BIBILIOGRAPHY

NAMUJHE, JOSEPHINE L. APRIL 2007. Incidence of Citrus Tatterleaf Disease in Kasibu, Nueva Vizcaya. Benguet State University, La Trinidad, Benguet.

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ABSTRACT

The study was conducted to pinpoint some of the areas of occurrence of the Citrus tatterleaf disease, establish characteristic symptoms of CTLV in the locality, and report the presence of Citrus tatterleaf disease in Kasibu, Nueva Vizcaya through detection by RT-PCR analysis and symptomatology.

The areas of occurrence of the CTLV are Antutot and Malabing. The probable spread of the disease was due to the exchange of budwoods among farmers and use of infected tools and implements.

The characteristic symptoms of CTLV observed in the locality are bud union bulging, bud union creasing, decline, stunted growth, and tattered leaf.

Seven from the twenty four trees tested for CTLV infection through RT-PCR analysis yielded positive results. This shows that CTLV is now present in Kasibu, Nueva Vizcaya.

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INTRODUCTION

Citrus is one of the most important fruit crops in the Philippines. With the wide range of climatic and topographic conditions suitable for citrus production, the Philippines could become one of the major producers of citrus in the world. Actually, citrus production was a flourishing industry in the late 1950's to early 1960's especially in Southern Tagalog, Bicol and Mindanao regions prior to its decline due to the presence of bud-transmissible diseases (Payot *et al.*, 1987).

Kasibu, Nueva Vizcaya ventured into citrus production in 1987 and has once again proven that citrus is a promising industry that has global market potential if proper technology can be established (Namujhe, 2006).

Although studies have been conducted on the diseases that affected Southern Tagalog, Bicol and Mindanao, none or inadequate studies were done in Nueva Vizcaya particularly in Kasibu. Despite the fact that the prevalence of citrus diseases was not yet overcome, Kasibu continues to produce citrus and is geared to further expansion.

Now around 1,000 hectares are planted to citrus where 30% are already in their productive stage. The varieties planted are Satsuma (60%), Ponkan (20%), sweet oranges (10%), and pomelos (10%). Given these statistics, Malabing Valley is renowned as the citrus capital of Luzon (Namujhe, 2006).

Dr. Hong-Ji Su of the Department of Plant Pathology and Microbiology National Taiwan University, Taipei, Taiwan recently confirmed the presence of tatterleaf disease of citrus in the area of Kasibu by RT-PCR analysis. This poses yet another threat to the industry especially if the problem is not contained immediately.

This graft and mechanically transmissible disease of citrus is moderately destructive. Although it may severely limit crop production especially when one is unaware of the symptoms, this disease is readily detected in propagative budwood, easily eliminated and its rapid spread curtailed.

It is imperative that farmers become aware of the tatterleaf disease that was detected in their locality to ensure that the booming citrus industry in Malabing Valley becomes sustainable. They should be familiar with the symptoms of the disease so that they could easily detect infected trees and apply proper control measure to prevent its eventual spread.

Farmers can use the result of this study as a guide in identifying Citrus tatterleaf disease in their farm. The pinpointed areas of occurrence of CTLV in Kasibu can serve as a reference point for further studies. This could also serve as a basis for further and more specialized research on CTLV in Kasibu, Nueva Vizcaya

This study was conducted to pinpoint some of the areas of occurrence of the Citrus tatterleaf disease, establish characteristic symptoms of CTLV in the locality, and report the presence of Citrus tatterleaf disease in Kasibu, Nueva Vizcaya through detection by RT-PCR analysis and symptomatology.

The field study was conducted in Kasibu, Nueva Vizcaya from December 2006 to February 2007. The RT-PCR confirmatory test was done in the laboratory of the Bureau of Plant Industry, Guisad, Baguio City.

Simultaneously, RT-PCR confirmatory test was conducted in Taiwan National University under the supervision of Dr. Hong-Ji Su using samples from the same trees tested in BPI, Guisad, Baguio City.

REVIEW OF LITERATURE

The Disease

The tatterleaf disease of citrus, induced by the Citrus Tatterleaf Virus (CTLV), was first described by Wallace and Drake (1962) as a transmissible disease that induced mottled and tattered leaves in *Citrus excelsa* indicator seedlings. Calavan, Christiansen and Roistacher (1963) first showed the destructive potential of this disease to citrange rootstock when tatterleaf-infected tissue was graft-inoculated to satsuma mandarin budded on Troyer citrange rootstock. Meyer (Beijing) lemon trees, which were first imported into the United States from Beijing (China) in 1908, were later found to contain the tatterleaf virus. Many Meyer lemon trees worldwide that originated from the 1908 introduction probably contain the virus, including many propagations and plantings of the original Beijing lemon in China (Zhang, Liang and Roistacher, 1988). The disease is endemic in mainland China and is widespread in Taiwan Province and in Japan (Zhang *et al.*, 1988).

Citrus tatterleaf virus expresses no symptoms in most citrus cultivars including sweet orange, mandarin, sour lemon, Meyer lemon and grapefruit. However, if virus-infected budwood is used as scion wood and grafted to citrange or trifoliate orange or any hybrids of trifoliate used as a rootstock, a brown bud-union crease will usually be evident, deep pits and grooves may develop in the rootstocks, and trees of these scionic combinations will usually decline.

The Pathogen

Physical and biochemical properties. The virus is a capillovirus (Nishio *et al.,* 1989; Namba, 1995) which is serologically related to apple stem grooving capillovirus and to a virus isolated from stunted and chlorotic lily (Lilium longiflorum) widespread in western parts of Japan (Inoue *et al.,* 1979). It has been suggested that, due to homologies in nucleotide sequences, it is now probably best considered to be a strain of apple stem grooving capillovirus rather than a distinct virus (Ohira *et al.,* 1994). This suggestion, however, has yet to be confirmed.

Filamentous and usually flexible rod particles (Figure 1) which are 650 nm long and 12 nm wide, with a helical construction of 3.4 nm pitch. The virus has a single RNA species of molecular weight 2.83 x 106 Da and produces a single protein band of molecular weight 27 x 103Da in SDS PAGE (Nishio *et al*., 1989).

