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Biological and serological techniques for detection of *Citrus tristeza* virus affecting *Citrus* species of Assam, India

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Infection of Citrus tristeza virus (CTV) in Assam was detected by double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS-ELISA). Field surveys were carried out in 8 citrus growing districts of Assam (Tinsukia, Lakhimpur, Jorhat, Kokrajhar, North Cachar Hills, Karbi Anglong, Golaghat and Kamrup). Altogether, 411 samples were collected from three different citrus species, viz., Assam lemon (*Citrus limon*), Gul nemu (*Citrus jambhiri*) and Khasi mandarin (*Citrus reticulata*) to test against CTV. Results of biological indexing showed Kagzi lime (*Citrus aurantifolia*) to be the better indicator plant (symptom expression 60%) than Assam lemon (symptom expression 30%). The symptom expression on the indicator host was observed from 5th to 9th week after inoculation. The inoculated plants again tested after 9th week using DAS-ELISA assay. The results revealed that percentage of positive plants were 90% in Kagzi lime followed by Assam lemon (80%). This indicated that even the symptomless plants after inoculation give the positive reactions in DAS- ELISA assay. 261 samples were found to be infected using ELISA with polyclonal antisera to CTV. The species Assam lemon (*C. limon*) were found to be susceptible to CTV with estimated disease incidence up to 76.47% followed by Khasi mandarin (*C. reticulata*; 61.18%) and Gul nemu (*C. jambhiri*; 52.03%). Different age groups (<10, 10 to 15 and >15 years) of the citrus trees indicated that prevalence of CTV was more in older plants.

Key words: ELISA, Citrus tristeza virus (CTV), Assam lemon, Khasi mandarin, Gul nemu.

INTRODUCTION

Citrus is considered to be one of the most remunerative fruit crops of India, having a lasting niche in the international trade and world finance. Most of the *Citrus* species are believed to be native to tropical and subtropical regions of Southeast Asia, particularly Northeastern India and the region between China and India (Ghosh, 2007). The Northeastern region of India is considered as the home of mandarin orange and many citrus fruits (Ghosh, 2007). Sub mountain and hilly tracts of states of Northeast, Assam, Meghalaya, Manipur, Arunachal Pradesh, Mizoram, Nagaland, Tripura, Sikkim and the Darjeeling hills of West Bengal grows excellent quality citrus fruits like, mandarin (Darjeeling, Khasi and Sikkim mandarin), sweet orange, lemons and limes. Khasi mandarin is the most important commercial cultivar of Northeast India, followed by Assam lemon, which is very popular in homestead gardens. Apart from these two cultivars, rough lemon (*Citrus jambhiri* Lush.), citron

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(*Citrus medica* L.), and sweet orange (*Citrus sinensis* Osbeck) are also grown in this region (Verma and Ghosh, 1979).

The global problem of citrus decline warranted special attention of agricultural scientists during the past few decades. The tristeza virus and viroid exocortis greening is caused by a fastidious bacterium in association with some fungi, nematodes and bacteria have been found to be involved in the citrus die back complex in India. Among the seven aphid species reported as vectors of *Citrus tristeza* virus (CTV), *Toxoptera citricida* has been found to be most efficient vector (Capoor and Rao, 1967). CTV is a phloem limited 2000 nm long filamentous virus having single stranded RNA genome (Karasev et al., 1995). The virus is genetically and biologically diverse and can cause field symptoms ranging from vein clearing, stem pitting, yellowing, slow decline and quick decline, or no symptoms depending on virus isolate, time of infection, root stocks, citrus cultivars and environmental conditions (Bar-Joseph et al., 1989).

Extremely limited information and experimental data are available on the occurrence and spread of CTV in the Northeastern region of India. However, a survey and indexing results recorded presence of tristeza in Khasi orange, Kagzi lime and Assam lemon in the Northeastern region in India (Bhagabati et al., 1989). The biological-indexing is relatively less reliable and time consuming, but double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS-ELISA) has revolutionized the detection test, making it feasible to test large number of samples (Bar-Joseph et al., 1979; Cambra et al., 1979; Chakraborty and Ahlawat, 2001). In recent years, PCR based diagnosis has been used for the rapid diagnosis of citrus virus (Biswas, 2010; Edson et al., 2008). ELISA test can provide rapid, sensitive and economical detection of CTV in crude extracts from citrus trees.

