus was transferred on cowpea through mechanical inoculation of infected sap from citrus species and to young sour orange plants by grafting. CVV produced local chlorotic and necrotic lesions on cowpea while systemic chlorotic mottling and vein banding in bean cultivars, Red Kidney and Bountiful. *Nicotiana tabacum* cv. White Burley and Samson NN and *Petunia hybrida* were also systemically infected (Table 4).

Citrus Exocortis Viroid (CEVd): The characteristic symptoms of CEVd such as bark scaling, exfoliation, stunting and epinasty in sweet orange trees was commonly observed in the NWFP. Symptoms were observed mostly in older trees of sweet orange and not on young plants. Mild to severe bark scaling, cracking and epinasty symptoms were observed in all sweet orange orchards (Table 2). In older citrus orchards, the incidence was higher as compared to new plantations. The incidence of CEVd ranged from 5 to 42% with an average incidence of 16% (Table 2). Lowest incidence of CEVd was found in newly planted citrus orchard in district Haripur whereas high incidence was reported (42%) in orchard at Timergara, district Dir.

Cachexia-xyloprosis: Older sweet orange trees developed mild to severe field symptoms of CXVd in all districts surveyed (Table 2). The symptoms were gumming on the inside of the bark, wood pitting, pegs of the inner face of the bark (Table 1), die-back, decline and stunting of trees. Like CEVd, C-XVd was predominant on older trees than in young citrus plantation. The incidence of CXVd was recorded as lowest (0-1%) of the viruses and viroid diseases recorded in citrus in the area surveyed. Haripur was found to be CXVd-free. In other locations, the incidence was variable. A maximum incidence up to 11 % was recorded in district Malakand (Table 2).

Prokaryotic Diseases

Citrus greening: Symptoms of greening such as late and un-even ripening of citrus fruits (sweet orange), mis-shapened, small sized, bitter in taste, poor in juice, have been recorded in citrus orchards in Peshawar, Nowshera, Swabi, Swat and Dir districts of the NWFP (Table 2). Maximum incidence up to 30% was recorded in Timergara. The incidence of mollicutes could be many fold higher than recorded in this paper if serological and molecular identification of the pathogen could be made. Discoloration of leaves beginning from mid rib and expanding towards internal areas were commonly observed in infected sweet orange trees in areas surveyed. In some instances symptoms of greening similar to micro-nutrients deficiency were observed and it was difficult to differentiate the greening symptoms from those of micro-nutrient deficiency.

Stubborn: The characteristic symptoms were only observed in a few sweet orange in Peshawar, Nowshera and Swabi districts. Bunchy growth, off-season flowering, excessive leaf drops, fruit of several sizes and seed abortion in seeds were the common symptoms observed and recorded. Nine location surveyed out of total 20 were found free from stubborn and 1-13% incidence was recorded in other 11 location surveyed in NWFP (Table 2).

ELISA-based indexing against CTV: ELISA based indexing of citrus trees revealed that the incidence of CTV is increasing with distribution of an infected rootstocks. The incidence ranged from 15 to 34% in NWFP. The highest incidence of CTV (34%) was recorded at Marghuz in Sawbi district, whereas lowest (15%) incidence was recorded in an orchard at district Haripur (Table 3). An average percent incidence of CTV detected in 10 major citrus growing districts of the NWFP was 24% (Table 3). The division-wise indexing of CTV also indicated that the average incidence of CTV was highest (29%) in Mardan division, whereas lowest (16%) in Hazara division (Table 3). Sweet orange is the major commercially grown citrus species in the province, so ELISA based indexing was done only in sweet orange orchards. In other citrus species such as sweet lime, rough lemon, sour orange, incidence of CTV was determined only on the basis of characteristic symptoms expression. The incidence of CTV was wide spread in sweet orange grafted on sour orange root stock, whereas low when rough lemon was used as root stock.

Transmission of CTV and CVV and symptomatology: CTV and CVV were transmitted through infected sap and graft inoculation on diagnostic hosts. CTV produced vein clearing and chlorosis on young leaves of *Citrus aurantium*, *C. lemon*, *C.*

sinensis three weeks after sap inoculation. The virus was transmitted through graft union of infected scion on healthy stock that produced severe vein clearing and chlorosis in *C. lemon* cv. Eureka and *C. sinensis* in four months (Table 4). CVV was readily sap transmitted on young seedlings of *C. aurantium* and *C. lemon* cv. Eureka. On *C. sinensis* severe variegation and crinkling symptoms were developed eight weeks after graft inoculation. *C. aurantium* produced leaf cupping, variegation, crinkling symptoms three to four weeks after mechanical inoculation. CVV produced local chlorotic lesions on cowpea, *Vigna ungiculata* and *V. sinensis*. The virus produced systemic chlorosis, mottling, vein banding and distortion on *Phaseolus vulgaris* cv. Red Kidney and Bountiful 2-3 weeks after inoculation. CVV produced thickening, vein banding and mottling in *Nicotiana tabacum* cv. White Burley and Samson NN and *Petunia hybrida* after two to three weeks of sap inoculation (Table 4).