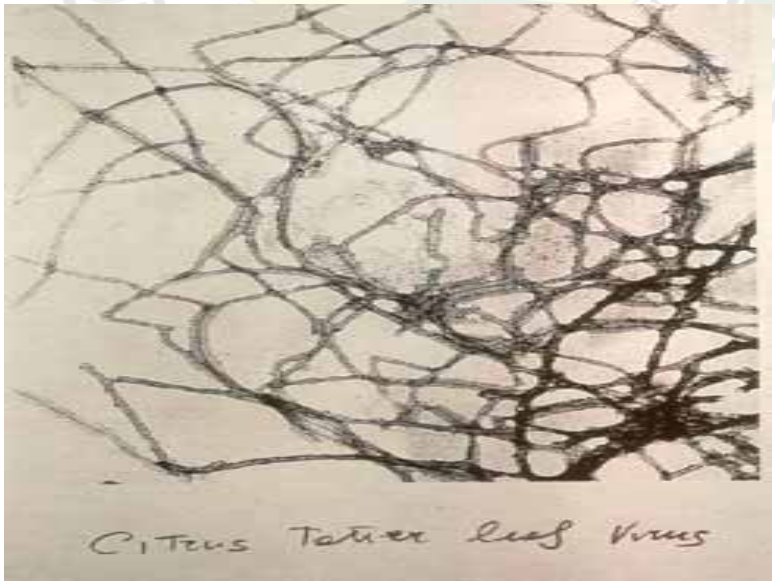

Figure 1. Studies by (Ohki *et al.,* 1989) and Nishio *et al*., (1989) indicated that the Citrus tatterleaf virus is a capillovirus and is closely and serologically related to the apple stem grooving virus

Biology

The major method of transmission from citrus to citrus is by grafting. Mechanical transmission by knife slashes and leaf-abrasion is easily achieved from infected *Nicotiana clevelandii* to citron (Garnsey, 1974) and from citron to citron (Roistacher *et al.,* 1980). However, a field trial which attempted to transmit the virus to 8-year-old mandarin trees by slashing their bark with a knife or sawing the branches gave a very low rate of infection

Seed transmission has been observed in *Chenopodium quinoa*, cowpeas and soyabeans but not in *Fortunella japonica* (Nishio *et al.*, 1982). No natural vector is known. These results suggest that natural transmission occurs only at a very low rate.

Symptoms

The common symptoms of Citrus Tatterleaf Virus is bud union necrosis or abnormality which develop when symptomless CTLV carrying citrus, such as sweet orange, sour orange, grapefruit (*Citrus paradisi* Macf.), mandarin (*C. reticulata* Blanco) or lemon (*C. limon* (l.) Burm.f.) is budded onto a trifoliate orange or a trifoliate hybrid rootstock. Deep fluting of the rootstock trunk and an extended bud union crease or gap can result as early as eighteen months after grafting. Chlorotic leaf symptoms are produced in *Citrus excelsa*, Rusk and Troyer citranges (*Poncirus trifoliata* x *Citrus sinensis*), Swingle citrumelos (*P. trifoliata* x *C. paradisi*) and other *P. trifoliata* hybrids. Leaves of *C. excelsa* may be deformed (so-called tatter leaf), stems of citrange plants may be deformed and have a zigzag growth pattern associated with chlorotic areas on the stem. Citranges and citrange hybrids are often pitted on their stem, affected plants

become stunted, chlorotic and overblooming, have early-maturing of fruit, and suckers often develop. In high winds, the scions may sever completely at the bud union (Herron *et al.,* N.D.).

Host Range

Almost all citrus plants can be symptomless hosts. *Poncirus trifoliata* is immune or highly resistant, but its hybrids can show symptoms after infection (Wallace and Drake, 1963).

The following plants are infected by the virus when inoculated mechanically (Nishio *et al.,* 1982): *Amaranthus tricolor*, *Catharanthus roseus*, *Chenopodium amaranticolor*, *C. quinoa*, *Cucurbita pepo*, *Dianthus barbatus*, *D. chinensis*, faba beans, *Gomphrena globosa*, *Nicotiana clevelandii*, *N. debneyi*, *N. glutinosa*, peas, *Petunia hybrida*, soyabeans, *Tetragonia tetragonioides*, tomatoes, *Vigna unguiculata*.

Geographical Distribution

CTLV was first found in *Citrus meyeri* in 1962 at Riverside, California, USA. The original tree was brought from China in 1908 (Wallace and Drake, 1962) and it is clear that the virus originated in China. Old budlines of *C. meyeri* which were imported from China into the USA and subsequently delivered to other countries were probably symptomless carriers (Wallace and Drake, 1962; Schwarz, 1966), so the virus may have a wider distribution. Su and Tsai (1990) also reported the Tatterleafvirus present in the Philippines, Thailand and Korea.

Economic Impact

Almost all citrus plants are symptomless if grown on their own roots or on a CTLV-tolerant rootstock. *Poncirus trifoliata* is immune or highly resistant to CTLV. However, when infected latent hosts are grafted on rootstock of *P. trifoliata* or its hybrids, a bud-union crease occurs and the tree becomes stunted or often dies (Calavan *et al.,* 1963). Yields of affected mandarins (*Citrus reticulata*) on *P. trifoliata* rootstock are 75% of those CTLV-free trees (Takahara *et al.,* 1988). Accordingly, *P. trifoliata* and its hybrids cannot be used in practice as rootstocks where CTLV is indigenous. Inserting a healthy interstock between the infected latent bud and the *P. trifoliata* rootstock only delays the problem. The scions grow normally for 1-2 years, but then become overblooming and yellow gradually and finally die within 5-6 years. These trees develop a crease at the bud-union between interstock and rootstock, and are occasionally dislocated at this point by strong winds.

Methods of Detection

CTLV can be detected through indexing to indicator plants such as Rusk, Troyer or Carrizo citrange, citremon and trifoliate stock. Inoculation to herbaceous host can also be used as an alternative method of detection (Miyakawa, 1978) although specialized inoculation chambers are needed to ensure reliable results. Reverse transcription PCR (RT-PCR) was formulated by using cDNA derived from the sequence of CTLV for detecting the virus more rapidly (Su *et al.,* 2005).

Control

CTLV-free budlines must be used for propagation. If latently infected scions were used for propagation without any therapy, *Poncirus trifoliata* or its hybrids cannot be used as the rootstock. *Citrus depressa* or *C. reshni* provide good results when used as rootstocks for CTLV-infected mandarins (*C. reticulata*) (Takahara *et al.,* 1988).

CTLV cannot be eliminated by shoot-tip grafting alone (Roistacher and Kitto, 1977). Heat treatment for 30 days at 35-40°C/30°C (day/night) followed by shoot-tip grafting can be an effective therapy (Koizumi, 1984). Incubation of budsticks on medium *in vitro* for 10-14 days at 32°C, followed by shoot-tip grafting can also produce CTLV-free plants with 30-50% success (Navarro *et al.,* 1989). Long-term heat treatment of affected plants for 90 or more days at 40°C/30°C (day/night) can eliminate CTLV (Miyakawa, 1980a). Mechanical transmission from citron to citron by knife-slashing is completely prevented by dipping the contaminated knife-blades into 1.05% sodium hypochlorite solution or 2% sodium hydroxide plus 5% formaldehyde solution, or merely by washing the blades with tap-water and drying, prior to slashing the receptor (Roistacher *et al.,* 1980).