The viral infection in plants alters the metabolism and physiology of the plant; hence, it is necessary to conduct an indexing of the viral disease to analyze the percentage of infection to control the disease in future. Diagnosis based on visible symptoms is generally unreliable and thus advanced diagnostic tools like ELISA and RT-PCR that has been used earlier in India (Chakraborty et al., 1992; Biswas, 2008), are very much essential for detection of CTV accurately. In the present study, advanced diagnostic tool that is, ELISA have been employed for detection of CTV. A comparative study on traditional method of diagnosis (that is, biological indexing) and antibody based detection technique (that is, DAS-ELISA) have been conducted and evaluated their efficiency.

MATERIALS AND METHODS

Survey of *Citrus tristeza* virus (CTV) infection and aphid population

Survey and investigation was conducted in four agroclimatic zones viz., Upper Brahmaputra Valley zone, Lower Brahmaputra Valley zone, North Bank Plain zone and Hills zone of Assam covering eight districts (Jorhat, Tinsukia, Golaghat, Lakhimpur, Karbi Anglong, North Cachar Hills, Kamrup and Kokrajhar) during 2009-2010. Three to five locations from each district were surveyed for CTV infected plants. Suspected CTV infected samples from Khasi mandarin (*Citrus reticulata*), Gul nemu (*C. jambhiri*) and Assam lemon (*Citrus limon*) were collected from both commercial and home stead gardens of citrus trees from the age group of <10 years, 10 to 15 years and above 15 years trees. Altogether, 411 leaf tissue samples were collected from three different citrus species (Table 1).

Although symptoms of greening were also observed in all the surveyed locations on the investigation primarily concentrated on the CTV infection. There may be a mixed infection of greening and tristeza because symptoms of both the diseases were prominent in almost all the locations. But for further confirmation of greening, disease molecular assay has to be performed (Figures 1 and 2).

Biological indexing

Bud wood from 30 trees, chosen at random and not necessarily showing symptoms, was used for indexing by the method of Roistacher (1991). Two indicator seedlings (*Citrus aurantifolia* that is, Maxican lime or Kagzi lime and *C. limon* to test as indicator hosts against CTV) were inoculated with buds obtained from CTV infected plants which were already tested as CTV positive using ELISA following the technique used by Wisler et al. (1996). Plants were grown in a greenhouse at about 27°C and inspected at least once each week for symptoms, beginning 15 days after inoculation. One bud was grafted onto each of the indicator plants. The plants were monitored for the symptom expression such as leaf yellowing and vein clearing. Fifteen (15) numbers of 1 year old seedlings of Assam lemon (*C. limon*) and 15 numbers of Kagzi lime or Maxican lime (*C. aurantifolia*) seedlings were grown inside the green house. Seedlings were tested by DAS-ELISA to test CTV negativity and were maintained in the greenhouse. The bud wood of 3 to 4 mm in diameter was budded at 20 cm height to each of the indicator seedlings. CTV infected scion of pencil thickness were collected from different fields nearby Jorhat, Assam. Trees from which bud sticks were taken were pre-tagged with CTV positive through DAS-ELISA. The samples were collected in the polypropylene bags. The scions were selected in such a way that every scion consisted of at least 2 to 3 swollen buds. Before putting the scion samples in polypropylene bags, these were wrapped in water soaked cotton so that they remain fresh during transportation. Then they were kept in refrigerator at 4°C until budding was performed and the process was completed within 48 h of their collection (Figure 3).

Double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS-ELISA)

The leaf midrib tissue samples from different citrus growing areas were tested using DAS-ELISA as described by Clark and Adams (1977). The antibodies were obtained from Bioreba AG, CH4153, Reinach BL1, Switzerland. CTV IgG (20 µl) were diluted in 20 ml of coating buffer to coat each 96 well ELISA plate at the rate of 200 µl per well. The plate was incubated for 4 h at 30°C followed by three times washing with phosphate buffered saline Tween 20 (PBS-T). One gram (1 g) of leaf vein tissue from each sample was ground using mortar and pestle in 1 ml PBS-T. A 200 µl of the sample were added to each well. The plates were incubated over night at 4°C. ELISA plate was then washed three times with PBS-T. Following this, the enzyme conjugate was added to each well and the plate was then incubated for 4 h at 30°C. The plate was then washed three times with PBS-T. After washing, 5 mg p-nitrophenol

Table 1. Number of suspected CTV infected citrus leaf samples collected from different districts during 2009-2010.