Table 3 Percent incidence and distribution of citrus tristeza closterovirus in major citrus growing areas of NWFP.

Division	District	Location	Plant tested	Plant Infected	% incidence
Peshawar	Peshawar	Malakandher	45	10	22
		ARI, Tarnab	55	14	25
	Charsadda	Tangi, Abazo	80	20	25
		Charsadda	70	16	23
	Nowshera	Manki Sharif	110	27	25
		Akora Khattak	90	24	27
Mardan	Mardan	Bakshali	194	60	31
		Palo Deri	190	56	29
	Swabi	Marghuz	180	61	34
		Dook	160	33	21
Hazara	Haripur	Haripur-1	162	27	17
		Haripur-2	154	23	15
Malakand	Malakand	Dargai, Dobandai	80	25	31
		Dargai, Kal Dera	80	17	21
	Swat	Barikot	80	16	20
		Fazalabad	100	20	20
	Dir	Timergara	62	14	23
		Khungi Payan	58	12	21
D. I. Khan	D. I. Khan	D. I. Khan-1	36	11	30
		D. I. Khan-2	44	9	20
Total			2030	495	Mean=24

(Based on DAS-ELISA detection of citrus tristeza closterovirus from leaf samples colected from fields).

Insect vector and virus transmission: *Myzus persicae*, *Aphis gossypii*, *A. nerii*, *A. fabae*, *Toxoptra auranti*, *Brachycaudus helichrysi* and *Diaphorina citri* were identified as insects associated with citrus plantation in the NWFP. The population density of *Myzus persicae*, *Aphis gossypii*, *A. fabae* and *Diaphorina citri* was found significantly high in some areas of Mardan, Charsadda and Peshawar districts during middle of March to April but generally low during May to October 2001-03.

Table 4 Bioassay of citrus tristeza and infectious variegation viruses.

Host species	Host reaction[1] CTV	CVV
1. Citrus species		
Citrus aurantium (L.)	VC, Chl	LF, Vg, Crk
C. limon (L.) Barm. cv. Eureka	VC, Chl	Vg, Crk
C. sinensis (L.) Osbeck	VC	Vg, Crk
2. Cowpea		
Vigna unguiculata Syn.	–	CL(L), NL(L)
V. sinensis (Tor.) Savi	–	CL(L),
3. Bean		
Phaseolus vulgaris		
cv. Red Kidney	–	CL(S), Mt, Vb
cv. Bountiful	–	CL(S), Mt, Vb
4. *Nicotiana* species		
Nicotiana tabacum L.		
cv. White Burley	–	Vb, Mt, Tk
cv. Samsun	–	Vb, Mt, Tk
5. *Petunia* species		
Petunia hybrida L.	–	CL(S), (Pk)

[1]VC: vein clearing; Chl: chlorosis; LC leaf cupping; LF: leaf flecking; Vg: variegation; Crk: crinkling (distortion); CL(L): chlorotic lesion (local); NL(L): necrotic lesion (local); chlorotic lesion (systemic); CL:(S); Mt: mottling; Vb:vein banding; Tk: Thickening; (Pk: puckering)

The transmission experimented conducted under controled conditions revealved that CTV successfully acquired and then transmitted to young *C. aurantium* plants by *Myzus persicae*, *Aphis gossypii*, *A. nerii*, *A. fabae*, *Toxoptra auranti*. Inoculated plants exhibited vein clearing and chlorosis after 4-6 weeks. The efficacy of different insects in transmission of CTV is given in Table 5. The results indicated that *A. gossypii* (66.6%) was the most efficient in transmission of CTV followed by *M. persicae* (50.0%). However, *T. aurantii* and *A. fabae* were also able to transmit CTV to indicator plants. The vector nature of the *A. nerii* and *B. helichurysi* could not be confirmed. *D. citri* was unable to transmit CTV in repeated experiments.