MATERIALS AND METHODS

Survey of suspected infected trees by symptomatology was done in the area. Trees that showed decline were examined and their symptoms were compared with the symptoms of tatterleaf disease described in the literature. Those trees with symptoms that coincided with the symptoms of tatterleaf were marked, digital images were taken and samples were collected for confirmatory tests.

Survey

Upon the observation of probable symptoms of CTLV in Antutot, Kasibu, a survey on other farms with trees showing the same symptoms was conducted. Farmers were asked if they have observed bud union bulging on their declining trees. After which, those who have such trees in their farm were visited and further observation was done.

The areas surveyed were:

Antutot, Kasibu. This is where the first batch of samples was collected. This farm belongs to Engr. Roy Bernardo and this is where the symptoms of CTLV were first observed. There were three trees which manifested the symptoms and they were all 9 year-old Ponkan budded to Trifoliate.

Namujhe Farm, Malabing, Kasibu. This is where the second batch of samples was collected. Ten trees were under observation with varying age, varieties and scion-stock combination.

Randomly selected farms. Samples for this batch were gathered from trees showing symptoms of CTLV from randomly selected farms in Malabing, Kasibu.

Observation and Documentation of Disease Symptoms

For the purpose of symptoms comparison, a list of known CTLV symptoms based on the available literature was prepared. This list of symptoms served as a basis for field diagnosis based on symptomatology. The table below represents the list.

Table 1. List of CTLV symptoms based on available literature

PLANT PART AFFECTED	SYMPTOMS
Tree	Stunted growth Decline
Trunk	Bud union bulging Bud union creasing Pitted stem
Leaves	Tatter leaf Chlorotic mottle
Flowering	Over blooming
Fruits	Early-maturing of fruit

After observing the trees and comparing the symptoms with the list, photo documentation followed. First, the overall appearance of the trees was taken. Then the bud union bulging of the trunk was captured. A portion of the bark within the bud union bulging was removed to reveal any deep creasing, this too was documented. Other characteristics such as tatter leaf and chlorotic mottle were noted when trees exhibit any of these symptoms.

Sample Collection

The shoots of the trees were collected as samples. Four shoots were gathered from the different sides of each tree. This was done to ensure a good representation of the whole tree and collected shoots were considered as a composite sample of the tree. The samples were placed in plastic bags and were labeled with the following information: a. Sample number, b. Variety/ scion-stock combination, c. age, d. location, e. farm owner.

The labeled samples were stored in the refrigerator prior to transport. Samples were kept under low temperature while in transit by placing them in styrobox with ice. They were again transferred into the refrigerator upon arriving in the laboratory.

Detection by Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

Confirmation was done through RT-PCR analysis conducted at the Bureau of Plant Industry, Guisad, Baguio City. Simultaneously, samples from the same trees were sent to National Taiwan University through Dr. Hong-Ji Su for counter testing and more accurate results.

RT-PCR Analysis conducted at the Bureau of Plant Industry, Baguio City.

Extraction of nucleic acids from citrus tissues. The procedure for extraction was adapted from RNeasy Plant Mini kit as produced by QIAGEN.

The samples were macerated using mortar and pestle. One 100 mg of the tissues were transferred to 1.6-ml tubes. RLT buffer (450 µl) was added to tissue samples and stirred vigorously.

Lysate were applied to the QIA shredder spin columns setting in 2-ml collection tubes and centrifuge at 11,000 rpm/3 min. Flow-through fractions were transferred from QIA shredders to new tubes.

One half volume (usually 225 µl) of ethanol (96~100%)was added to each cleared lysate and was mixed well by pipetting. Samples (usually 225 µl) were applied onto RNeasy mini spin columns set in 2-ml collection tube. Samples were ran in the centrifuge at 11,000 rpm for one minute. Flow-through were discarded and the collection tubes were re-used.

One volume of Buffer RW1 (700 µl) was added to each RNeasy columns and were spun at 11,000 rpm for one minute to wash. Flow-through were discarded and the collection tubes were re-used.

This step was repeated using 500µl of buffer RPE. Again, the flow-through were discarded and the collection tubes were re-used.

A final volume of 500 µl buffer RPE was added to each RNeasy columns and were spun in the centrifuge at 11,000 rpm for 3 minutes. RNeasy columns were transferred into new 1.5-ml collection tubes. Approximately 30~50 µl of RNase-free water was added directly into each RNeasy columns. The columns were spun in the centrifuge at 11,000 rpm for 2 minutes to elute. The flow-through were kept as RNA templates for RT-PCR.

RT-PCR conditions. Each PCR reaction contained the components in Table 2 and should have a total of 25 µl. Amplification was carried out in a BIOMETRA Personal Cycler (PC 20 with heated lid) using a cDNA primer pair. The forward and reverse

primers have the following respective sequences (reference): CGA AGA CTC ACA TAG ACC CG and TAC TCT CCG AAC CTG CCT C.

Table 2. Components of the RT-PCR reaction

COMPONENT	**AMOUNT (µl)**
ddH_2O	10.0
5X Superscript II buffer	2.5
10X Taq buffer	2.5
100mM DTT	1.25
10mM dNTPs	2.0
Taq polymerase (BRL or BerTaq)	0.2
Superscript II polymerase (BRL)	0.25
CTLV primer pair (10 pmol/µl each)	1.0
RNA template	0.25

The amplification schedule began with one initial cycle at 50 and 94ºC for 35 and two minutes. The next step consisted of 10 cycles at 94, 56 and 68 ºC for 30, 30 and 45 seconds, respectively. This step was followed by 25 cycles with the same parameters as the previous step but with 5 seconds increasing ramp. The final cycle was at 68 ºC for seven minutes.

The negative control came from BPI citrus foundation greenhouse while the positive control was unavailable.

Electrophoresis analysis of PCR products. A 1.4% agarose gel plate in TAE buffer (0.04 M Tris-acetae, 0.001 M EDTA, pH 8.0) was prepared.

A 3-5 µl of PCR products (CTLV DNA extract) was loaded into each well, and the gel was run in an electrophoresis chamber at 100 volts for about 30 minutes when the markers reached near the bottom line. (Running buffer: 0.5 X TAE buffer, pH 8.0).

The gel was stained with Ethidium bromide (0.5 ug./ml) solution for 3-5 min, and was soaked in water for several minutes.

The amplified CTLV-DNA band (at 632 base pair) was observed under UV-chamber and a picture of the electrophoretic plate was taken.

The following were composition of the buffers used computed per 1000mL:

50 X TAE buffer:

242g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0).

Loading buffer:

0.25% Bromophenyl blue

30% glycerol (1:4).

