PURIFICATION AND SEROLOGY OF BANANA BUNCHY TOP VIRUS

By

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THESIS

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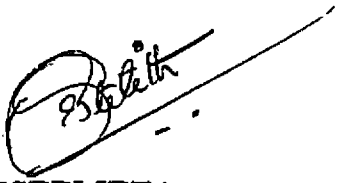
Dedicated to my beloved father Sukumaran

DECLARATION

I hereby declare that the thesis entitled '**Purification and serology of banana bunchy top virus**' is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.



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CERTIFICATE

Certified that the thesis entitled `**Purification and serology of banana bunchy top virus**' is a record of research work done independently by **Smt.S.Estellita**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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INTRODUCTION

The bunchy top disease caused by banana bunchy top virus (BBTV) is considered to be the most important disease of the crop. The primary spread of the virus is through the infected suckers which are used as planting materials and the secondary spread is by the viruliferous banana aphid *Pentalonia nigronervosa*. Suckers are the common planting materials, though micro propagated plants are also available on a limited scale; and the infected suckers serve as the main source of inoculum. Even the micro propagated plants derived from untested source plant may carry the infection, and hence they can also be used as planting materials only with a caution.

Serological tests provide rapid and convenient methods for the detection and identification of virus in the suckers in a large scale. Even the micropropagated plants are also to be indexed for virus before large scale multiplication. This is particularly important when they are obtained from the regions where BBTD is commonly seen. The meristem tip culture, commonly adopted in micropropagation, is normally conceived as generating disease free plants as the apical shoots contain very little virus concentration, but they cannot be ensured as virus-free for obvious reasons.

Field level quarantine measures are essential to prevent the spread of the virus diseases in the crop. Staining techniques suggested by Summanwar and Marathe (1982) are not giving consistent results for this purpose.

In this context, a study was designed and carried out to purify the banana bunchy top virus, to identify the nucleic acid of the virus and to produce antisera for developing a serological technique for the presymptomatic detection of the virus

infection in banana planting materials. Electron microscopy of the purified virus preparation was also done to determine the particle morphology of the virus.

This is the first attempt of successful purification of BBTV in India and to develop antiserum for detection of the virus. This method will help to evolve purified virus preparation which is useful for inoculation of test plants, probe preparation or molecular cloning. This will also help in developing a strategy for presymptomatic detection of virus present in the extensively used planting materials, even upto 200-60 µg of plant material according to the ELISA technique used.

Though the study was carried out in a systematic way as programmed, further studies on nucleic acid hybridisation can be taken up to provide highly sensitive and specific assays for known viruses. The time and resource constraints of the student researcher as well as the non-availability of the DNA probe were the limiting factors in taking up such investigations along with the present study.

Information on the basic aspects of virus-vector relationship is necessary for better understanding of the virus. Therefore, preliminary studies were conducted on virus-vector relationship before entering into the finer aspects of the virus.

The report of this research project is presented here in six chapters. Following this chapter, the review of literature contains an overview of the past studies conducted on these lines. The next chapter describes the methodology followed in carrying out the project. The results of the experiments are described in fourth chapter, which are interpreted and discussed in the fifth chapter. The last chapter contains the summary and conclusions of the study.

Review of Literature

REVIEW OF LITERATURE

Banana bunchy top disease (BBTD) which is also called 'strangles', 'curlytop' or 'cabbage top disease' is the most important virus disease infecting bananas in India. By 1940, it was introduced to South India from Ceylon. The disease has spread to Karnataka, Tamil Nadu, Andhra Pradesh, Orissa, Maharashtra, Bihar and Assam. In Kerala, appearance of the disease became conspicuous in 1943 in Kottayam district. By 1950, the disease had spread over an area of about 9000 sq. km. in Kerala state and was then estimated to cause an annual loss of about Rs.40 millions (Mehta *et al.*, 1964). In the hills of Madurai district in Tamil Nadu, Hill banana Virupakshi (Syn. Sirumalai and Vella vazhai), which is grown in more than 10,000 ha as a rainfed perennial crop, was affected by the disease.

Kesavamurthy (1980) estimated that more than 60 per cent of this area was completely damaged by banana bunchy top disease. He also reported that the incidence of BBTD was found higher in Kerala (5-90 per cent), followed by Assam (0-19 per cent) and Tamil Nadu (2.0-18.2 per cent).

2.1 Symptomatology

The symptoms of BBTD appear at any stage of growth. One of the most important symptoms for early diagnosis is the occurrence of prominent dark green streaks in the petioles and along the leaf veins (Magee, 1940). In a severely infected plant, leaves bunch together at the apex. The younger plants show marked stunting. Since the leaf stalks fail to elongate, the leaves become more erect. In an initially healthy plantation, the distribution of the disease at first is sporadic. The diseased plants do not immediately wilt or die. In the case of a new secondary infection, leaves show irregular, dark-green streaks. The markings range from a series of

dark-green dots to a continuous dark-green line. Affected leaves may be slightly chlorotic and smaller with a wavy margin of lamina.

The first leaf affected shows green streaks on the secondary veins on the under side of the lamina and on the midrib and petiole. The streaks are about 0.75 mm wide and vary in length up to 2.5 cm. A powdery bloom covers the midrib and petiole, if this is rubbed off the dark green streaks can be clearly seen. These streaks vary from a series of dark green dots to a continuous dark green line with a ragged edge. Subsequent leaves show the same symptoms as the first one affected and are progressively dwarfed; they also show marginal chlorosis and curling. The leaves are brittle in texture and the petioles incompletely elongated. Pale, whitish streaks may be seen along the secondary veins when the newly emerged or emerging leaf is still tightly rolled. Transverse wrinkling occurs along the length of the lamina. In subsequent leaves the unfurling process is premature but slow so that several leaves are unrolling at the same time. The leaves become smaller and eventually the crown of the plant becomes composed by stunted leaves - the 'rosette' or 'bunchy top' which gives the disease its name. The opening bunches are constricted or choked by the pseudostem and may split instead of emerging in the normal fashion. The bunches themselves are reduced in size and the fruits quite unsaleable. The lower hands of the bunch often die off.

In primarily infected plants, leaf symptoms may be observed as soon as the first leaf appears above ground. These leaves are small, rosetted, profusely streaked and possess highly chlorotic margins slightly rolled upward. Growth is slow and fruits are rarely produced, or if produced does not ripen. The plants remain as such for atleast one or two years.

Bunchy top is often accompanied by secondary root decay caused by fungi and bacteria and in other cases a secondary heart rot may occur, especially

during the wet season. None of these rots is characteristic of bunchy top and only the presence of broken dark green streaks along the secondary veins of the lamina, or along the midrib or petiole, is a definite and reliable symptom of acute infection (Wardlaw, 1972).

In most cultivars, the initial symptoms are the appearance of a few dark green flecks on the petiole of the youngest emerged leaf. This petiole flecking becomes more pronounced on all subsequent leaves and these flecks often coalesce to become dark green streaks. These petiole symptoms do not appear in all cultivars. Marginal yellowing of the lamina becomes evident on the third or fourth leaf. Subsequent leaves also showed marginal yellowing and become progressively smaller and narrower. These leaves showed dark green streaking of the midrib and dark green flecks and streaks on the leaf blade. Infected leaves tend to remain upright, thereby giving the plant the characteristic bunched appearance after which the disease is called. The plant shows various degrees of stunting and the lower leaf petioles may separate from the pseudostem (Frison and Putter, 1990).

The symptoms of the disease are quite distinctive but not necessarily obvious to the untrained observer. In advanced infections with severe symptoms, the leaves become narrow with marginal yellowing and become upright and brittle giving the plant a 'bunchy top' appearance. A closer inspection reveals the characteristic pattern of dark green streaks in the leaf veins and midribs and on the petioles and the pseudostem. Plants are also stunted and if infected early, do not produce a bunch. The suckers are also affected. Sometimes after infection, the cultivar and environmental conditions can affect the appearance of symptoms and, in many instances, the symptoms will be less severe than those described above (Iskra, 1990).

2.2 Virus

The hypothesis of the existence of a viral agent responsible for BBTD was put forward by Magee (1927). This virus is named as banana bunchy top virus (BBTV), Banana Virus I or Musa Virus I. As the virus had never been isolated, it was classified in 1975 as a possible luteovirus (Shepherd *et al.*, 1975), for reasons of symptomatology, transmission and location in the plant. A synthesis of various current researchers showed a virus to be associated with BBTD and that it might be the agent responsible, although this had not yet been demonstrated.

The name luteovirus (Latin luteus = yellow) was coined because infected plants tend to show yellowing symptoms. The concentration of virus particles within the plant is low (less than 100 µg/l of sap) and particles have been observed only in phloem tissues. Luteoviruses are transmitted neither by mechanical inoculation nor through seed, but are transmitted by aphids in a persistent (circulative non-propagative) manner. Most members showed a high degree of vector specificity and some luteoviruses can act as helpers for the aphid-transmission of associated viruses.

Very little information was available regarding BBTV itself prior to 1986. Virus particles had not been purified and therefore there was no biochemical data available. Recent information based on the properties of virus - like particles purified from BBTV infected plants has resulted in confusion over the classification of BBTV (Iskra, 1990).

2.2.1 Properties of luteovirus particles

When negatively stained most luteovirus particles have a diameter of 25-28 nm; they are usually angular in outline and not penetrated by stain. Thermal

inactivation points range from 45°C-75°C. The particle mass of Beet western yellow virus (BWYV) is 6.5 x 10^6, as determined by optical diffraction and scanning transmission electron microscopy (Chevallier and Engel, 1983). The protein subunits of a luteovirus particle are probably in a T = 3 arrangement, the M.wt. of 180 protein subunits plus the M.wt. of the RNA gives a total very close to that obtained for the whole particle mass of BWYV. Hewings and D'Arcy (1986) reported that purified preparations of a legume isolate of BWYV consisted not only the usual nucleoprotein particles but also particles made of the viral protein subunits but devoid of nucleic acid.

2.3 Host

Menon and Christudas (1963) identified *Costus* sp. as one of the hosts for the banana aphid, while the other collateral hosts of the vector include *Colocasia* and *Alocasia*, and in the absence of banana these hosts help in the multiplication of the vector. Rao (1977) found that *Amomum* sp. also harbour this vector.

Su *et al.* (1993) reported that *Hedychium coronarium* Koenig and *Canna indica* Linn. were also found to be the intermediate hosts of BBTV. They also reported that the concentration of the virus in cavendish banana plants showed seasonal fluctuation and varied with different virus strains.

2.4 Vector

The major vector is the banana aphid, *Pentalonia nigronervosa* Coq. The aphids are most numerous during the wet season. They are usually found around the lower part of the mat. When they are numerous they are found on the apex of the pseudostem, round the heart leaf and at the petiole bases. The aphid is mostly confined to the genus *Musa*. Generally the aphids may spread by contact transfer

from plant to plant by flight, by movement of aphid infected suckers and by translocation of soil on the tools, shoes and clothes of workers (Magee, 1927).

Banana bunchy top virus is not transmitted mechanically. The virus is transmitted by the banana aphid, *Pentalonia nigronervosa* Coq. In eastern U.P., presence of the aphid has been recorded by Khurana (1971). The vector is required to feed on the infected plant for about 17 h. The incubation period of the vector may range from 90 min to 48 h and vector remains infective for about 13 days. The aphid attacks the basal portion of the pseudostem, upper leaf sheaths and petioles. The colonies are commonly seen in the heart or crown of the plant which provide ideal conditions for feeding and protection.