Discussion

The results obtained during the two years research confirmed the wide spread infection of virus, viroid and prokaryotic pathogens in all citrus growing areas of the NWFP surveyed. Citrus tristeza virus, exocortis viroid and greening posed a real threat to citrus cultivation, not only in Pakistan but also in most citrus producing areas of the world (Catara *et al*., 1988; Bar-Joseph *et al*., 1989; Lee *et al.,* 1994; Rocha-Pena *et al*., 1995). In NWFP, CTV was reported in a few locations with low intensity during 1987-88 (Catara, 1987; Catara *et al*., 1988), however, greening was reported to be potential threat to citrus in this area (Catara *et al*., 1988). During the course of these investigations, comprehensive and systematic surveys and indexing of mother plants revealed that the average incidence of CTV has gone up to 27% and is almost prevalent in all areas surveyed. The common practice of grafting of sweet orange on sour orange could be one

of the reasons for its wide spread occurrence in the NWFP. Sour orange is susceptible to viruses, viroids, and prokaryotic pathogens. The unjudicious use of infected bud scion could also be the cause of its rapid spread in the target areas. This practice provides both virus and host to grow and result in citrus decline in almost 8-10 years depending on host genotype, virus strains and suitable environmental conditions. These infected trees also served as reservoir hosts for viruses and its vectors. Thermotherapy based technique has recently been developed for the elimination of CTV from infected bud-wood (Arif *et al*., 2005). This technique can be used for cleaning desirable citrus germplasm. A third reason for its wide spread occurrence is the abundance of aphid vectors. CTV is transmitted by many aphid species. The most important aphid vector, *Toxoptera citricida* (Kirdaldy) of CTV elsewhere in the world, however, has not been reported in Pakistan (Catara, 1987). However, the virus is also efficiently transmitted by *Aphis gossypii* and *A. citricola* in NWFP, Pakistan (Catara, 1987). Small scale transmission studies indicated that only single *A. gossypii* can efficiently transmit CTV from sour orange to sweet orange and back from sweet orange to sour orange. *A. gossypii* was found in abundance during March and April on succulent citrus shoots. The efficiency of *A. gossypii* as a vector of CTV has been well documented (Roistacher, 1984; Roistacher & Bar-Joseph, 1987). A number of other insect species were also found associated with citrus plantation in the areas surveyed but their role in transmission of virus and virus-like pathogens has not been determined and exploitation of research in this area, is needed.

The incidence of viroids (CEVd, CXVd) infecting citrus has also been rising especially in older trees. The possible reason for viroid spread in field is unhygienic cultural practices. A common practice in the orchards has been observed that a pruning specialist is being hired from Agriculture Extension Department, who pruned hundreds of trees in different orchards at different locations with a single knife without sterilizing it and same is being practiced in budding. So viroids are disseminated through the contaminated pruning and budding tools. Similar reasons have also been reported in several other countries for the dissemination of viroids in citrus (Roistacher *et al.*, 1969). The transmission of CEVd and CXVd can be prevented by dipping the tools in bleach prepared by diluting 1½ points of bleach (containing 5.25 percent sodium hypochlorite) in one gallon of water. The symptoms of viroid diseases were observed generally in older trees that suggest that transmission occurred through pruning practices rather than budding. The spread of viroids in citrus orchards has also been reported during pruning particularly in wet climate (Garnsey & Jone, 1967; Garnsey, 1968; Roistacher *et al*., 1969; Timmer *et al*., 2000).

Based on the wide spread and quick decline syndrome, citrus greening was previously reported as potential threat (Catara *et al*., 1988). No doubt, greening is playing pivotal role in the deterioration of citrus industry in Pakistan and also elsewhere in the world, but citrus decline seems to be a complicated disease syndrome and involvement of CTV, CEVd and *Spiroplasma citri* or other pathogens could also be possible. Detailed studies are required for isolation and characterization of the pathogens associated with the quick decline syndrome of citrus.

Based on these preliminary investigations, the following recommendations are made for successful production of citrus in this country:

1. Prevention of introduction of virus, viroid and prokaryotic organisms through exchange of and movement of infected nursery plants/ bud-wood from out side into the NWFP and Pakistan. This could be accomplished by strict quarantine regulations.

2. Continued education and awareness about the problem and its prevention of the farming community, researchers, extenionists, and quarantine officials etc., are suggested. Eradication and immediate disposal of source of inoculum where the number of infected trees are a few and are restricted to a well-defined location.
3. Local and long distance movement of infected citrus germplasm/ propagative materials should carefully be handled and if possible be avoided.
4. Use of sour orange, *C. aurantium* for ornamental purposes should be discouraged.
5. Rootstocks other than sour orange should be searched and tested for future plantation.
6. Transmission and dissemination of viroids such as CEVd and CXVd could be prevented by treating budding knives, pruners, clippers in sodium hypochlorite solution.
7. Insect vectors viz., *Aphis gossypii, A. citricolla, Ciculifer tenellus, Scaphytopius nitridus, S. acutus* should carefully be monitored and controlled through insecticidal sprays.
8. An integrated approach could be adopted by using pathogen-free bud-wood, avoidance sources of infection, proper and timely use of insecticides, implementation of quarantine regulations and the use of resistant/ tolerant root stocks.

This preliminary information could be beneficial and of great interest for the researchers and growers of the citrus fruits and this work will certainly serve as a base line to exploit research on citrus pathology in Pakistan and elsewhere in the world.

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