RT-PCR analysis done at the National Taiwan University, through Dr.Hong-Ji Su.

Extraction of nucleic acids from citrus tissues. A 0.3g of tissue from each samples were ground in liquid nitrogen and homogenized in 3ml TRIzol reagent buffer. The samples were spun in the centrifuge at 12,000xg at room temperature for 10 min. The supernatants, about 1ml each, were transferred into new tubes. A volume of 200 l chloroform was added to each sample. The tubes were shaken vigorously using the vortex

for 30 sec. The samples were again spun in the centrifuge at 12,000xg at room temperature for 10 min. The aqueous phase were transferred into new tubes using a pipette. A 300 l volume of isopropanol and 0.8 M sodium citrate/1.2 M NaCl (about 300 l), ½ volume of the aqueous phase each, was added and mixed by gentle inversion. The samples were again ran in the centrifuge at 12,000xg at 4°C for 15 minutes. After which the supernatants were discarded and the pellets were washed with 500ul of 75% ethanol. The pellets were briefly dried for about 10-12 minutes. Finally 80 l (or 50 l) ddH2O was added to each samples.

The TRIzol reagent buffer (pH5) consisted of 38 % Phenol , 0.8 M Guanidine thiocyanate, 0.4 M Ammonium thiocyanate, 0.1 M Sodium acetate , 5% Glycerol.

RT-PCR conditions. Each PCR reaction contained the components in Table 3 and has a total volume of 25 µl. The forward and reverse primers have the following respective sequences (reference): CGA AGA CTC ACA TAG ACC CG and TAC TCT CCG AAC CTG CCT C.

The amplification schedule began with one initial cycle at 50 and 94ºC for 35 and two minutes. The next step consisted of 10 cycles at 94, 56 and 68 ºC for 30, 30 and 45 seconds, respectively. This step wasfollowed by 25 cycles with the same parameters as the previous step but with 5 seconds increasing ramp. The final cycle was at 68 ºC for seven minutes.

Electrophoresis analysis of PCR products. A 1.4% agarose gel plate in TAE buffer (0.04 M Tris-acetae, 0.001 M EDTA, pH 8.0) was prepared.

Table 3.Table showing RT-PCR components.

COMPONENTS	**AMOUNT (µl)**
ddH_2O	11.5
5X Superscript II buffer	2.5
10X Taq buffer	2.5
100mM DTT	1.25
25 mM $MgCl_2$	1.25
10 mM dNTPs	2
Taq polymerase (BRL or BerTaq)	0.25
Superscript II polymerase (BRL)	0.25
CTLV primer pair (10 pmol / ul each	1
RNA template	2.5

A 3-5 µl of PCR products (CTLV DNA extract) was loaded into each well, and the gel was run in an electrophoresis chamber at 100 volts for about 30 minutes when the markers reached near the bottom line. (Running buffer: 0.5 X TAE buffer, pH 8.0).

The gel was stained with Ethidium bromide (0.5 ug./ml) solution for 3-5 min, and was soaked in water for several minutes.

The amplified CTLV-DNA band (at 632 base pair) was observed under UV-chamber and a picture of the electrophoretic plate was taken.

The following were composition of the buffers used computed per 1000mL:

50 X TAE buffer:

242g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0).

Loading buffer:

0.25% Bromophenyl blue

30% glycerol (1:4).

The data gathered were:

a. Digital images of the actual field symptoms of Citrus Tatterleaf disease was recorded and compared with those listed in the literatures,
b. Results of RT-PCR analysis from BPI Baguio and National Taiwan University which confirmed the presumed presence of CTLV infection based on the symptoms observed , and
c. Map of Kasibu where the areas detected with CTLV infected trees were indicated.

RESULTS AND DISCUSSION

Areas of Occurrence

According to the owner of the farm in Antutot, Kasibu, where the trees manifesting symptoms of Citrus Tatterleaf Disease was first observed, the scions of the Ponkan he budded to Trifoliate came from the trees brought by his uncle from Japan. It is a logical presumption that the initial inoculum of CTLV came from these trees whose scion came from Japan and did not undergo proper Quarantine procedures when brought here.

The eventual spread of the disease in the area, until it reached Malabing, Kasibu where some trees also showed symptoms of CTLV, probably occurred when farmers started propagating their own planting materials and most of them exchanges bud woods with each other to acquire new varieties.

The map of Kasibu is presented in Figure 1, which shows the occurrence of CTLV in Kasibu and the probable movement of inoculum spread. The green dot represents Antutot and the yellow dot is Malabing. The lines represent the probable movement of the inoculum from the two sources through exchange of budwoods among the farmers.

Symptoms of Citrus Tatterleaf Disease

The comparison of the observed symptoms of Citrus Tatterleaf Disease and the previously reported symptoms is summarized in Table 4. The symptoms for the flowering stage and the fruits were not observed in Kasibu since the duration of the study