Siddappaji and Reddy (1972) reported that the aphid occurring on banana was typical *P. nigronervosa* and the one on cardamom (*Elettaria cardamomum*) Salisb. and colocasia (*Colocasia esculenta* (L.) Schott. was *P. nigronervosa* var. *caladii* v.d. Goot. Colonies of brown aphids are found in the crown of the plant, around the base of the pseudostem and between the sheath of the outer leaf and the pseudostem. The aphids are often accompanied by ants (Wardlaw, 1972).

Tsai *et al.* (1986) reported that the populations of apterae of *P. nigronervosa* on banana suckers in southern Taiwan increased from October and decreased after February and were highest in December-January. The alate aphids appeared in banana fields in September-April. Banana plants infected with bunchy top disease were found mostly in July and August in the fields where suckers had been planted in March-May. About 69 per cent of the aphids migrated away from the sucker when detected in the mother plant before the leaves become dried out; the remaining were dead *in situ*. All aphids on unflowering banana plants were

killed when Furadan (carbofuran) was injected into the pseudostem, but aphids on suckers emerging from infected mother plants remained alive.

Yang (1991) studied the biology of *P. nigronervosa* a vector of BBTV and reported that the pest had 4 generations in a year having 4 nymphal instars. The peak population of the aphids were during April and September-October. The morphology of the aphids was also described.

The wingless aphids reproduce for 7-10 generations which is more than that of alate adults. These are similar to the wingless generation in colour and have prominent dark brown to black wing veins. The alate aphids are disposed to new host plants usually of *Musa* spp. (Basu and Giri, 1993).

2.5 Mechanism of transmission

BBTV is associated with BBTD. The virus is transmitted in two ways. Firstly, it is transmitted by the black banana aphid, *Pentalonia nigronervosa* coq., in a persistent manner. This mechanism of transmission has important implications for the epidemiology of the disease.

The second mechanism for the transmission of BBTD is through plant parts. Once a banana becomes infected, the virus moves through the entire plant and therefore any propagating material taken from an infected plant is most likely to be infected with BBTV. This include suckers, parts of the corm, and tissue culture material including explants and plantlets derived from explants.

Both of these mechanisms are extremely important for the spread of the virus. For instance, aphids very effectively move the virus from one banana to another within a plantation particularly within a growing season. Usually this

movement is only over a short distance (Allen, 1978b). However, aphids can be and have been, recorded as transmitting the virus over quite long distances but primarily within a region. Infected planting material is often responsible for the introduction of BBTV into a plantation; this is the most common mechanism of introduction into a region and is almost certainly the only mechanism for intercontinental movement of BBTV (Iskra, 1990).

Luteoviruses are transmitted in a persistent (Circulative, non-propagative) manner. Aphids acquire and transmit luteoviruses while feeding on phloem sieve tube elements of host plants. Minimum feeding access times reported for aphids to acquire or inoculate different luteoviruses range from 0.1-4.0 h and 0.2-1.0 h respectively (Cockbain and Costa, 1973; Elnagar and Murant, 1978). These reported times include the time required for the aphid's stylets to penetrate the phloem tissues. Efficient transmission of most luteoviruses requires acquisition and inoculation feeding access times each of 24 h. The minimum latent period (usually defined as the time from the start of the acquisition feeding period until the insect becomes able to infect plants with the virus) is normally between 12 and 24 h (Duffus, 1979; Elnagar and Murant, 1978).

Aphids that have acquired luteoviruses do not cease to transmit it after moulting and studies of Barley yellow dwarf virus (BYDV) transmission by *Rhopalosiphum padi* indicate that luteovirus particles pass by cellular transport through the hindgut into the aphid's haemocoel. The particles then circulate in the haemolymph throughout the aphid. Current evidences indicate that luteoviruses do not replicate in their vectors although Weidemann (1982) suggested that they may do so to a small extent, but aphids that have begun to transmit a luteovirus may continue to do so for 2-3 weeks without re-acquisition. Ultrastructural studies on BWYV, BYDV and PLRV suggested that they were transmitted following the endocytosis of virus particles from the haemocoel into the aphids accessory salivary

gland. These particles were then transported within coated vesicles through the salivary gland secretary cell and released by exocytosis into the salivary duct (Gildow, 1985; Gildow and Rochow, 1985; Gildow and Harris, 1987).

Wu and Su (1990a) conducted transmission of banana bunchy top virus by aphids to banana plantlets from tissue culture. When *P. nigronervosa* were incubated on diseased plantlets at 16° for one month and then transferred to healthy plants, the virus was not transmitted. Similarly, when *P. nigronervosa* were incubated on diseased plantlets at 16° for 2 or 24 h, all the plants developed bunchy top symptoms. Transmission rate of BBTV was directly correlated with the number of viruliferous aphids feeding on the plantlets and is inversely correlated with age of the host plantlets. For one month old plants from tissue culture, the shortest incubation period was 15 d with the conclusion that banana plantlets were ideal for transmission studies as they were easy to obtain in large quantity and required less space and time compared to suckers.

2.6 Virus-Vector relationship

A characteristic feature of the luteoviruses is their high degree of vector specificity. Each luteovirus is transmitted efficiently by only one or a few species of aphid. Vector specificity is believed to result from interaction between the particle protein and membrane surfaces in the aphids accessory salivary gland, these interactions regulate transport of virus particles to the salivary duct (Gildow 1983). Vector specificity is not absolute, however, and some luteoviruses are transmitted inefficiently by species other than the main vector. Transmission efficiency has also been reported to vary with the age of the aphid (Zhou and Rochow, 1984) and among distinct populations or clone of a single aphid species (Gildow and Rochow, 1985). Genetic variability of aphids, as well as of the viruses evidently influences vector specificity and transmission efficiency.

2.7 Vector control

Johnson (1963) and Sun *et al.* (1992) studied the biology and control of the aphid, *Pentalonia nigronervosa*. Wellings *et al.* (1994) reported the introduction and establishment of a polyphagous parasitoid *Aphidius colemani* Viereck into Tonga to control. *P. nigronervosa*, which is a serious pest of *Musa* spp. because of direct feeding damage and more importantly as a vector for banana. About 32,000 parasitoids were released at Tongatapu Island, Tonga between April 1990 and November 1991. The studies showed that *P. nigronervosa* was readily parasitized in the absence of ants (Formicide).

2.8 Disease control

Allen (1987) conducted studies on epidemiological factors influencing control of banana bunchy top disease and evaluation of control measures by computer simulation. He reported that the basic infection rate of BBTV in established plantings averaged 0.0342 new infections/ infectious plant daily, but varied seasonally with a maximum in summer.

A practice of removing apparently healthy plants within 5 m of plants detected with bunchy top disease symptoms in 5 or more leaves was found to locate 30 per cent of the remaining undetected infected when disease was first detected in a plantation. However, its use as a routine control measure was ineffective in reducing the number of inspection required to maintain control or in reducing the risk of the disease spreading to adjoining plantations. Removal of apparently healthy plants within 5 m had some bearing on disease control when applied around plants with symptoms on 2 leaves or less, but also caused a significant loss of healthy plants.

2.9 Purification of BBTV

Research progress on BBTD has been very slow in comparison with other serious virus diseases because of the inability to purify the virus, although numerous attempts have been made by many different workers (Dale *et al.*, 1986; Wu, 1987; Iskra *et al.*, 1989; Wu and Su, 1990a; Harding *et al.*, 1991 and Thomas and Dietzgen, 1991).

BBTV is considered to be a possible luteovirus by Matthews (1982) on the basis of its disease characteristics, its transmission in the persistent manner by aphids and by inducing phloem damage.

Dale *et al.* (1986) used the method similar to that of Morris and Dodds (1979) for the extraction of ds RNA from banana plants. Whole plants were harvested, frozen in liquid nitrogen and ground to a fine powder. Hundred gram ground tissue was shaken for 1 h at 4°C in 400 ml 40 mM Tris HCl pH 7.0, 100 mM NaCl and 1 mM EDTA (STE), 200 ml water saturated phenol containing 0.1%, 8-hydroxyquinoline, 200 ml chloroform, 50 ml 10% SDS and 5 ml 2-mercapto ethanol. The mixture was centrifuged at 7000_g for 15 min and the aqueous phase was collected. Ethanol was added to it while stirring to give a final concentration of 15%. Whatman CF-11 cellulose was then added (0.15 g/g of original tissue) and the suspension was stirred under vacuum for 15 min at room temperature. The suspension was then poured into a chromatography column and washed with STE containing 15% ethanol for each gram of tissue extracted. Double stranded RNA was then eluted by washing with STE alone and precipitated by adding 2.5 vol. ethanol and kept at -20°C overnight. The precipitated ds RNA were collected by centrifuging at 10000_g for 30 min, dried in vacuum and resuspended in sterile distilled water.

The method of Rowhani and Stace-Smith (1979) used for purification of potato leaf roll virus was modified and used for extraction and purification of BBTV by Wu and Su (1990a). Diseased leaf tissues were sliced into small pieces and were placed in liquid nitrogen for 1 min and pulverized in a coffee grinder for 1 min. The pulverized powder was mixed with 0.1 M potassium phosphate buffer, pH 7.4 containing 0.2% (v/v). 2-mercaptoethanol and 0.1% (w/v) sodium diethyl dithiocarbamate and stirred for 30 min at 10°C. The mixture was filtered through two layers of cheese cloth and chloroform:n-butanol (1:1) was added gradually to 10% (v/v). The mixture was emulsified for 1 h with a magnetic stirrer, incubated for 10 min at 4°C and centrifuged at 7,000 rpm for 10 min. The supernatent was centrifuged in a Beckman 45 T rotor at 36,000 rpm for 2.5 h and the resulting pellet was resuspended in 0.07 M phosphate buffer, pH 7.2. The suspension was stirred for 2 days, then stored for 2 days at 4°C. The suspension was subsequently clarified by low speed centrifugation at 7,000 rpm for 10 min and the virus was concentrated by high speed centrifugation at 36,000 rpm for 2.5 h. The pellet was resuspended in 0.07 M phosphate buffer, pH 7.2 (2 ml/100 g tissue). The virus was further purified by sucrose density gradient centrifugation (2.5-30% w/v) in a Beckman SW 28-1 rotor at 26,000 rpm for 3.5 h. The tube contents were fractioned and collected with a fraction collector. Ultraviolet absorption at 260 nm of each fraction was determined with a spectrophotometer and those containing virus were pooled and diluted with three volumes of 0.07 M phosphate buffer, pH 7.2, centrifuged at 36,000 rpm for 3 h and the pellet was resuspended in the same buffer (1/600 volume of the original extract). The ultraviolet absorption spectrum (200-400 nm) of the virus preparation was made in a spectrometer. The virus concentrations in purified preparations were estimated using an extinction coefficient ($E_{260}^{0.1\%}$) of 8.6 (Takanami and Kubo, 1979).