S/N	Districts	Month of Survey (2009 - 2011)	Number of samples									Total
			Assam lemon (years)			Gul nemu (years)			Khasi mandarin (years)			
			<10	10 - 15	>15	<10	10 - 15	>15	<10	10 - 15	>15	
1	Golaghat	April, 2010 and September, 2010	13	10	2	9	1	11	8	4	20	78
2	Jorhat	December, 2009	8	10	-	3	8	1	5	3	8	46
3	Tinsukia	September, 2009	12	8	-	4	8	2	14	4	10	62
4	Lakhimpur	April, 2010	12	3	3	3	13	2	2	5	8	51
5	Karbi Anglong	April, 2010	7	2	2	3	5	1	3	2	16	41
6	N.C. Hills	April, 2010 and August, 2010	6	5	4	6	6	4	-	4	6	41
7	Kamrup	May, 2010	2	2	10	1	8	6	5	5	5	44
8	Kokrajhar	December, 2009 and July, 2010	2	3	10	5	5	8	4	4	7	48
Total			62	43	32	34	54	35	41	31	80	411

(DAS-ELISA).

Table 2. Symptoms expression in biological - indexing of *Citrus tristeza* virus (CTV) in indicator hosts.

Indicator host	No of symptomatic plant/no. of plant inoculated	No of asymptomatic plant/no. of plant inoculated	%Symptom expression	Symptoms produced (number of plants)		Symptoms appeared after weeks of inoculation
				VC	LY	
KL	6/10	4/10	60	4	4	5 - 9 week
AL	3/10	7/10	30	2	3	7 - 9 week

LY, Leaf yellowing; VC, vein clearing; KL, Kagzi lime; AL, Assam lemon.

phosphate substrate tablets were dissolved 20 ml of diethanolamine buffer and 200 µl of substrate was added in each well and observed for change of colour in the wells. CTV IgG, enzyme conjugate and substrates were purchased from Bioreba (USA). ELISA plate was then read by the ELISA plate reader (Bio Rad) using 405 nm wavelength after 1 h of addition of substrate. Plants were considered infected with CTV if the ELISA reading was four times higher the average reading of the healthy samples (usually ≥0.1) (Azzam et al., 2001). Data were analyzed and disease incidence was recorded.

RESULTS AND DISCUSSION

Biological indexing

The results of biological indexing assay on two indicator hosts viz., Kagzi lime or Maxican lime *(C. aurantifolia*) and Assam lemon (*C. limon*) are shown on Tables 2 and 3. Both leaf yellowing and vein clearing symptoms were observed on both the hosts from 5th to 9th weeks after inoculation (Table 2). The results revealed that among the two species of citrus used as indicator hosts, Kagzi lime (*C. aurantifolia*) showed better expression of symptoms (60%) compared to Assam lemon (30%). The inoculated plants after 9th week (63 days) again tested using DAS-ELISA assay. The results revealed that percentages of positive plants were 90% in Kagzi lime followed by Assam lemon (80%). The comparison analysis between biological indexing and immunological indexing further indicated that although biological indexing was accepted as one of the reliable method of virus identification, the DAS-ELISA assay had been found more accurate and quick in confirming virus incidence (Table 3).

Identification of plant tissues as source of virus

DAS-ELISA of leaf samples from eight districts of Assam on three different *Citrus* species was performed successfully and ELISA value (Virus titre values) ranges were recorded (Table 4). The results of DAS-ELISA showed 261 trees, out of 411 tested, infected with CTV indicating 63.50% CTV disease incidence in Assam. Results revealed presence of CTV in all the surveyed districts of Assam showing a high incidence of 78.04% CTV in Karbi Anglong district followed by 76.92% in Golaghat district (Table 5). Among the three different *Citrus* species, Assam lemon showed maximum incidence of CTV with 76.47% followed by Khasi mandarin (61.18%) and Gul nemu (52.03%). The results also revealed that in all the three different *Citrus* species Assam lemon, Gul nemu and Khasi mandarin the percentage of CTV infection was more in the higher age

Table 3. Comparison of biological - indexing and double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS-ELISA) against CTV on indicator hosts.

Citrus species	Percentage of infection after 9 weeks (63 days)	
	Biological - indexing	DAS-ELISA
Assam lemon (AL)	30	80
Kagzi lime (KL)	60	90

Table 4. Accumulation of *Citrus tristeza* virus (CTV) titer in CTV infected *Citrus* leaf samples in different citrus growing districts of Assam.