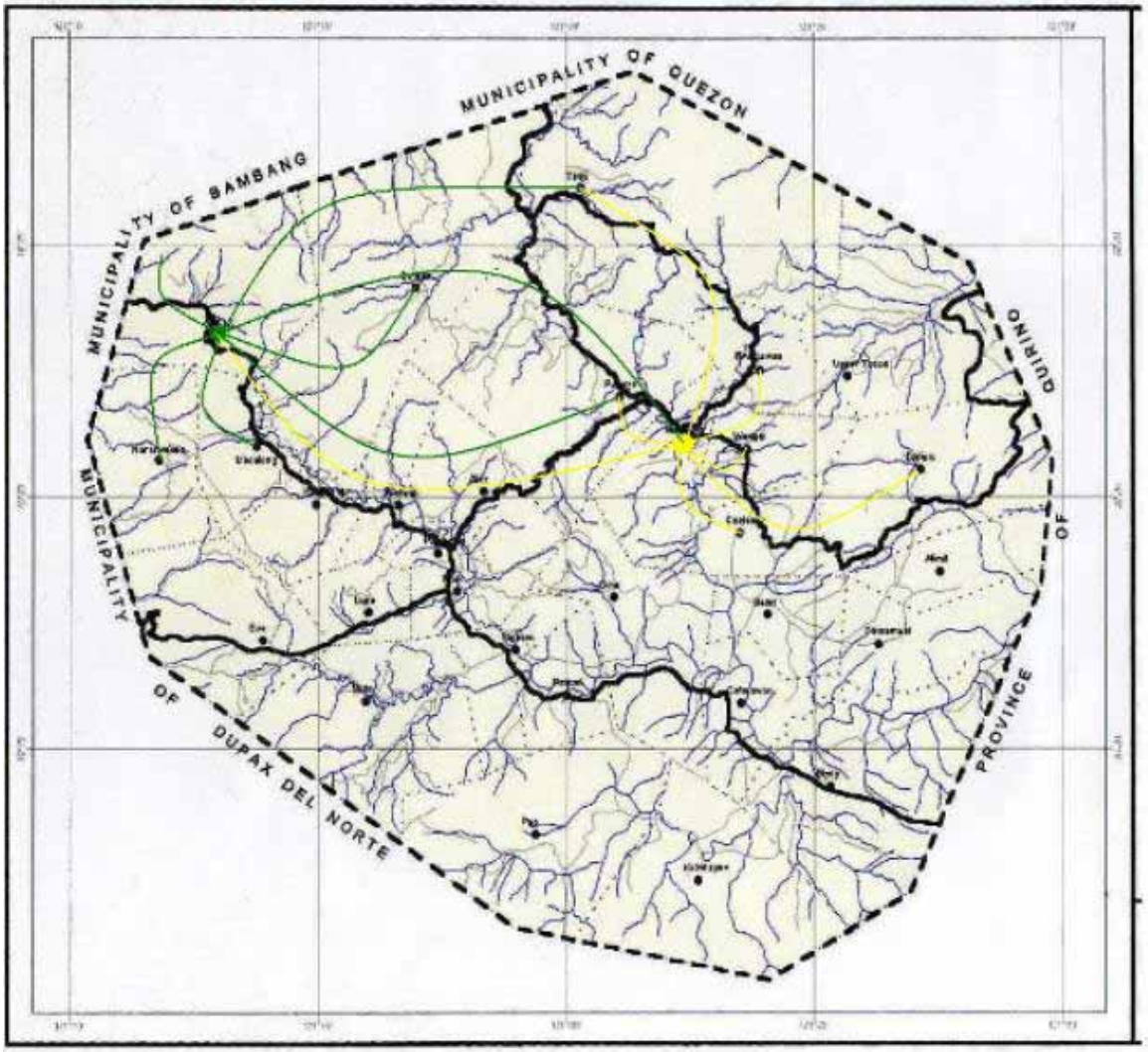

Figure 2. Map of Kasibu showing the occurrence of CTLV in the area and the probable movement of inoculum spread through budwood exchange among farmers with green dot representing Antutot while the yellow dot representing Malabing

did not cover the said stages. Some of the symptoms like pitted stem and chlorotic mottle were not observed because the variety of citrus under observation does not manifest such symptoms.

The symptoms manifested by the infected trees in Kasibu coincided with that of the CTLV as described in the literatures and was confirmed by Dr. Su during his visit in the area last February 2-4, 2007.

Table 4. Comparison of the symptoms of CTLV from the literature and those observed in Kasibu, Nueva Vizcaya

CHARACTERISTIC SYMPTOMS OF CTLV BASED ON THE LITERATURE	**CHARACTERISTIC SYMPTOMS OF CTLV OBSERVED IN KASIBU**
Trees: Stunted growth (Figure 4)	Stunted growth (Figure 3)
Decline (Figure 4)	Decline (Figure 3)
Trunk: Bud union bulging (Figure 6)	Bud union bulging (Figure 5)
Bud union creasing (Figure 8)	Bud union creasing (Figure 7)
Pitted stem (Figure 8 B)	-do-
Leaves: Tattered leaf (Figure 10)	Tatterleaf(Figure 9)
Chlorotic mottle	-do-
Flowering: Over blooming	Duration of study did not cover the stage
Fruits: Early-maturing of fruit	Duration of study did not cover the stage

The characteristic symptoms of Citrus Tatterleaf Disease are:

Stunted growth. The tree is relatively small for its age regardless of its variety and scion-stock combination (Figure 3 and 4).

Decline. This is manifested by poor growth of the plant; relatively small leaves, brittle and yellowish; some defoliation and dieback (Figure 3 and 4).

Bud union bulging. This is the most typical characteristic symptom of CTLV infection that is manifested by a swelling around the bud union area (Figure 5 and 6)

Bud union creasing. It is the formation of a line, which seems to fold inwards, along the bud union. In severe cases, the crease is so deep that the scion tends to sever from the rootstock (Figure 7 and 8).

Pitted stem. This symptom is characterized by depressions on the stem of the trees (Figure 6B).

Tattered leaf. The leaves are deformed and the edges has a tattered appearance (Figure 9 and 10), for which the disease was actually named.

Chlorotic mottle. This symptom appeared as yellowing of the leaves with the boundaries of light and dark variegated areas diffused.

Deep creasing at the bud union causes decline and stunted growth due to the disruption of the translocation of synthesized food as well as the movement of water and nutrients from roots to the leaves.

The trees in Figure 3 shows the stunted growth and decline in Antutot, Kasibu, while the trees in Figure 4 shows the same stunted growth and decline in trees from Zhejiang Province, China. You will notice that the trees from both areas are relatively small for their age.

Figure 3. Infected 14-yr old trees in Antutot, Kasibu showing stunted growth and decline

Figure 4. Fifteen-year old trees in Zhejiang Province, China showing stunted growth and decline due to CTLV infection; photo illustration by Roistacher C.N.

The bud union bulging symptom is clearly depicted in Figure 5, observed in Kasibu, which is similar to Figure 6 (A), observed at the Citrus Research Station in Riverside, California.

Figure 5. Bud union bulging as a typical symptom of CTLV observed in the infected trees in Kasibu

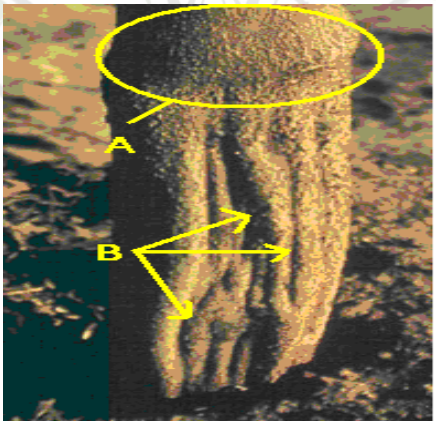

Figure 6. Symptoms bud union bulging (A) and pitted stem (B) observed at the Citrus Research Station in Riverside, California; photo illustration by Calavan E.C.

Another symptom of CTLV infection is bud union crease as shown in Figure 7, observed in infected trees from Antutot, Kasibu, similar with those shown in Figure 8, observed in trees from Kutchinotsu, Japan and at the Citrus Research Station in Riverside, California.