Thomas and Dietzgen (1991) purified BBTV from midrib and petiole tissue according to a modification of the method of Su and Wu (1989). Tissue was

sliced into pieces less than 1 cm in diameter, immediately placed into liquid nitrogen, then pulverized in a commercial coffee blender and processed immediately or stored at -70°C for up to 12 months. The powder was thawed in 2 vol. extraction buffer (0.2 M potassium phosphate pH 7.4, containing 0.5% w/v Na_2SO_3) and then stirred for 30 min at 5°C. The extract was filtered through cheese cloth and the fibres were reextracted with one volume of extraction buffer, using a mortar and pestle and acid-washed sand. Chloroform butanol (1:1, 0.1 vol.) was added gradually to the combined filtrates, the mixture was stirred for 1 h at 5°C and then centrifuged at 8000_g for 10 min. The aqueous phase was centrifuged at 170000_g for 90 min. The pellets were resuspended in 0.07 M sodium phosphate pH 7.2 (PB) (at least 1 ml/5 g original tissue), stirred for 2 days and then allowed to stand for a further 2 days at 5°C. The extract was then clarified by centrifugation at 8000_g for 10 min prior to ultracentrifugation at 270000_g for 60 min. Pellets were resuspended in about 1 ml PB/100 g original tissue, layered on to 10 to 40% sucrose density gradients in PB and centrifuged at 70000_g for 4 h. The zone containing virus particles was located and removed using an Isco density gradient fractionator and U.V. monitor, diluted in PB and concentrated by ultracentrifugation. Virus particles were further purified by equilibrium centrifugation in CS_2SO_4 (3 ml gradient of initial density of 1.325 g/ml, overlayered with paraffin oil) at 35000 rpm (Beckman SW 41 Ti rotor) for about 20 h. The virus zone was located with a vertical beam of light and removed by puncturing the side of the tube with a needle. The virus preparation was diluted in PB and concentrated by ultracentrifugation and the final pellet was resuspended in a small volume of PB. Alternatively, after sucrose density gradient centrifugation, some preparations were centifuged in gradients of 30 to 60% (w/v) Nycodenz (Nycomed) at 55000 rpm (Beckman TLS 55 rotor) for 3 h at 15°C. The gradients were fractionated by carefully removing 100 µl aliquots from the top. Virus concentrations were estimated assuming $E_{260} = 3.6$, based on that of subterranean clover stunt virus (SCSV) (Chu and Helms, 1988).

2.10 Electron microscopy

Kang (1984) reported that thin sections of plants infected by BBTV contained virus - like particles (20-22 nm in diameter) in phloem parenchyma cells, although these particles were indistinguishable from ribosomes. Double-stranded ribonucleic acid (ds RNA) has also been extracted from BBTV infected bananas but not from comparable healthy plants (Dale *et al.*, 1986). Wu (1987) treated BBTV infected banana tissues with RNase to eliminate ribosomes and found distinct virus - like particles (20-22 nm in diameter) in groups or scattered in cytoplasm of phloem parenchyma cells. These particles were not detected in similarily treated healthy tissue. Iskra *et al.* (1989) detected isometric virus particles of 28 nm diameter in BBTD afected plants. The method he tried was reproduceable and gave 66-170 µg purified virus/kg infected leaves. Wu and Su (1990a) isolated and purified BBTV from banana seedlings of Giant cavendish (*Musa acuminata* Colla) naturally infected with BBTV. Harding *et al.* (1991) purified virus like particles of 18-22 nm from banana midrib tissues as described by Su and Wu (1989) and Wu and Su (1990a). Thomas and Dietzgen (1991) isolated isometric virus - like particles of 18 nm in diameter from banana (*Musa* spp.) affected by BBTD in Australia.

2.11 Gel electrophoresis of nucleic acid and protein

Dale *et* al. (1986) analysed nucleic acid of BBTV by electrophoresis in polyacrylamide gels consisting of a 10% resolving gel and a 5% stacking gel using the buffer system of Laemmli (1970) but with SDS omitted from all buffers. The gels were electrophoresed at 150 V for 16 h and then stained either with ethidium bromide (50 µg/ml for 10 min) or with silver nitrate (Merril *et al.*, 1981). Molecular weights of the different ds RNA species were estimated by comparison with the ds RNA of sugarcane Fiji disease virus (FDV) (Reddy *et al.*, 1975) and ds RNAs

extracted from *Helminthosporium maydis*, *Penicillium chrysogenum* and *Penicillium stonoliferum*.

Double stranded RNA extracts from equal fresh weights of healthy banana plants and those with bunchy top disease were compared by electrophoresis. A number of ds RNAs were present in extracts from infected and not from healthy plants. These ds RNAs were very low in concentrations and would only be detected when the total ds RNAs obtained from 75 to 100 g infected tissue were electrophoresed in one lane of a 0.75 mm thick gel. Four major ds RNA bands were present in all seven different extracts of infected bananas harvested within 5 days after symptoms had arisen. The largest of these BT-1, had a molecular weight of 4.4×10^6 with BT-2 at 1.35×10^6 and a pair of bands at 0.50×10^6 (BT-3) and 0.48×10^6 (BT-4). Two less-intense bands, BT-x (Mol. wt. 0.86×10^6) and BT-y (Mol. wt. 0.69×10^6) were discernible in four of the seven extracts. Other very minor bands were present in some BBTD ds RNA preparations.

The double stranded RNA was proven by incubating ds RNA preparations with ribonuclease A (1 µg/ml) in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), or in 2 x SSC or with deoxyribonuclease (10 µg/ml) in 50 mM Tris HCl pH 8.0 and 10 mM $MgCl_2$. The nucleic acids were incubated with the nucleases at 37°C for 1 h and then proteinase K was added to a final concentration of 20 µg/ml. Incubated them for 30 min and shaken with phenol : chloroform (1:1) and centrifuged. The ds RNAs of FDV were used as controls. All bands both major and minor thought to be BBTD specific ds RNA were resistant to RNase in 2 x SSC and DNase but not to RNase in 0.1 x SSC ,demonstrating that these bands were ds RNA. There was some high molecular weight material in the BBTD ds RNA control but this was digested by RNase in 0.1 x SSC and 2 x SSC but not by DNase suggesting that this material was single-stranded RNA.

Wu and Su (1990a) isolated nucleic acid of BBTV as per the method of Hewings and D'Arcy (1986). BBTV was incubated in TMS dissociation buffer consisting of 0.1 M Tris HCl, pH 8.0, 5% 2-mercaptoethanol and 4% sodium dodecyl sulphate at 37°C for 1 h. Electrophoresis was carried out in 1.5% agarose cylindrical gels at 2 mA per gel for 280 min. After electrophoresis, the gels were incubated in a solution containing 0.5 µg/ml of ethidium bromide for 1 h, and destained in deionized water for another 1 h before examination with UV light. The type of BBTV nucleic acid was determined by Morris and Dodds (1979) method. The gel was incubated in a citrate buffer (0.03 M sodium citrate, pH 7.0) containing 0.3 M Nacl and 50 µg/ml RNase (Sigma, St. Louis MO) for 1 h before examination with UV light. TMV RNA and DNA markers were used as controls.

After incubation in TMS dissociation buffer and agarose gel electrophoresis, BBTV and TMV each showed a prominent band of nucleic acid with similar mobility, indicating that the Mr of BBTV RNA was about 2.0×10^6. There was a faint diffuse of faster migrating material on each gel attributable to fragments of RNA. BBTV and TMV RNAs were digested by treatment of gels with RNase in 0.3 M NaCl solution, although the DNA marker was unaffected with the confirmation that the RNA of BBTV is single stranded.

Wu and Su (1990a) determined the relative molecular mass (Mr) of BBTV coat protein subunits by SDS-12% polyacrylamide gel electrophoresis using the system of Laemmli (1970). Samples were prepared by the addition of an equal volume of 2x 'treatment buffer' consisting of 0.125 M Tris-Hcl, pH 6.8, 4% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 10 M urea and 0.001% bromophenol blue, to either BBTV or standard protein solutions (Bio-Rad Laboratories, Rockville Center, N.Y.) followed by incubation in boiling water for 3 min. Electrophoresis of samples was carried out in a dual slab cell (Bio-Rad Laboratories, Richmond, CA). The gels were stained in a solution consisting

of 0.25% coomassie brilliant blue, 10% (v/v) acetic acid and 50% (v/v) methanol and destained in a solution of 7% (v/v) acetic acid and methanol. The Mr of BBTV coat protein was estimated by interpolation from plots of the Mr of the standard proteins against their relative mobilities in the gel. Standard proteins and their Mr were lysozyme, 14,300, lactoglobulin, 18,400, α-chymotrypsinogen, 25,700, ovalbumin, 43,000, bovine serum albumin, 54,000, phosphorylase b, 97,400, myosin (H-chain), 200,000.

After dissociation and electrophoresis of BBTV, the viral protein showed a single major band with Mr 21,000.

Harding *et al.* (1991) extracted nucleic acid of BBTV with a slightly different method. Virus preparations in 0.07M sodium phosphate buffer pH 7.2 (PB) were mixed with an equal volume of 0.02M Tris HCl, 0.001M EDTA and 4% SDS, pH 9.0 and incubated at 60°C for 15 min. The mixture was then emulsified with an equal volume of phenol:chloroform (1:1), centrifuged and the aqueous phase was reextracted with an equal volume of chloroform. Nucleic acids were precipitated with 70% ethanol and resuspended in sterile distilled water. Nucleic acid preparations were incubated at 37°C for 1 h with (i) DNase (0.2 µg/µl Boehringer Mannheim (BM) in 50 mM Tris HCl, 10 mM $MgCl_2$, pH 8.0, (ii) RNase (20 µg/µl, BM) in water or (iii) S1 nuclease (1 unit/µl, BM) in 0.2M NaCl, 50 mM sodium acetate, pH 4.5, 1 mM $ZnSO_4$.

Nucleic acids were electrophoresed in 1 to 1.5% agarose gels in Tris-acetate-EDTA buffer pH 7.8 (Maniatis *et al.*, 1982), and visualized by ethidium bromide staining. For Mr estimation, nucleic acids were denatured with glyoxal and electrophoresed in 1% agarose gels as described by McMaster and Carmichael (1977). A 0.24 to 9.5 Kb RNA ladder (Bethesda Research Laboratories) and a Bg/l/Hint I digest of $_p$BR 328 DNA (BM) were used as Mr standards. Proteins were

denatured in 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and analysed in discontinuous SDS - polyacrylamide gels. The separated proteins were stained with silver nitrate (Blum *et al.*, 1987). A low Mr protein calibration (pharmacia) was used for Mr determinations.

Agarose gel electrophoresis showed that extracts from sucrose gradient purified VLPs contained a single low Mr band and diffuse stained material of lower Mr. Nucleic acid extracted from caesium sulphate purified VLPs was also analysed with or without prior treatment with nucleases by agarose gel electrophoresis. The untreated sample contained discrete low Mr band and in some extracts, a small amount of lower Mr material. This discrete band was digested by DNase I and S1 nuclease but not digested by RNase A confirming the nucleic acid of BBTV as ssDNA of about 1 kb and one major protein of Mr 20100.

Thomas and Dietzgen (1991) extracted nucleic acid of BBTV as described by Francki and Randles (1973) in 1% (w/v) agarose Tris-borate-EDTA buffer pH 8.3 and visualised by staining with ethidium bromide. CMV RNAs and 0.24 to 9.5 Kb RNA ladder (BRL) were used as markers. Total RNA was extracted from healthy banana midrib tissue as described by Rezaian *et al.* (1983). Digestion with 1 µg/ml of boiled ribonuclease A (Sigma) or 10 µg ml DNase I (Promega Biotec RQI DNase) were carried out in 0.01 M Tris HCl pH 7.6 containing 0.01 M NaCl, 0.006 M $MgCl_2$, 0.1 M $CaCl_2$ and 100 µg/ml proteinase K (Sigma) for 30 min at 37°C (Tullis and Rubin, 1980). Digestion with 50 units/ml of S1 nuclease was in 0.033 M sodium acetate pH 4.5, containing 0.3 M Nacl and 0.001 M $ZnSO_4$, for 30 min at 37°C. Subsequently, samples were extracted with phenol : chloroform (1:1) and the nucleic acid was precipitated from the aqueous phase with 2 vol. of ethanol and 1/20 vol. of 4 M sodium acetate pH 6.0.