District	Range of OD_{405} values	OD_{405} values in healthy control
Jorhat	0.66 - 1.16 (3 - 5)	0.24
Tinsukia	1.12 - 2.59 (3 - 7)	0.38
Golaghat	1.07 - 1.46 (2)	0.63
Lakhimpur	0.84 - 2.39 (3 - 9)	0.28
Karbi Anglong	0.45 - 0.86 (2 - 4)	0.24
N.C. Hills	0.59 - 1.88 (2 - 8)	0.25
Kamrup	0.28 - 1.14 (1 - 5)	0.25
Kokrajhar	0.55 - 1.29 (3 - 6)	0.21

OD value was measured at 405 nm. Data in parentheses represent CTV titer in folds compared to healthy control.

Table 5. Over all *Citrus tristeza* virus (CTV) infection in leaf midrib tissues of different citrus species in Assam using DAS-ELISA.

S/N	Districts	Locations	% Infection			
			Assam lemon	Khasi mandarin	Gul nemu	Average
1	Golaghat	Bokeal Buragohain Khat, No. 2 Chatiana (Bogijan), Naojan, Bhilgaon, Namlagua gaon, Missamora	23/25 (92.0)	24/32 (75)	13/21 (61.90)	60/78 (76.92)
2	Jorhat	Nagajanka, AAU Orchard, Choladhara	12/18 (66.6)	9/16 (56.26)	6/12 (50.00)	27/46 (58.69)
3	Tinsukia	Kachijan, Borgaon, Kherjan, Hatigarh, Kakapathar	12/20 (60.0)	13/28 (46.42)	8/14 (57.14)	33/62 (53.22)
4	Lakhimpur	Hatilong, Town Bantow, Nowboisha, Khelmati, Chutiakari, Johing	16/18 (88.8)	12/15 (80)	11/18 (61.11)	39/51 (76.47)
5	Karbi Anglong	Singnot Engti Gaon, Merabheti Kaliani, Thengal Gaon (Nilip Block), Diphu	6/11 (54.54)	20/21 (95.23)	6/9 (66.66)	32/41 (78.04)
6	N.C. Hills	Haflong, Sub centre Area, Guruling area	10/15 (60.0)	5/10 (50)	7/16 (43.76)	22-41 (53.65)
7	Kamrup	HRS (Kahikuchi), Mirza, Sonapur	13/14 (92.8)	0/15 (0.00)	4/15 (26.66)	17/44 (38.64)
8	Kokrajhar	Tilapara, Debargaon, Gossaigaon, Kachikotha Hainary, Chirang	12/15 (80.0)	10/15 (66.66)	9/18 (50.00)	31/38 (64.58)
Total Infection (%)			104/136 (76.47)	93/152 (61.1)	64/123 (52.03)	261/411 (63.5)

groups viz., > 15 years and 10 to 15 years followed by lower age group (<10 years old plants) (Table 6).

CTV infection was detected in all the surveyed locations. This is an indication that CTV is prevalent in this region. Typical symptoms of CTV infection was observed in most of the orchards and gardens. Prevalence of the vector *Toxoptera* spp. was noticed in all the surveyed locations. The results of DAS-ELISA

Table 6. Status of infection of *Citrus tristeza* virus (CTV) in different citrus hosts with age of the plant.

Citrus species	Age group (years)	Percent CTV infection	Average percent infection
Assam lemon	Up to 10	41/61 (67.2)	104/136 (76.47)
	10 to15	35/41 (85.3)	
	Above 15	28/32 (87.5)	
Gul nemu	Up to 10	10/31 (32.2)	64/123 (52.03)
	10 to 15	30/51 (58.8)	
	Above 15	24/32 (75.0)	
Khasi mandarin	Up to 10	11/38 (28.9)	93/152 (61.18)
	10 to 15	18/29 (62.0)	
	Above 15	64/80 (80.0)	