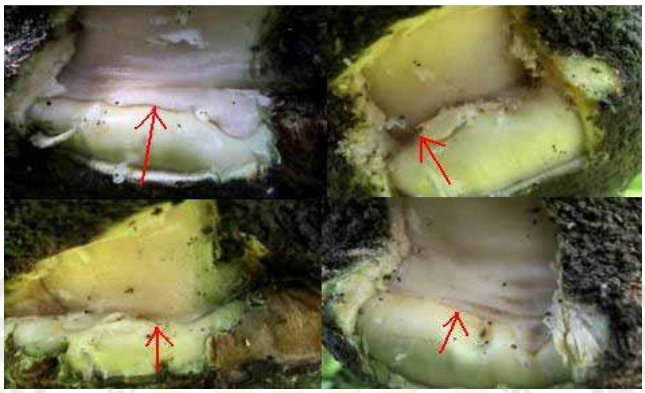

Figure 7. Bud union bulging and deep creasing manifested by the CTLV infected trees in Antutot, Kasibu

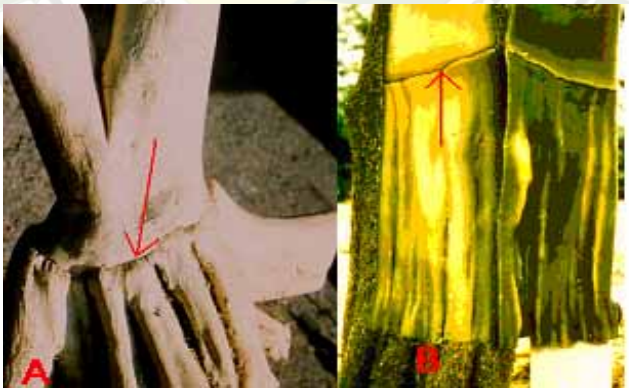

Figure 8. A specimen showing a severe bud union crease and incompatibility of a Satsuma mandarin grafted on Trifoliate rootstock that had been infected with the Citrus tatterleaf virus at Kutchinotsu, Japan(A); severe bud union crease (B) a typical reaction with trifoliate and its hybrid rootstock observed; photo illustration by Calavan E.C.

The tatterleaf symptom shown in Figure 9, observed in Antutot, Kasibu, is similar to Figure 10, from the literature. Manifestation of the severity of this symptom may vary depending on the scion-stock combinations.

Figure 9. Tattered leaves of infected trees in Antutot, Kasibu

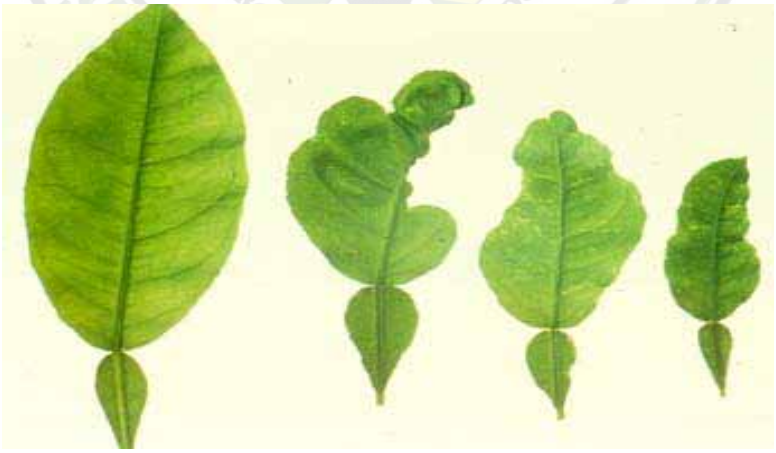

Figure 10. Tattered leaf symptoms (first three leaves from right side) for which the disease was named and the uninoculated control leaf (leftmost); photo illustration by Roistacher C.N.

After knowing the symptoms of CTLV infected trees, it is imperative that the distinction between the symptoms of infected trees and that of natural incompatibility should be taken into consideration. Some trees with scion-rootstock incompatibility may have larger base (rootstock) than the scion trunk but it does not affect the growth and yield of the tree. Other healthy trees have natural bud union crease and brown line that are merely superficial as shown in Figure 11, observed in Malabing Kasibu and Figure 12 that was taken from the literature.

Figure 11. Natural bud union brown line in virus-free trees in Malabing, Kasibu

Figure 12. Natural incompatibility of a virus-free Meyer lemon scion budded on trifoliate rootstock (A) with the deep brown line extending into the wood (B); photo illustration by Roistacher C.N.

RT-PCR Results

The RT-PCR confirmatory test was conducted simultaneously at BPI, Baguio under the supervision of Senior Pathologist Juliet M. Ochasan and at the National Taiwan University, Department of Plant Pathology & Microbiology under Dr. Hong-Ji Su.

The RT-PCR results from the BPI, Baguio (Figure 13) were not satisfactory due to some technical problems encountered during the process. Some of the most likely reasons for ineptness were the poor state of the equipment used, such as the fluctuating voltage delivered by the electrophoresis machine and the limit of the micropipettes available which delivered inaccurate amounts of reagents. The total amount of the PCR mixture, which did not actually coincided with the stated amount required, could also be a factor. Another probable reason for poor results, as suggested by Dr. Su who is an expert from National Taiwan University laboratory, was the state of the samples. Since RNA easily degrades, he advised that using fresh samples be recommended for better RNA yield.

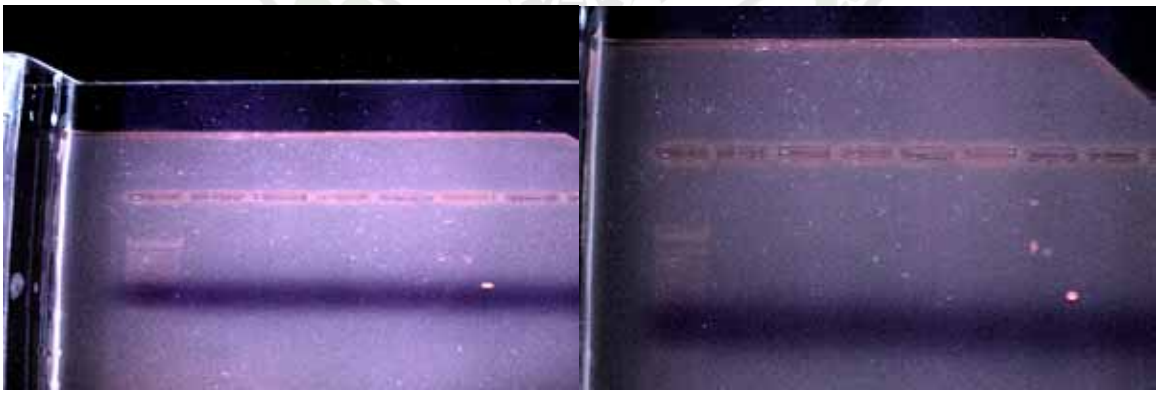

Figure 13. Electrophoresis reading of the RT-PCR analysis from BPI, Baguio laboratory

Since RT-PCR is a very sensitive test that requires precision, top of the line equipment and experience, the researcher sent some samples from the trees under study to NTU Laboratory through Dr. Su to ensure that reliable results.