Purified BBTV preparations were denatured in 3% (w/v) SDS at 5% (v/v) 2-mercaptoethanol and analysed in discontinuous 12% SDS-PAGE. After electrophoresis the separated proteins were either visualized by staining with silver nitrate (Wedrychowski *et al.*, 1986) or transferred electrophoretically to nitrocellulose membrane (0.45 µm pore size, Bio-Rad) after which the blot was incubated in PBS overnight at 37°C. Skim milk (5% w/v) in rinse buffer was used for blocking. Immunoblots were probed with BBTV MAb F10 (1:5 diluted culture supernatent) or polyclonal antiserum (1:1000) and processed as described by Deitzgen and Francki (1988) using nitroblue tetrazolium phenazine methosulphate-5-bromo-4-chloro-3-indolyl phosphate as an alkaline phosphatase substrate.

Nucleic acid extracted from purified BBTV preparations migrated as a discrete band of about 1.0 kb when compared to ssRNA size markers. This band was found sensitive to digestion by DNase I and S1 nuclease but not to RNase A. Single stranded DNA of about 1 kb as well as ssRNA smaller than 0.45 kb was found associated with the particles. A single coat-protein of Mr 20500 was identified with antibodies to BBTV particles from Australia.

Wu (1994) reported two circular ssDNAS associated with BBTD. Nucleic acids extracted from partially purified BBTV consisted of 20 Kb DNA, 0.9-1.1 Kb DNA and 0.3 Kb RNA. Partially purified BBTV preparations predigested with DNase and RNase before particle disruption and nucleic acid isolation yielded only the 0.9-1.1 Kb DNA, but no corresponding nucleic acid band was obtained in total nucleic acid isolated from healthy banana tissue. Analysis of 2 BBTV cDNA clones showed that clone 1 consisted of 287 nucleotides and clone 2 contained a 1.0 Kb DNA insert. Clone 1 is not part of clone 2. When 2 pairs of primers, each pair in opposite orientation, were used to amplify BBTV DNA by PCR using the total DNA from diseased banana tissues or DNA encapsidated in

BBTV particles as the template, a DNA product of 1.1 Kb was generated by both indicating that the BBTV DNAs are circular. Additional results suggested that BBTV contained 2 circular ssDNAs designated BBTV cssDNA I (containing clone 1 nucleotide sequence) and BBTV cssDNA II (containing clone 2 nucleotide sequence).

Wu *et al.* (1994) studied the nucleotide sequence of two circular single stranded DNAs associated with banana bunchy top virus. Two circular single stranded DNAs designated DNA I and II of the BBTV genome were cloned and sequenced. The sequence of BBTV DNA I consisted of 1106 nucleotides and contained 4 open reading frames (ORFs) potentially encoding proteins with molecular masses ranging from 5.4 to 33.18 KDa and 1 putative stem loop structure of 29 nucleotides. The sequence of BBTV DNA II had 1096 nucleotides and contained 4 ORFs encoding putative proteins with sizes ranging from 5.6 to 32.77 Kda and 1 putative stem-loop structure of 32 nucleotides. The largest ORF (V_2) in DNA II encoded a putative replicase with the nucleotide triphosphate-binding motif (GGEGKS) DNAs I and II had a 54.3% identity in total sequence and 2 conserved regions with >80% sequence identity. The 2 BBTV DNA sequences showed no close relationship with gemini virus DNA sequences but some similarity with the coconut foliar decay virus (CFDV) DNA sequence (0.39% identity). DNAs I and II showed considerable similarity with BBTV component 1, with 50.3 and 52.9% identity respectively. The loop sequences of putative stem loop structure in both BBTV DNAs were highly homologous and also had considerable similarity with those of gemini virus, CFDV DNAs and BBTV component 1.

Yen *et al.* (1994) reported the genome characterization and identification of viral-associated dsDNA component of BBTV. The single stranded (ss) and double stranded (ds) viral-associated DNAs (VADs) of BBTV were characterized. The ss VADS were shown to be the viral genomic DNA and the ds VADS are the

likely replication forms. Both ds VADs and ss VADs contain DNA species that cross-hybridize to each other. Under EM both circular and linear DNAs were observed from these VAD species. Libraries were constructed from VADs to further characterize them. Two overlapping VAD clones were isolated and their combined sequence indicated that their corresponding VAD, named as BBTV component 2, is circular with a size of 1095 nt. The largest ORF spans from nucleotides 82 to 390, which is sandwiched by a putative TATA box, 54 nt upstream from the first ATG codon, and a AATAAA motif, 7 nt downstream from the UAA codon. Although the sequence of this VAD is different from the previously reported BBTV component 1 and the genome of CFDV stretches of highly homologous sequences were found between BBTV component 2 and the other 2 viral genomes by computer analysis. The sequence of BBTV component 1 is highly homologous to one of the clones which hybridized with ss viral genomic DNAs but not with PBTs 26 and BBTV component 2, indicating that the viral genome consists of 72 components.

An additional 5 circular ss DNA components of BBTV which were designated components 2, 3, 4, 5 and 6 were cloned, sequenced and analysed by Burns *et al.* (1995). These components were present in all BBTV infections tested. Of these components 4 components (3, 4, 5 and 6) had 1 large ORF in the virion sense located 3' of a stem-loop structure. Each ORF had a potential TATA box and 1 or 2 potential polyadenylation signals associated with it and each polyadenylation signal had an associated GC-rich region containing the trinucleotide sequence TTG. A number of ORFs were identified in component 2 but none of these had appropriately located potential TATA boxes and polyadenylation signals associated with them. None of the ORF amino acid sequences nor the functional DNA sequences of any of the components had significant sequence identity with any known protein or nucleic acid sequences. However, the ORF of component 4 encoded a 30 residue hydrophobic domain which suggest that this ORF encoded a

transmembrane protein. Further the ORFs of components 3 and 5 potentially encoded proteins of C.20 KDa the size of the BBTV coat protein. There were 2 regions of sequence identity between the 5 components described and the previously described component 1. Each component contained a conserved stem-loop structure and a non nucleotide potential TATA box which was 5' of the virion (CR-SL) of 69 nucleotides which was 62% identical between components. All 6 BBTV components also contained a major common region (CR-M) which was located 5' of the CR-SL in each component, in the non-coding region and was 76% identical over 92 nucleotides. Each CR-M contained a near complete 16 nucleotide direct repeat and a GC-box which was similar to the right ward promoter element found in wheat dwarf gemini virus.

According to the authors, it was concluded that BBTV belonged to an undescribed plant virus group which could also include subterranean clover stunt virus, coconut foliar decay virus, faba bean necrotic yellows virus and milk vetch dwarf virus.

The Hawaiian isolate of BBTV was purified from infected banana cv. Williams by Xie and Hu (1995). Three ssDNA components named 1, 3 and 4 were cloned and sequenced. Components 1 was 1110 nucleotides in length and shared 98% nucleotide sequence identity with the BBTV DNA component 1 of the Australian isolate. This component contained 2 ORF capable of encoding a protein of 33.5 KDa, which may function as 2 replicase, and a protein about 15.2 KDa, with unknown functions. Component 3 was 1057 nucletodies in length and did not contain any ORFs of >10 KDa. Component 4 was 1017 nucleotides in length and potentially encoded a protein of 18.9 KDa. All 3 ss DNA components shared the same stem-loop sequence and had a conserved noncoding region. The sequence of each of these 3 components was different from that of BBTV DNA components of 2 Taiwanese isolates. BBTV-specific clones were used in dot-blot hybridization

assays for detection of BBTV in plants using radioactive and nonradioactive probes. A polymerase chain reaction (PCR) assay was developed for detection of BBTV in banana samples and single aphids. Dot-blot hybridization assays were as sensitive as ELISA, while PCR was 1000 times more sensitive than dot-blot and ELISA assays for detection of BBTV in banana.

2.12 Antisera

Antiserum to BBTV was produced in a Newzealand white rabbit following two intramuscular (150 µg in 0.3 ml or day 0 and 10 µg in 0.3 ml on day 21) each emulsified with an equal volume of Freund's adjuvant. Blood taken 20 days after the last injection was used throughout. The antiserum titre was determined by gel diffusion (Thomas, 1984). Immunoglobulin (Ig) and IgG purification and preparation of alkaline phosphatase conjugates was essentially as described by Clark and Adams (1977). The sources of other antisera were: Taiwanese BBTV MAb 3D12 and 2H6 and 3D13 alkaline phosphatase conjugate (Wu and Su, 1990b) and Australian BBTV MAb F10 (Thomas and Dietzgen, 1991).

2.13 Serological methods

Serological methods was attempted by Rajagopalan (1980) for the detection of virus infection in plants. He succeeded in developing the serum containing polyclonal antibodies and tested the antigenic properties of the virus by tube precipitation test and gel diffusion methods. But according to him these methods involved large quantity of antiserum.

2.14 ELISA

Two forms of ELISA were conducted with either Nunc Masinch or Dynatech M1 29B microtitre plates. The double antibody sandwich (DAS) form of direct ELISA was essentially as described by Clark and Adams (1977). Coating was with polyclonal Ig at 3 μg/ml or MAb 3D12 at 0.5 μg/ml. Banana leaf tissue and aphids were prepared in purification extraction buffer while purified virus preparations were diluted in extraction buffer or phosphate buffer. For routine detection, banana tissue was extracted at the rate of 1 g/4 ml. BBTV polyclonal Ig-alkaline phosphatase conjugates (1:500) or MAb 3D12 conjugate (1:4000) were incubated in 0.01M phosphate buffered 0.8% (w/v) saline pH 7.4 (PBS) containing 0.05% (w/v) Tween 20 (PBST), 2% (w/v) polyvinyl pyrrolidone (PVP) and 1% (w/v) skim milk powder. In the plate trapped antigen (PTA) form of indirect ELISA, antigen was bound to plates in the presence of 0.05M carbonate buffer pH 9.6. Bound antigen was detected with 1 to 10 μg/ml of BBTV MAb 2H6 in PBST-PVP. Rabbit anti-mouse IgG conjugated with alkaline phosphatase in PBST-PVP was used at a dilution of 1:1000 to 1:2000. ELISA reactions were considered positive when the A_{405} value was greater than the mean plus two standard deviations of appropriate control samples.

Micropropagated banana plants (cv. Cavendish) all originally derived from one source and shown to be free of BBTV by DAS-ELISA were used for the studies. Groups of 20 aphids, five either infective or non-infective colonies were caged on individual plants for an inoculation access period of 48 h. The aphids were then removed from the plants and assayed for BBTV by DAS-ELISA (coating with polyclonal Ig, MAb 3D12-alkaline phosphatase conjugate). After inoculation as each new leaf emerged it was checked for BBTD symptoms and then the apical half was sampled and stored at -70°C prior testing by DAS-ELISA (coating with polyclonal Ig, polyclonal IgG and MAb 3D12-alkaline phosphatase conjugation.

According to Wu and Su (1990b) purified BBTV was used for the production of antiserum, two intraperetonial injections 2 weeks apart were made on 6-8 weeks old Balb/ mice, each with 15-26 μg purified BBTV in 0.15 M saline solution mixed with the same volume of complete Freund's adjuvant. One week after the second injection, the mice were given a final injection of the same amount of purified virus without adjuvant in the tail vein. Three days later they were killed and their spleen were removed aseptically for cell fusion. The cell fusion and hybridoma cloning were performed following the procedure described by Hsu *et al.* (1994) except that the myeloma cell line NS-1 was used. The cell suspensions of spleens of BBTV immunized mice were mixed with myeloma-cell suspensions at the ratio of 5:1 in cell number, and centrifuged at 1000 rpm for 10 min. Cell fusion was achieved by cropping 1 ml of prewarmed 50% PEG (M.W.1500) in RPMI-1640 medium (R medium) to the pellet over a period of 45 sec in the centrifuge tube in a water bath at 37°C with gentle agitation. After incubation for 75 sec and dilution with R medium, the suspension was centrifuged. Cells in the pellet were resuspended in HAT medium (R medium supplemented with foetal bovine serum, Na-pyruvate, L-glutamine, penicillin, streptomycin, hypoxanthine, aminopterin and thymidine) distributed in 96-cell culture plates, and incubated in Co_2 incubators. One week later, HT medium (HAT medium without amino protein) was added. Once established, the hybridomas were grown in R-S medium (R medium supplemented with 15% foetal bovine serum, 1 mM Na-pyruvate, 1 mM L-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin).