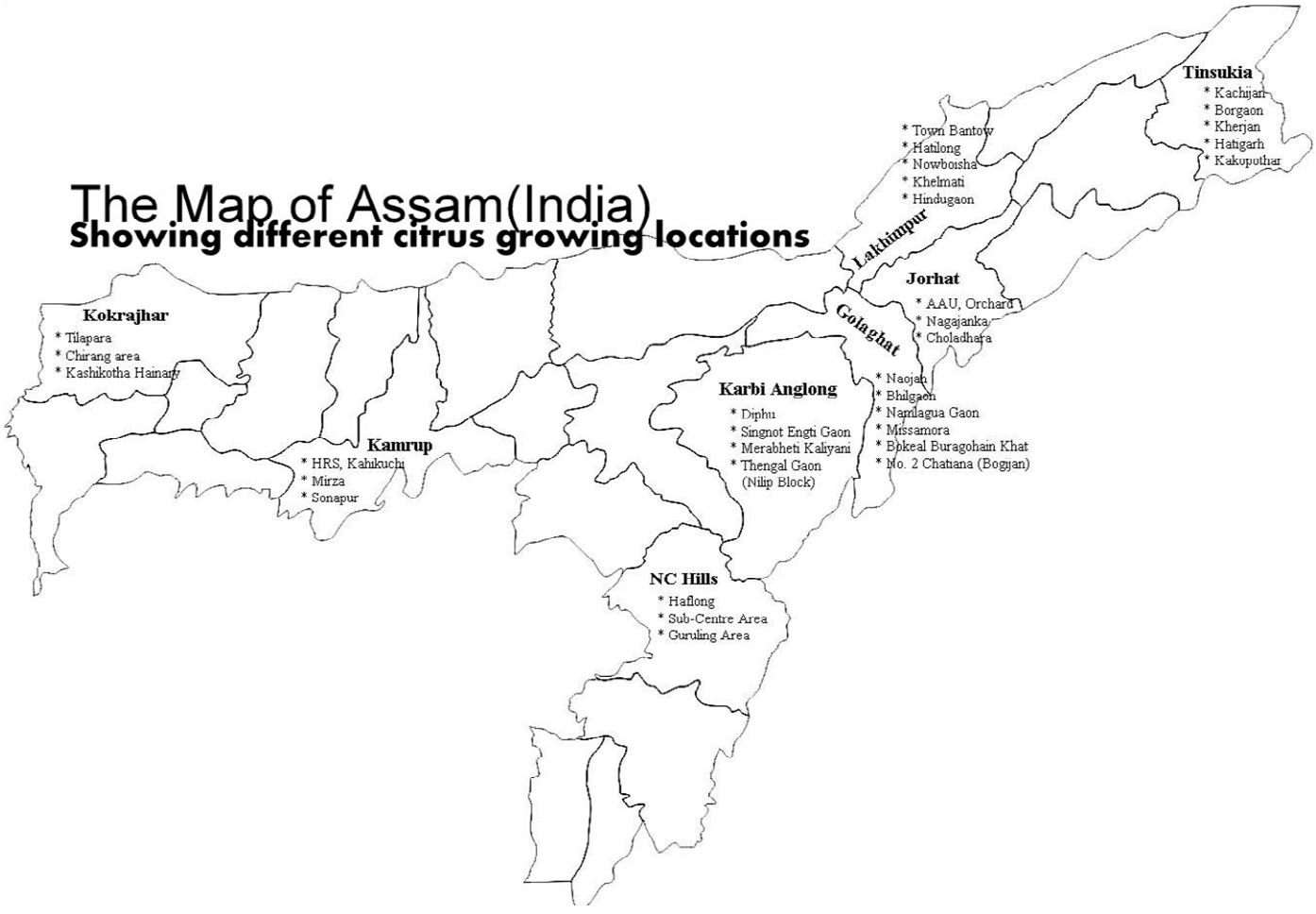

Figure 1. Map of Assam (India) showing different citrus growing locations.

showed 63.50% CTV disease incidence in Assam at different level of incidence in different survey sites. DAS-ELISA results also revealed that in all the three different *Citrus* species Assam lemon, Gul nemu and Khasi mandarin, the percentage of positivity is more in the higher age group. This indicates that incidence of CTV is more in matured and older plants. The results further confirm the earlier report of incidence of CTV in the citrus

Figure 2. Symptoms of CTV in Khasi mandarin: **a,** >10 years old plant; b, <10 years old plant; c, insect vector *Toxoptera citricida* in the field.

Figure 3. Process of biological indexing: a, Indicator host *Citrus aurantifolia*; b, Performing budding process; c, Indicator plants after budding.

growing areas of Assam (Bhagabati et al., 1989). Similar type of studies were carried out by Kishore et al. (2010) who studied the assessment of CTV incidence in mandarin of Sikkim, estimated by DAS-ELISA. Biswas (2008) also diagnosed CTV in Darjeeling Hills through molecular tools and reported CTV infection up to 90.00% in mandarin trees at varying altitudes.

Conclusion

CTV is the most important and widely distributed disease in Assam. Laboratory based ELISA test could be successfully used to detect CTV infection in the field. Thus, findings of the research would be helpful in initiating future strategies on CTV certification, eradication, rejuvenation and management programme.

Conflict of Interest

The authors declare no conflict of interest.

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