The results from the NTU laboratory are as follows:

First Batch from Antutot, Kasibu. The samples are all positive of CTLV infection (Table 5, and Figure 14). The result confirmed the symptoms shown in Figures 3, 5, 7 and 9 as CTLV infection, which were quite severe.

Table 5. Results of the RT-PCR test from NTU on samples from Antutot, Kasibu

SAMPLE NUMBERS				
1	2	3	Disease-check	Healthy-check
+++	++	++	++++	-

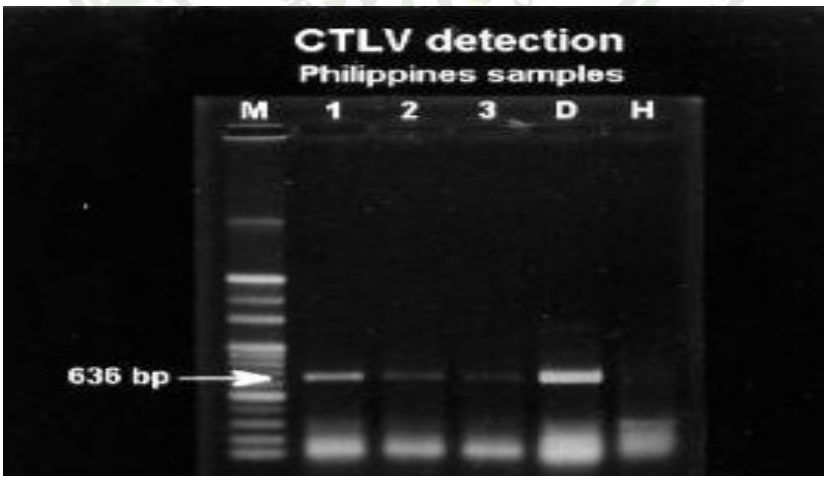

Figure 14. Electrophoresis reading of the RT-PCR analysis of samples from Antutot Kasibu showing all samples positive to CTLV infection with emphasis on the white line along the 636bp

Second batch of samples from Namujhe farm. As shown in Table 6, only two samples from this batch were positive for CTLV infection. Samples Fi-3 (Figure 15 and 16) and Fi-4 (Figure 15 and 17) showed decline and bud union bulging.

Table 6. Citrus samples for CTLV indexing (Namujhe)

MATERIAL NO.	*CULTIVAR*	**CTLV/ DETECTION (RT-PCR)**
Fi-1 (No. 4)	Ponkan	-
Fi-2 (No. 7)	Ponkan	-
Fi-3 (No. 9) (Figure 16)	Ponkan	+
Fi-4 (No. 10)(Figure 17)	Ponkan	+
Fi-5 (No. 1)	Satsuma	-
Fi-6 (No. 2)	Satsuma	-
Fi-7 (No. 3)	Satsuma	-
Fi-8 (No. 4)	Satsuma	-
Fi-9 (No. 5)	Satsuma	-
Fi-10 (No. 6)	Satsuma	-

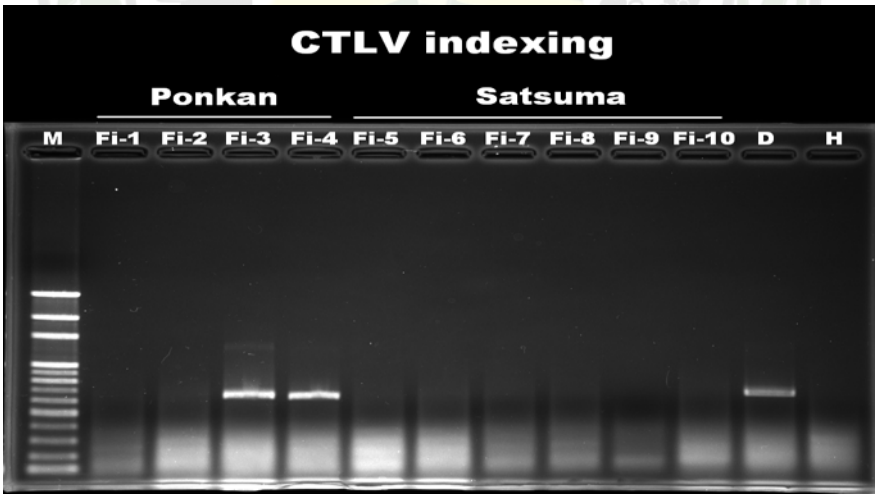

Figure 15. Electrophoresis reading of the RT-PCR analysis for second batch of samples from Namujhe Farm at Malabing, Kasibu with only Fi-3 and Fi-4 showing a white line along with the disease check that indicates positive CTLV infection

Figure 16. Sample Fi-3 (sample 9) which was confirmed positive of CTLV infection both by symptomatology and RT-PCR test with the tree showing decline (A) and bud union bulging and creasing (B)

Figure 17. The declining tree where sample Fi-4 (sample 10) was gathered

Third batch of samples randomly gathered from Malabing, Kasibu. The samples of this batch (Table 7, Figure 18) were randomly gathered from trees showing decline and bud union bulging from different farms in Malabing, Kasibu. It can be noted that only two samples were positive for CTLV infection, H/Fi-4 and H/Fi-6 respectively. These trees were the ones shown in Figure 19 and 20. The scion budded to sample H/Fi-6 (Figure 20) came from sample H/Fi-4 (Figure 19).

Table 7. Results of the RT-PCR test for the third batch of samples that were randomly gathered from various farms in Malabing, Kasibu

SERIAL NO.	*CULTIVAR*	*PLACE*	**DISEASE INDEXING** CTLV/RT-PCR
G/Fi-1	Satsuma-Fi-1 (>10 years)	Malabing Farm-1	-
G/Fi-2	Pummelo-Fi-1 (5 years)	Malabing Farm-1	-
H/Fi-3	Satsuma-NH-3 (>6 years)	Namujhe Farm-1	-
H/Fi-4	H / Sat-NH-4(>20 years) (Figure 19)	Namujhe Farm-1	++
H/Fi-5	H / Sat-NH-5 (>20 years)	Namujhe Farm-1	-
H/Fi-6	H / Sat-NH-6(7-8 years) (Figure 20)	Namujhe Farm-1	++
G/Fi-7	Ponkan-Fi-1 (>6 years)	Namujhe Farm-1	-
G/Fi-8	Satsuma-Fi-2 (>6 years)	Namujhe Farm-1	-
G/Fi-9	Satsuma-Fi-3 (10 years)	Malabing near PF-foundation	-
G/Fi-10	Satsuma-Fi-4	Malabing near PF-foundation	-
G/Fi-11	Pummelo-Fi-2	Malabing Farm-3	-