Hybridomas secreting specific antibody were selected using plate trapped antigen (PTA) ELISA (indirect ELISA) as described below and cloned by limiting dilution to single cell. Immunoglobulin biotype of monoclonal antibodies was determined by the agar gel double diffusion test. Each glass slide was layered with 3 ml of 1% Bacto agar with 0.1% sodium azide, 1 central and 4 surrounding wells on the gel were made using a gel punch. Filtrates from cell cultures were added to

the central well and antisera to mouse IgG1, IgG2a, IgG3 (Zymed Labs, South San Fransisco, CA 94080) were added to the surrounding wells. Immunodiffusion was done at 10°C and result recorded after 2 days.

To produce antibodies in ascitic fluid, Balb/mice were primed intraperetonially with Pristane (2, 6, 10, 14-tetramethyl pentadecane) 10 days before intraperetoneal injection of 10^7 cells of hybridoma. The ascitic fluid was removed within 2 weeks, and centrifuged at 3,000 rpm for 20 min. For purification of Ig from cell culture supernatent fluid or ascitic fluid, the ammonium sulphate precipitation method was applied, DEAE-cellulose column chromatography of the Ig was performed according to the method described by Johnston and Thorpe (1982), except that PBS was used as the starting buffer. The protein yield was calculated from A_{280} assuming $E_{280}^{0.1\%} = 1.4$ and the antibody activity was determined by PTA ELISA.

Plate trapped antigen ELISA was used to detect antibody-producing hybridoma purified virus antigen (Wu and Su, 1990b) diluted to a concentration of 0.5-1.0 µg per ml of 0.05 M carborate buffer, pH 9.6. Partially purified preparation from healthy tissue was concentrated to 1/10 of the original volume, mixed with the same volume of carbonate buffer and used as a healthy control. Antigen coating was carried out by adding 50 µl of antigen preparation to each well of the 96 well microtitre plates. After an incubation of overnight at 4°C, plates were rinsed 6 times with PBS-Tween (PBS with 0.05% Tween 20), and 30 µl of 0.2% bovine serum albumin (BSA) in carbonate buffer was then added to each well. After incubation for 2 h at 37°C, plates were rinsed as before. For detecting antibody production by hybridomas, 50 µl of supernatent of hybridoma culture was added to a well (Dietzgen and Sander, 1982). Diluted antiserum was used as positive control and supernatent of myeloma cell culture as negative control. After incubation and rinsing as before, 50 µl of alkaline phosphatase-labelled goat anti-mouse

immunoglobulins IgG and IgM (Zymed Labs) in diluted PBS-Tween supplemented with 0.1% BSA was added to each well and the plates were incubated at 37°C for 2 h. Following final rinsing the wells were treated with 100 µl of 0.2% p-nitrophenyl phosphate (Sigma) in a solution containing 0.02% NaN_3 and 9.7% diethanol amine, pH 9.8 for 1 h at 37°C. The A_{405} nm of each well as measured with a Bio-Tek ELISA plate reader.

For detection of BBTV in diseased tissues, 1 g of banana leaf tissue was ground in a mortar with 2 ml of 0.1M potassium phosphate buffer pH 7.4 containing 0.2% (v/v) 2-mercaptoethanol and 0.1% (w/v) sodium diethyldithiocarbamate. The mixture was centrifuged at 7000 rpm for 10 min and resultant supernatent was used as the plant crude extract. Virus preparations purified as described previously (Wu and Su, 1990a) to the step before sucrose density gradient centrifugation were used as partially purified BBTV, while those purified to the step after density gradient centrifugation were used as purified BBTV. When the PTA ELISA was used to detect BBTV in infected banana tissues, plates were coated with antigens in crude extracts, partially purified or purified virus preparations, and the purified Ig produced by hybridoma 2H6-Mc5 at the concentration of 10 µg/ml was substituted for cell culture supernatent fluids. Antibody trapped antigen (ATA) ELISA (also known as direct ELISA) was also used to detect BBTV in infected banana tissues. The microtitre plates were precoated with 200 µl of purified anti BBTV Ig (2H6-Mc5) at the concentration of 5-10 µg/ml. The immunoglobulin coated wells were used to trap BBTV from plant crude extract, partially purified or purified preparations. Alkaline phosphatase-labelled anti BBTV Ig (2H6-Mc5) was diluted to 1/400-1/1600 of stock before use.

Mariappan and Mathikumar (1992) prepared partially purified virus as per the method of Gordan and Nault (1977) with slight modification. This partially purified virus was used for immunizing the rabbit. The antiserum was prepared by

following the methods of Hobbs *et al.* (1987). The serum obtained was cross absorbed and concentrated by dialysis. The serum containing immunoglobulin was tested by ELISA methods.. Among the several methods of ELISA tested, the direct antigen coating (DAC) method has been found to be quite suitable in detecting the presence of virus even at a very high dilution of 1:1000. The OD value at 405 nm for the dilutions of 10^{-3} for antigen and 1:500 for antiserum has been found to be high (0.412) for infected outermost sheath covering the rhizome as against 0.004 to 0.007 in healthy.

Zhang *et al.* (1995) conducted an indirect ELISA for the detection of banana bunchy top disease with monoclonal antibody of BBTV. Leaf sap from diseased plants reacted positively with the MAb when diluted to 1:1280, while sap from healthy plants reacted negatively. Samples of test tube seedlings and of field plants in Guangdung and Guangxi showed 8% and 76.5% infection respectively. The virus was systemically distributed in diseased plants, with similar concentration in the midribs and leaf blades. The concentration of BBTV in older leaves with moderate symptoms was lower than that in younger leaves with obvious symptoms.

Materials and Methods

MATERIALS AND METHODS

The present studies on banana bunchy top virus were carried out during 1993-98. The experiments were conducted at the Biochemistry laboratory and the Centre for Plant Biotechnology and Molecular Biology of College of Horticulture, Vellanikkara, Microbiology Department of College of Veterinary and Animal Sciences, Mannuthy, Kerala Forest Research Institute, Peechi and Indian Institute of Science, Bangalore.

3.1 Survey, collection and maintenance of bunchy top infected plants

During 1994-96, survey was conducted to study the symptomatology and to record the incidence of BBTD and aphid population in the major banana growing areas of Thrissur district in Kerala. Observations were taken from six locations where six plots were selected in each location. A total number of 15,000 Nendran variety plants were observed and the percentage of disease incidence was calculated. Various symptoms of BBTD and aphid populations in these area were also recorded during the survey. Diseased plants were collected randomly from the field based on symptom variation and were planted both in pots and in the field of College of Horticulture, Vellanikkara.

The virus has been maintained in the glass house by time to time inoculation on tissue culture plants of Nendran through the vector banana aphid, *Pentalonia nigronervosa* Coq., for various experiments.

3.1.1 Maintenance of test plants

Tissue culture plants, rhizome buds (100-250 g weight), 3-4 months old suckers were planted in small pots and polythene bags to maintain the test plants for the various studies.

3.1.2 Collection and rearing of vector

Banana aphids are mainly seen in the heart or crown of the plants, in the outersheath of the basal portion of the pseudostem, on the axils of the petiole and on the lower side of the leaf midrib. Aphids were collected from the healthy banana plants. The insects were gently tapped and disturbed and then collected with the help of a camel hair brush in Petri dishes lined with moistened filter paper. These aphids were then released into the pseudostem portions or to the leaf axils of the healthy plants, grown in glass house condition for multiplication. Field collected aphids were initially screened through transmission test to detect disease inoculum carried by them, if any. Disease free aphids thus reared in the glass house were used for the transmission studies.

3.2 Transmission studies

3.2.1 Transmission through planting material

Suckers were collected from both infected and healthy plants during three seasons such as August-November, December-March and April-July and planted in pots of size 75 cm x 30 cm. These plants were sprayed with insecticide and kept in the glass house for 1-2 months for symptom expression.

3.2.2 Mechanical transmission

Mechanical transmission was conducted with infected leaf extract which is prepared by grinding the midribs of young leaves of bunchy top affected plant with suitable buffer (2 ml/g tissue). This inoculum was rubbed on to the upper surface of the leaves of banana and also on *Hedychium coronarium* Koenig, *Canna indica* Linn and *Colocasia esculenta* (L.) Schott. which are reported to be the hosts of BBTV, using carborundum powder as abbrassive. Excess inoculum was washed

away with tap water and inoculated plants were kept in insect proof glass house for the symptom appearance. Plants inoculated with healthy leaf extract served as control. The following buffers were used for the transmission studies

1. 0.1M sodium phosphate pH 7.2
2. 0.1M sodium phosphate pH 8.0
3. 0.1M sodium phosphate + 1% 2-mercaptoethanol pH 7.2
4. 0.1M sodium phosphate + 1% 2-mercaptoethanol pH 8.0
5. 0.01M sodium phosphate pH 7.2
6. 0.01M sodium phosphate pH 8.0
7. 0.01M sodium phosphate + 1% 2-mercaptoethanol pH 7.2
8. 0.01M sodium phosphate + 1% 2-mercaptoethanol pH 8.0
9. Deionised water pH 6.5
10. Distilled water pH 7.0

3.2.3 Insect transmission

Insect transmission studies were conducted with the nymphs of banana aphid. Banana plants showing conspicuous bunchy top symptoms were used as the source plant for the acquisition. Twenty five disease free nymphs were collected and allowed to feed on infected plant for 24 h to acquire the virus. After the acquisition the viruliferous aphids were collected and released on to three months old tissue culture plants, rhizome buds, 3-4 months old suckers and 5-6 months old plants of Nendran for inoculation access for 24 h. These aphids were then killed by spraying with 0.1% Quinalphos 25 EC. Aphid transmission was also conducted on *Hedychium*, *Canna* and Colocasia at four leaf stage in the same manner and were kept in the insect proof glass house for symptom expression. Back inoculation was done to confirm the findings.

3.3 Virus-vector relationship

3.3.1 Relative efficiency of single nymph and adult on the transmission of BBTV

Nymphs and adults of banana aphids (25 each) were allowed to feed on infected plant for 24 h. After the acquisition access, adults and nymphs were released singly and separately on different test plants mentioned earlier for inoculation feeding for 24 h and plants were sprayed with insecticide and kept for further observation.

3.3.2 Effect of number of infective aphids on the transmission of BBTV

In order to find out the effect of number of infective aphids on the transmission of BBTV, three months old tissue culture plants were inoculated with viruliferous nymphs and adults (25 each), which had been given 24 h access to the source plant. Adults and nymphs were employed singly or in batches of 3, 5, 10, 20, 30, 50, 75 and 100 on the test plants for 24 h. Inoculated plants were kept under observation after spraying with 0.1 per cent Quinalphos 25 EC.

3.3.3 Effect of pre-acquisition fasting on transmission of BBTV

Group of 25 nymphs and adults of banana sphids were starved at different intervals of 0, 10 min, 1, 3, 6 and 12 h. After the fasting, adults and nymphs were released separately on to the infected plant for acquisition. After the acquisition of 24 h, each group of adults and nymphs were released on to healthy tissue culture plants to test the infectivity of the vector. Inoculated plants were sprayed with insecticide and kept in the glass house for symptom expression.

3.3.4 Effect of acquisition access period on the percentage of transmission of BBTV

Disease free nymphs and adults were released on infected plants seperately to have acquisition access periods of 10 min, 30 min, 1, 3, 6, 12, 18 and 24 h. The time was recorded as soon as the insects settled and seemed to attain feeding posture. Nymphs and adults were then removed and transferred to test plants at the rate of 25 insects per plant and allowed to feed for 24 h. The inoculated plants were kept in the glass house and examined regularly for symptom appearance.

3.3.5 Effect of inoculation access period on the percentage of transmission

Viruliferous aphids (nymphs and adults) in group of 25 were given an acquisition access period of 24 h on source plant and were allowed different inoculation access periods lasting 10 min, 30 min, 1, 3, 6, 12, 24 and 48 h on three months old tissue culture plants. The inoculated plants were kept in the glass house for symptom expression after spraying with insecticide.

3.3.6 Incubation period of virus in the vector

Nymphs and adults of *P. nigronervosa* were given 24 h acquisition access to the diseased plants separately. A group of 25 aphids were transferred regularly at intervals of 30 min, 1, 3, 6, 9 and 12 h to the test plants. The inoculated plants were examined for the symptom appearance.

3.4 Purification of BBTV

Materials

BBTD samples

Healthy samples

Liquid N

Potassium phosphate buffer (0.2M pH 7.4)

Sodium sulfite

Mortar and pestle

Acid washed sand

Chloroform

Butanol

Sucrose (Molecular grade)

Procedure

Attempt was made to purify BBTV from field infected and aphid inoculated plants according to Thomas and Dietzgen (1991). Fresh tissues (midribs, petiole, leafsheath and rhizome) of diseased plants were used for the study. Healthy plant tissues served as control. Tissues (100 g) were sliced into small bits and immediately placed into liquid N, and were extracted with 2 vol. of extraction buffer (0.2M potassium phosphate pH 7.4, containing 0.5% w/v Na_2SO_3) using a precooled mortar and pestle with acid washed sand. The extract was stirred for 30 min at 5°C. It was filtered through muslin cloth and the fibres were re-extracted with one volume of extraction buffer. Chloroform : butanol (1:1, 0.1 vol.) was added gradually to the combined filtrates. The mixture was stirred for 1 hour at 5°C and then centrifuged at 8000_g for 10 min. The aqueous phase was centrifuged at $1,70,000_g$ for 90 min. The pellets were resuspended in 0.07M sodium phosphate buffer (PB) pH 7.2 at least 1 ml/5 g original tissue), stirred for two days and then allowed to stand for further two days at 5°C. The extract was then clarified by

centrifugation at 8000_g for 10 min, prior to ultracentrifugation at $2,70,000_g$ for 60 min. Pellets were resuspended in about 1 ml phosphate buffer/100 g original tissue. It was then subjected to density gradient centrifigation. The gradient columns were prepared by layering 7, 7, 7, 7 and 7 ml of 10, 20, 25, 30 and 40 g/100 ml of sucrose in phosphate buffer, 0.07M and then stored overnight at 4°C before use. Five ml of virus preparation was layered on each gradient column and centrifuged at 70000_g for 4 h in SW60 Ti rotor. The light scattering zone was not obtained and the fraction containing virus particles was located and removed using a pasteur pipette and stored at 4°C for the serological and EM studies. The concentration of virus was estimated at 260 and 280 nm on U.V. monitor.

Purification of BBTV from different sources (tissue culture plants, 3-4 months old suckers and rhizome buds) and from different portions in various seasons were also attempted. Efforts were also made to purify BBTV from midribs of leaves from different positions.

3.4.1 Infectivity assays of BBTV

Purified BBTV from density gradient fractions were resuspended in 20% sucrose and placed between two parafilm membranes (American Can Company, Marathon, Wisconsin). The aphids were allowed to feed through the membrane for 24 h. Afterwards, they were released to tissue culture plants (20 aphids/plant) and allowed to feed for 24 h. Exposed plants were sprayed with 0.1% Quinalphos 25EC.

3.4.2 Determination of concentration of purified virus

The purified virus from diseased plant was used for the U.V. absorption studies in a spectrophotometer. The final pellet dissolved in 1 ml buffer was diluted to 1:10, 1:50, 1:100, 1:500 and 1:1000. The U.V. absorption of 1:10 dilution was

taken from 200-340 nm at 20 nm interval. In the other dilutions the U.V. absorption at 260 nm and 280 nm were recorded. The concentrations of the purified virus in 1 ml was calculated by using the formula A_{260} x dilution factor assuming an extinction coefficient of ($E_{260}^{0.1\%}$) of 3.6 based on that of subterranean clover stunt virus (SCSV) (Chu and Helms, 1988). The A_{260}/A_{280} ratio was also calculated for the purified virus.

$$\text{Concentration of the purified virus} = \frac{A_{260} \text{ x dilution factor}}{\text{Extinction coefficient}}$$

3.4.3 . Particle morphology of BBTV using electron microscopy

The purified virus preparations were negatively stained with 1% (v/v), Ammonium molybdate pH 5.8 and 2% aqueous Uranyl acetate and viewed in a Jeol 100c X II transmission electron microscope operating at 80 KV. Grid preparation and particle size measurements were done as described by Hitchborn and Hills (1965) and Thomas (1986). An ocular micrometer was used to measure the diameter of isometric particles in millimeter which was converted into the equivalent value of nanometer. It was divided by the corresponding magnification to get the size of a virus particle (Basu and Giri, 1993).

$$\text{Size of virus particle} = \frac{\text{Diameter in mm x } 10^6}{\text{Magnification}}$$

3.5 Estimation of nucleic acids of banana plants

The nucleic acid estimation method followed was as described by Schneider (1945) and Ogur and Rosen (1950). The methods involved the following properties of nucleic acids.

1. Insolubility of nucleic acids in cold perchloric acid
2. Insolubility in various organic solvents like alcohol and ether
3. Ultraviolet absorption spectrum of nucleic acids
4. Property of RNA to be hydrolysed in cold PCA while DNA is not
5. Specific reaction of RNA and DNA with Orcinol and Diphenylamine

Materials

1. Refrigerated centrifuge
2. Mortar and pestle
3. Perchloric acid (PCA)
4. Ethanol
5. Ether

The samples, both healthy and bunchy top affected plants (1 g) were weighed first and then homogenised in 10% perchloric acid (PCA) at 0°C in a glass homogeniser. Centrifuged the homogenate at 5°C for 15 min at 8000_g and the extracts were discarded. The residue was resuspended in cold 5% PCA and centrifuged again. The supernatent was discarded. The residue was then sequentially washed with 70% ethanol, 95% ethanol and finally with ethanol:ether (3:1) twice and then with cold 0.2N PCA, centrifuged immediately for 10 min and the residue was suspended in cold 2N PCA and stored at 5°C for 18 h. The suspension was centrifuged and the supernatent was collected. The residue was again washed with cold 2N PCA. After centrifugation the two supernatents were combined and made up the volume to 20 ml with distilled water. This part will contain RNA fraction and samples from this supernatent could be used for quantitative estimation of RNA. The residue left was then suspended in 1N PCA and heated at 70°C for 20 min and then centrifuged. The supernatent was retained. The residue was again extracted with hot 1N PCA. The two supernatents were

combined and made up the volume to 20 ml. This portion comprised DNA fraction and could be used for the estimation of DNA.

RNA and DNA in PCA extracts were estimated by measuring absorption at 260 nm. The nucleic acid contents were calculated on the basis of values of Ep (extinction per mole of phosphorous), 10,800 at 260 nm for RNA and 8780 at 260 nm for DNA. DNA was also estimated by diphenylamine reaction (Burton, 1956; Abraham *et al.*, 1972) and RNA by Orcinol reaction.

3.5.1 Determination of DNA by diphenylamine method

DNA content was determined by employing the diphenylamine test for deoxyribose.

Reagents

Diphenylamine	- 1.5 g
Glacial acetic acid	- 100 ml
Conc. H_2SO_4	- 1.5 ml
Aqueous acetaldehyde	- 16 mg/ml

Dissolved 1.5 g of diphenylamine in 100 ml of glacial acetic acid and added 1.5 ml of Conc. H_2SO_4 and stored the diphenylamine reagent in the dark. On the day of use, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent required.

Method

1. Took 1 ml of PCA extract
2. Added 2 ml of diphenylamine reagent
3. Kept them at room temperature for 16 h in the dark
4. Determined the absorbance at 595 nm

3.5.2 Orcinol reaction for RNA

Quantitative estimation of RNA by measuring pentose sugar.

Reagent A

1. 1 g orcinol
2. 375 mg $FeCl_3$ 6 H_2O
3. Diluted to 25 ml with water
4. Cooled them in an ice bath at 4°C

Reagent B

500 ml of concentrated HCl to 100 ml water

5. Added 475 ml HCl reagent B
6. Stored in a dark bottle in a freezer

Three ml of butanol:pyridine:water (4:1:1) was added to remove the precipitation. The tubes were shaken and centrifuged and used the upper phase for assay.

3.6 Isolation of DNA from banana plants

DNA was isolated from diseased and healthy plants as per the procedure of Hattori *et al.* (1987).

Materials

Extraction buffer pH 8.0

Tris HCl (0.1M)	15.76 g
EDTA (0.05M)	18.612 g
SDS (1%)	10 g
Distilled water	1 L

TE Buffer pH 8.0

Tris HCl (10 mM)	1.576 g
EDTA (1 mM)	0.3723 g
Distilled water	1 L

All the buffers were sterilized by autoclaving.

Procedure

1. Midribs, petiole, leafsheath and rhizome portions of diseased and healthy plants were taken for DNA isolation. Hundred gram sample was ground in liquid nitrogen with the aid of a sterilised mortar and pestle. Fifty ml of extraction buffer was added and freezed the sample with liquid nitrogen. This step was repeated until a homogenous paste was obtained.
2. Extraction buffer was added to a final volume of about 200 ml (2 ml/g) and transferred to sterile polypropylene centrifuge tubes.
3. Extracted with 40 ml phenol centrifuged at 10000_g for 10 min at 4°C and separated the aqueous and phenol phases.
4. The phenol phase was back extracted with 40 ml extraction buffer.
5. The two aqueous phases were combined and extracted with (a) 40 ml of phenol and centrifuged. Phenol phase was removed and added. (b) 40 ml of Phenol:sevag (chloroform:isoamyl alcohol 24:1) in a separating funnel, centrifuged and phenol phase was removed. Then added (c) 20 ml of diethyl ether.
6. The ether from the aqueous phase was chased by placing the centrifuge bottles in a waterbath at 60°C for 10-15 min.
7. Mixed with insoluble PVP (500 mg) for a few minutes at room temperature and centrifuged at 10000_g for 10 min.
8. Added 5 M NaCl to a final concentration of 0.5 M and PEG 6000 to 10% (w/v). The solution was left overnight at 4°C.

9. The pellets were collected by centrifugation at 10000_g for 10 min at 4°C. The centrifuge tubes were drained thoroughly and removed PEG.
10. The pellets were precipitated with 70% ethanol and centrifuged at 8000_g for 20 min at 4°C.
11. Drained the centrifuge tubes thoroughly and resuspended them in a small volume of TE buffer (5 ml) and read at 260 and 280 nm.
12. Enzymes DNase free RNase and RNase free DNase (50 µg/ml) were added to the sample and incubated for 30 min at 37°C.
13. 20 µl of the samples, both diseased and healthy along with marker DNA (lambdaphage DNA digested with Hind III) was loaded on a minigel in TAE buffer (IX) and checked quickly for the presence and size of DNA.
14. The gel was placed over a U.V. transilluminator and photographs taken.