Samples

2 : G/Fi-2
3 : H/Fi-3
4 : H/Fi-4
5 : H/Fi-5
6 : H/Fi-6
7 : H/Fi-7
8 : G/Fi-8
9 : G/Fi-9
10 : G/Fi-10
11 : G/Fi-11

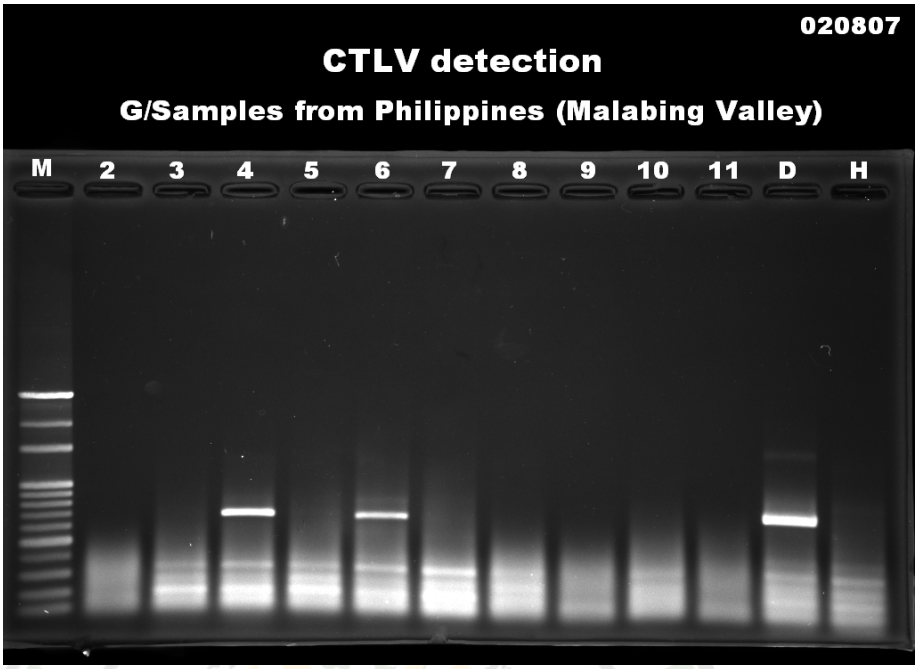

Figure 18. Electrophoresis reading of the RT-PCR analysis for third batch of samples with emphasis on the very distinct white line of samples 4 and 6 in line with the white line in the disease check indicating positive reaction to CTLV infection

Since all citrus varieties are symptomless carriers of CTLV there are instances that the tree may manifest bud union bulging but no creasing may occur, just like some of the trees observed in Malabing, Kasibu where Satsuma was budded to Calamandarin. A 20 year-old tree (Figure 19) showed bud union bulging but there was no creasing and decline of the tree since Calamandarin is not susceptible to CTLV. However, when scion from one of the trees was budded to a Trifoliate rootstock the tree showed decline (Figure 20).

Figure 19. A 20-year old Satsuma tree (A) budded to Calamandarin, observed in Malabing, Kasibu showing bud union bulging (B and a) but no creasing (C and b) and decline (A)

Figure 20. The declining 7-year old tree (A) whose scion came from the infected Satsuma tree budded to Calamandarin, in Trifoliate rootstock with emphasis on the bud union bulging (B)

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

Kasibu, Nueva Vizcaya has once again proven the potential of the citrus industry here in the Philippines. With the vast expanse of land dedicated to citrus farms and with the continuous development of proper technology, Kasibu is geared towards producing quality citrus that can compete in the global market.

However, some trees showing decline were observed in Antutot, Kasibu. Aside from stunted growth and decline, another distinct symptom manifested by the trees was bud union bulging. This particular symptom arouses the suspicion that these trees were infected with Citrus Tatter Leaf Capillovirus. To make sure that the presumptions were true, the areas of occurrence were observed and mapped out, a comparison of the field symptoms with the typical symptoms of CTLV as described in the literature was done. RT-PCR test was conducted at the Bureau of Plant Industry, Baguio laboratory at the same time samples from the same trees were sent to National Taiwan University for a more accurate RT-PCR analysis.

The areas of occurrence were in Antutot, Kasibu and Malabing Kasibu. The farm in Antutot has three trees showing the bud union symptom. While in Malabing twenty one trees were observed but only four were actually infected.

The symptoms observed in the field coincided with the symptoms described in the literature and was personally confirmed by Dr. Hong-Ji Su of the Department of Plant Pathology and Microbiology National Taiwan University Taipei, Taiwan, when he visited the area last February 2-4, 2007.

The results of the RT-PCR analysis conducted by Dr. Hong-Ji Su confirmed the presence of tatterleaf disease of citrus in some of the areas in Kasibu.

Conclusion

The probable source of the inoculum in Antutot was the scion of the Ponkan from Japan that did not underwent proper quarantine procedures. The eventual spread of the disease could be attributed to the exchange of budwoods among farmers when they started propagating their own seedlings. This could escalate into an epidemic if farmers are unaware of the presence of the disease and continue propagating their own seedlings without indexing their source of budwoods. Continuous use of Trifoliate as rootstock will also contribute to its rapid spread.

The established symptoms of CTLV in the locality can be used as a guide for field diagnosis. The characteristic symptoms of CTLV are bud union bulging, bud union creasing, decline, stunted growth, and tattered leaf.

The RT-PCR test confirmed that the disease observed in Kasibu was indeed caused by Citrus Tatter Leaf Capillovirus.

Recommendations

It is highly recommended that farmers be informed of the presence of CTLV in their locality so that they will become aware of its symptoms and its economic impact. Information materials about CTLV must be made available to farmers.

Farmers are encouraged to eradicate positively tested trees to eliminate inoculum source and prevent spread of the disease. At the same time, use of clean certified planting

materials to start their farm or replace eradicated trees is very essential. It is important that indexing for CTLV should be included in any program for establishing primary foundation trees since many citrus species and commercial cultivars are symptomless carriers and the virus is highly mechanically transmissible.

Use of clean farm tools and implements by disinfecting pruning shears in 5% sodium hypochloride will help ensure the cleanliness of your implements. Proper sanitation in the farm should be part of the management program.

Since CTLV is already present in the area, avoiding the use of Trifoliate and its hybrids as rootstock will help prevent the manifestation of bud union creasing which is the symptom deleterious to the tree.

Finally, for studies on CTLV in Kasibu, it is highly recommended that the researcher should undergo intensive laboratory training to be familiarized with the protocol. It is also advisable to adopt the protocol from the laboratory of National Taiwan University. Adequate and appropriate laboratory equipment, apparatus, and reagents should be available since the test requires utmost precision. All these will help ensure accurate results.

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