3.7 Extraction of nucleic acid of BBTV

Materials

Proteinase K (Sigma)

SDS (5%)

Phenol

Chloroform:isoamyl alcohol (24:1)

Potassium acetate

Ethyl alcohol

TE Buffer

Tris HCl (0.02M)

EDTA (0.001M)

Magnesium chloride (10 mM)

Tris HCl (50 mM)

NaCl (0.2M)

Sodium acetate (50 mM) pH 4.5

$ZnSO_4$ (1 mM)

DNase I (Promega Biotec RQI DNase)

RNase A (Sigma)

S1 Nuclease (Sigma)

1. 500 µl of purified virus preparations were treated with 50 µl proteinase K and 50 µl SDS (5%).
2. Incubated at 37.5°C for 1 h.
3. Centrifuged with 300 µl saturated phenol and 300 µl chloroform:isoamyl alcohol (24:1) at 8000_g for 10 min.
4. Aqueous phase was taken and the phenol phase removed.
5. Repeated the phenol, chloroform:isoamyl alcohol centrifugation at 8000_g for 10 min thrice.
6. 50 µl potassium acetate and 100 µl ethanol was added.
7. Kept in the refrigerator for 1 h or overnight.
8. Centrifuged at 8000_g for 10 min.
9. Ethanol was removed and drained well.
10. Pellet was taken and added 100 µl TE buffer.
11. Nucleic acid preparations were incubated at 37°C for 1 h with (i) DNase (0.2 µg/µl (BM) in 50 mM Tris HCl, 10 mM $MgCl_2$, pH 8.0), (ii) 1 µg/µl boiled RNase A, (iii) S1 nuclease (1 unit/µl, BM) in 0.2M NaCl, 50 mM sodium acetate pH 4.5, 1 mM $ZnSO_4$.

3.8 Gel electrophoresis of nucleic acid of BBTV

Materials

1. Electrophoretic tank with tray and comb
2. Power pack
3. Microwave oven/hot plate
4. UV transilluminator or UV torch

5 Micropipette

6. Microfuge tubes, tips etc.

7. Agarose

8. Ethidium bromide

9. TAE buffer

10. Bromophenol blue

Stock solution

1. TAE (50 X)

Tris base	- 242 g
Glacial acetic acid	- 57.1 ml
0.5 M EDTA (pH 8.0)	

Made up to 1000 ml with distilled water

2. Ethidium bromide - 10 µg/ml in distilled water

3. Loading dye (6 X)

Bromophenol blue - 0.25%

Ficoll 400 0.15% in distilled water

Protocol

1. Sealed the edges of an electrophoresis gel tray with adhesive tape and placed on a levelled surface. Fixed the comb at one end of the tray.
2. Weighed agarose (1.2%) in a conical flask
3. Added IX TAE buffer to the required percentage (w/v)
4. Boiled for 2 min on a hot plate or for 1 min in microwave oven and swirled gently for dissolving the agarose
5. Cooled to 50°C

6. Added Ethidium bromide from stock solution to a final concentration of 0.5 µg/ml gel. Mixed thoroughly by swirling
7. Poured into the gel tray with the comb and allowed the gel to set
8. After gelling, added a few ml of IX TAE buffer and removed the comb gently and placed the gel tray in an electrophoresis tank containing IX TAE buffer after removing the adhesive tape
9. Loaded 20 µl of the sample after mixing with the loading dye. Marker DNA was also loaded in one side of the gel
10. Closed the lid of the tank and electrophoresis was carried out at 90-100 v
11. Removed the gel tray when the dye reached at the aniodic end of the gel
12. Examined the gel under UV light in a transilluminator and recorded the observations

3.9 Characterization of proteins by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (PAGE-SDS)

The method of PAGE described by Laemmli (1970) and Bock *et al.* (1977) was used in the study with slight modifications.

Materials

1. Antigen: 1. BBTV purified in 10-40% sucrose gradient, (2) Midrib, petiole, leafsheath and rhizome portions of diseased and healthy plants.

2. 2X sample buffer

Tris base (0.125M)	0.15 g
Distilled water	4 ml
pH was adjusted to 6.8 with HCl	
10% SDS	4 ml

10% Glycerol	1 ml
2-mercaptoethanol	0.4 ml
Bromophenol blue	20 mg

Made up the volume to 10 ml with distilled water

3. Acrylamide stock

Acrylamide	30 g
Bisacrylamide	0.8 g

Made upto 100 ml with distilled water, filtered and stored in a dark bottle at 4°C.

4. 2X Stacking gel buffer

Tris base (0.25M)	3 g
Distilled water	90 ml

pH adjusted to 6.8 with HCl

10% SDS	4 ml

Made upto 100 ml with distilled water.

5. 4X Resolving gel buffer

Tris base (1.5M)	16.95 g
Distilled water	90 ml

pH adjusted to 8.8 with HCl

10% SDS	4 ml

Made upto 100 ml with distilled water.

6. 10X Reservoir buffer stock

Tris base (0.25M)	30.25 g
Glycine	144 g
Distilled water	850 ml

pH	8.3
10% SDS	100 ml

Made upto 1000 ml with distilled water.

7. Coomassie brilliant blue stain

Coomassie brilliant blue (R-250)	0.2 g
Methanol	50 ml
Acetic acid	10ml
Distilled water	40 ml

The stain was filtered through Whatman No.1 filter paper before use.

8. Destaining solution

Destaining solution I (for rapid destaining)

Methanol	250 ml
Acetic acid	50 ml
Distilled water	200 ml

Destaining solution II (for rehydration and storage)

Methanol	25 ml
Acetic acid	50 ml
Distilled water	425 ml

9. Molecular weight markers (Sigma)

Carbonic anhydrase	29,000
Ovalbumin	45,000
Bovine serum albumin	66,000

Protocol

1. PAGE plates were cleaned well first in water followed by 10% SDS, water, ethanol and water. The cleaned plates were air dried.
2. Prepared the stopping gel by mixing 3.5 ml of acrylamide, 1.25 ml of resolving gel buffer, 5 ml of distilled water, 35 µl of TEMED (N, N, N-tetramethyl diamine) and 1.3 ml of 1.5% ammonium persulphate.
3. The cleaned plates were attached to each other and separated by lightly greased spacer. The bottom (2 cm) of the plates were then sealed by pouring the stopping gel and allowed to polymerize.
4. Mixed 20 ml of 30% acrylamide/bisacrylamide with 15 ml of 4x resolving gel buffer and 100 µl of 1.5% ammonium persulphate and 30 µl of TEMED. The volume was made upto 60 ml with distilled water to give 10% gel.
5. The resolving gel was then poured into the prepared plates without making any air bubbles. Then a few millimeters of 2X stacking gel buffer was gently layered on top to form a flat surface and the gel was allowed to polymerize.
6 After 1 h the upper unpolymerized liquid was poured out. A top stacking gel was prepared by mixing 1 ml of 30% acrylamide/bisacrylamide, 4 ml of distilled water, 5 ml of 2X stacking gel buffer, 10 µl of 1.5% ammonium persulphate and 5 µl TEMED. Enough of this stacking gel mixture was poured to fill the upper 3 cm of the gel. Then the sample position forming comb was inserted and the stacking gel was allowed to polymerize. The stacking gel was added to sharpen the bands and to increase the resolution.
7. The gel was then attached to the electrophoresis unit (LKB). Then 500 ml of 1X reservoir buffer stock was added to both the top and bottom chambers of the unit and the leak, if any, was checked.
8. The sample wells were cleaned by pipetting the reservoir buffer in and out of each.

9. 20 µl samples were separately mixed with equal quantity of 2X sample buffer. The samples were then heated to 95°C for 3 min in a steaming water bath and applied on the gel with a micro-syringe. A similarly treated mixture of molecular weight markers was also included in the run.
10. The gel was then run at a constant voltage of 100 V until the dye front was near the stopping gel (6 h).
11. The gel was carefully removed from the glass plates and stained overnight in Coomassie brilliant blue and destained in first destaining solution. The destaining solution II was used to destain and rehydrate the gel.

Calculation of molecular weight of proteins

The molecular weight of proteins was calculated by plotting the log molecular weight of the markers against their mobility (measured in mm) in the gel. Similarly the mobility of the unknown proteins was also measured. The corresponding log molecular weight was found out from the graph and the molecular weight of unknown proteins was calculated.

3.10 Estimation of proteins of banana plants

Protein content in diseased and healthy plants were estimated by Lowry's method (Lowry *et al.*, 1951) and expressed in percentage.

Reagent A

Sodium carbonate 2% in 0.1N sodium hydroxide.

Reagent B

Copper sulphate ($CuSO_4.5H_2O$) 0.5% in 1% potassium sodium tartarate.

Reagent C

Reagent A 50 ml and Reagent B 1 ml was mixed prior to use.

Reagent D

Folin ciocalteus' phenol reagent was diluted to 1:2 (reagent:water).

Stock standard

Weighed accurately 50 mg of Bovine serum albumin (Fraction V) and dissolved in 0.1N NaOH and made up to 50 ml.

Working standard

Diluted 10 ml of the stock solution to 50 ml with 0.1N NaOH in a standard flask and 1 ml of this solution contains 200 µg protein.

Procedure

To this supernatent 3 ml of 10% Trichloroacetic acid (TCA) was added. Centrifuged for 20 min and poured out the supernatent. To the protein which was precipitated down, 5 ml of 0.1N NaOH was added which dissolved the protein and this solution was used for the estimation of protein.

Pipetted out 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard into a series of test tubes and sample extracts were added to each tube. Made up the volume to 1 ml in all the test tubes and a test tube with 1 ml of distilled water served as blank. Added 5 ml of reagent C to each tube including the blank. Mixed well and were allowed to stand for 10 min. Reagent D 0.5 ml was added and mixed well and were incubated at room temperature in the dark for 30 min. Blue colour was developed and the reading was taken at 660 nm using a UV Vis spectrophotometer. A standard graph was drawn and calculated the amount of protein in the sample and expressed in percentage.

3.11 Serological studies

Materials required

Virus sample

Freund's adjuvants, complete and incomplete (Difco)

Merthiolate, 1:100 stock in distilled water

Phosphate buffered saline, 0.01M pH 7.2

Agarose, SRL

Sodium azide, AR

Sodium chloride AR

Microscopic slides

Whatman filter paper No.1

Amido black stain

Amido black 10B - 1 g

Sodium acetate - acetic acid buffer - 1000 ml

(0.2M, pH 3.6)

Decolourizer I

Methyl alcohol - 45 ml

Glacial acetic acid - 10 ml

Distilled water - 50 ml

Decolourizer II

Ethyl alcohol - 40 ml

Glacial acetic acid - 10 ml

Distilled water - 50 ml

3.11.1 Production of antiserum

1. Injected rabbits intramuscularly with 2 ml of antigen incorporated with Freund's adjuvant (Difco)
2. Given totally 3 injections at weekly intervals, Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant for the subsequent injections.
3. Seven days after the last injection, test bleed the rabbits from the ear vein and separated the serum. Then tested for its antibody level.
4. If sufficient concentration of antibodies were detected, bled rabbits intracardially.
5. After separation of the serum, it was clarified and stored at - 20°C with merthiolate to a final concentration of 1:10,000.

3.11.2 Determination of antibody titre

3.11.2.1 Agar gel diffusion test (AGDT)

Two fold dilutions of antiserum viz., 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 and 2048 were prepared in 0.01M potassium phosphate buffer pH 7.2

containing 0.14M sodium chloride (PBS). Agar gels were prepared in petri dishes by pouring 15 ml of 0.85 per cent agar prepared in PBS. Sodium azide was added to the molten agar to a final concentration of 0.02 per cent to prevent fungal and bacterial contamination. At the centre of each petriplate, wells were cut in the agar with the help of a gel cutter with 6 tubes 3 mm in diameter at 4 mm apart. The bottom of the wells were smeared by pouring a drop of molten agar. 20 µl of antiserum dilutions were placed in the outer wells and 20 µl of purified virus (concentration 1 mg/ml) was put in the centre well. The petriplates were then incubated at 37°C for diffusion of antigen and antiserum.

Staining

Following the required incubation period of 12 h, the slides were dipped in normal saline for 24 h with frequent changes and subsequently in distilled water for about 24 h. After this period, the gels were dried by placing a sheet of filter paper over the surface of the gel and kept at 37°C for 24 h. After complete drying, the filter paper was removed and cleaned the slides for few seconds in running water to remove the adhering particles of filter paper. Stained the slide in amido black stain for about 15 min. Washed the slides in decolourizer I. Then destained the slide using decolourizer II until the background was decolourized. When complete decolourization was obtained, dried the slides and recorded the observations.

3.11.2.2 Ring interface precipitin test

Antibody titre was also determined by ring interface precipitin test as described by Reddy and Black (1966). Two fold dilutions of antiserum was prepared in PBS as mentioned in the double diffusion test. To each dilution glycerin was added to a final concentration of 30 per cent. Afterwards the serum dilutions

were placed in the bottom of the serological tubes (4 mm inside dia and 40 mm length) at the rate of 200 µl per tube. Holding the tubes at a sharp angle, approximately 30° from horizontal, 100 µl antigen (purified virus at a concentration of 1 mg/ml) was carefully layered without mixing with the antiserum dilutions at the bottom of each tube. Glycerin was added to the antiserum to increase its density so that it could support the antigen without mixing. The tubes were then incubated at room temperature and observations were taken at every 1 h interval for the development of precipitate in the form of a ring at the junction of antiserum and antigen.

3.11.3 Antigen detection

3.11.3.1 Chloroplast agglutination test

A drop of virus infected sap squeezed through a muslin cloth and diluted with 0.01M potassium phosphate buffer pH 7.2 containing 0.14M sodium chloride was mixed with same amount of homologous antiserum with a tooth pick on a clean washed microscopic slide. Where ever virus was present, chloroplasts in the sap aggregated together forming a precipitate which became very distinct in transmitted light or observed under a steriobinocular microscope for agglutination. If the reaction was delayed, the slide was incubated at 25°C for 5 min and gently warmed. Similarly 1:10, 1:50, 1:100, 1:500 and 1:1000 dilutions of the purified isolates were mixed separately with the antiserum on microscopic slides. Normal rabbit serum and buffer were mixed in each case as control in all the tests conducted.

3.11.3.2 Tube precipitin tests

The diseased and healthy plant sap used was centrifuged in separate tubes at 7,000 rpm for 15 minutes. The preciptin tube tests were conducted in thick

walled serological tubes of 1 cm in diameter. Various two fold dilutions each of antiserum and normal serum as well as of the antigen (healthy and diseased plant sap extracted as in the first experiment of purified virus preparations) were prepared in 0.85 per cent saline (0.14M NaCl). One ml of antiserum was mixed in serological tubes with an equal volume of antigen. The tubes were shaken and placed immediately in an electrically operated, temperature controlled, serological water bath at 37.5°C. The tubes were observed after 15 min, 30 min, 1 h, 2 h, 4 h and 6 h. Tests with normal serum and healthy plant sap served as control.

3.12 Enzyme Linked Immunosorbent Assay (ELISA)

Indirect method

Materials

1. Coat buffer
2. Wash buffer
3. Extraction buffer
4. Conjugate buffer
5. Substrate buffer
6. PBS-Tween
7. 3M NaOH
8. Polystyrene Microtitre plates (Dynatech Laboratories)
9. BSA fraction V/Alkaline phosphatase (ALP) type VII-S Sigma No.P-5521.
10. Polyvinyl Pyrrolidone MW 44,000 (BDH chemicals)
11. Photometer for measuring absorbance at 405 nm.

Reagents

1. Purified immunoglobulin, diluted in coating buffer
2. Enzyme labelled antirabbit immunoglobulin (Genei, Bangalore) diluted in PBS - Tween containing 2% PVP + 0.2% ovalbumin

3. Enzyme substrate, p-nitrophenyl phosphate (Sigma Chemicals), 0.6 mg/ml in substrate buffer
4. Test and control samples extracted or diluted in PBS - Tween containing 2% PVP

Samples

1. BBTV infected banana
2. Apparently healthy looking banana plants (Field collection)
3. Healthy banana

One gram of infected portion from different samples Viz., midrib, petiole, leafsheath and rhizome were extracted in 10 ml PBS-T-PVP separately and diluted, 10^{-1} to 10^{-5}. The extracts were taken with the help of mortar and pestle.

Buffers

1. Coat buffer (freshly prepared every time)

0.05M carbonate buffer pH 9.6

Sodium carbonate - 1.59 g

Sodium bicarbonate - 2.93 g

Distilled water to 1 L

pH adjusted to 9.6

2. Stock buffer

Phosphate buffered saline (PBS) pH 7.4 (IX)

Sodium chloride 8.0 g

Disodium hydrogen phosphate 1.44 g

Potassium dihydrogen ortho phosphate 0.20 g

Potassium chloride 0.20 g

Sodium azide 0.20 g

Distilled water to 1 L

Stored at 4°C

3. Wash buffer

Phosphate buffered saline -Tween (PBS-T) pH 7.4

PBS - 1000 ml

Tween-20 - 0.50 ml

Stored at 4°C

4. Tissue Extraction buffer

PBS-T-PVP pH 7.4

PVP 40 - 20 g

PBS-T - 1 L

Polyvinyl pyrrolidone (MW 40,000) - 20.0 g

Stored at 4°C

5. Enzyme conjugate buffer

PBS-T-PVP-BSA

PBS-T-PVP - 100 ml

Bovine serum albumin (BSA) - 0.20 g

6. Substrate buffer

Diethanol amine buffer pH 9.8

Diethanol amine - 97 ml

Sodium azide (NaN_3) - 0.20 g

Distilled water to 1 L

Adjusted pH to 9.8 with 1N Hcl (approx. 6.7 ml)

Covered the bottle with aluminium foil and stored at room temperature in the dark.

7. Stopping solution (freshly prepared solution)

NaOH 3 g in 25 ml

3.12.1 Purification of Immunoglobulins (IgG) from antiserum

Reagents

PBS(IX)

Ammonium sulphate

Antiserum

Double distilled water

Protocol

1. To 1 ml of antiserum of BBTV, added 9 ml of double distilled water.
2. Added 10 ml of saturated Ammonium sulphate solution (7.67 g Ammonium sulphate dissolved in 10 ml of water at 25°C gave a saturated solution)
3. Stirred and left at room temperature for 2 h
4. Centrifuged at 10,000 rpm for 15 min and discarded the supernatent
5. Dissolved the precipitate in 2 ml 0.5 x PBS
6. Dialysed against 0.5 x PBS for 24 h with four changes of buffer
7. Stored the dialysed solution in eppendorf tubes at 4°C

3.12.2 Preparation of DEAE cellulose columns

1. Took 1 g of DEAE cellulose (Sigma-D 8382) in a beaker and added 20 ml of double distilled water.

2. Transferred to a funnel and washed with 1M NaOH, double distilled water, 0.5N HCl, distilled water, and made sure that the pH of the elute is same as that of water. Washed the filter cake with about 25 ml of 0.5X PBS.
3. Prepared a column using a 10 ml pipette cut at the top. Plugged the bottom with fine glass wool and connected the tubing with a stop cock valve. Packed the column with washed DEAE cellulose slurry carefully to avoid trapping of air bubbles. Equilibrate the column with four bed volumes of 0.5X PBS. Adjusted the flow rate of the column to about 20 ml/h. Never allowed the column to get dry. The column could be stored at 4°C for a few days.

3.12.3 Fractionation of antiserum

1. Brought the column to room temperature. Loaded carefully 2 ml of the salt fractionated antiserum on the top of the column. Followed it with 0.5X PBS. For running the sample, reduced the flow rate to about 6 ml/h.
2. Collected 20 fractions of 0.5 ml each in eppendorf tubes placed on an ice bucket.
3. Bound proteins from the column can be eluted off using 2 bed volumes of 2M NaCl in 0.5X PBS. The column was then washed with 0.5X PBS regenerated and again washed with 0.5X PBS containing 0.02% sodium azide. This column was stored at 4°C.
4. The fractions had to be evaluated for the presence of IgG protein.
5. Absorbance readings were taken in spectrophotometer at 280 nm and adjusted to 1 mg/ml in 0.5X PBS (1 mg protein/ml has O D 1.4 at 280 nm) 0.5X PBS could used as blank.

3.12.4 Indirect ELISA Procedure

The indirect ELISA employed was similar to that described by Barbara and Clark (1982).

1. Added 200 µl aliquots of BBTV extracted in PBS-T-PVP to microtitre plates, covered the plates and incubated overnight at 4-6°C.
2. Washed the plates by flooding with PBS-T and soaked for 3 min. Repeated the wash twice then emptied the plates and removed the residual liqud.
3. Added 200 µl aliquots of purified anti BBTV immunoglobulin diluted in PBS-T-PVP-BSA to each well. Covered the plates and incubated for 3 h at 37°C.
4. Repeated washing as in step 2
5. Added 200 µl aliquots of anti-rabbit immunoglobulins conjugated with the enzyme, alkaline phosphatase to each well. Covered the plates and incubated for 3 h at 37°C.
6. Repeated washing as in step 2
7. Added 200 µl of enzyme substrate, p-nitrophenyl phosphate and incubated the plates for 30 min at room temperature.
8. Added 50 µl of 3M NaOH to stop the reaction
9. Agitated to ensure thorough mixing. Plates read at 405 nm. Yellow colour indicated positive reaction.

Results

RESULTS

Purification of banana bunchy top virus (BBTV) transmitted by the banana aphid (*Pentalonia nigronervosa* coq.), identification of nucleic acid of the virus, electron microscopy of the purified virus preparation for the determination of particle morphology of the virus and production of antisera for the presymptomatic detection of the virus infection in banana plants besides the basic studies on virus-vector relationship were done and the results are presented under the following heads.

4.1 Survey, Collection and maintenance of bunchy top infected plants

4.1.1 Symptomatology

4.2 Transmission studies

4.2.1 Transmission through planting materials

4.2.2 Mechanical transmission

4.2.3 Insect transmission

4.3 Virus-vector relationship

4.3.1 Relative efficiency of single nymph and adult on the transmission of BBTV

4.3.2 Effect of number of infective aphids on the transmission of BBTV

4.3.3 Effect of Pre-acquisition fasting on transmission of BBTV

4.3.4 Effect of acquisition access period on the percentage of transmission of BBTV

4.3.5 Effect of inoculation access period on the percentage of transmission of BBTV

4.3.6 Incubation period of virus in the vector

4.4 Purification of BBTV

4.4.1 Infectivity assays of BBTV

4.1 Survey, collection and maintenance of bunchy top infected samples

A survey was conducted to assess the incidence of banana bunchy top disease from the most important banana growing areas of Thrissur district of Kerala during 1994-'95. The symptoms on infected banana were recorded while collecting the diseased samples. During the course of the survey the per cent of BBTD incidence and aphid population were recorded and the data are presented in Table 1. The data revealed that banana bunchy top disease incidence and aphid population varied with the seasons. The maximum incidence of BBTD (6.5%) and aphid population (205nos.) was observed during August-November. Thereafter both disease incidence and aphid population were found to be decreasing tremendously. The disease incidence and aphid population during this period

Table 1. Incidence of BBTD and aphid population on Nendran banana during 1994-95

Period	District	Place	Per cent incidence of BBTD	Aphid* population
August-November	Thrissur	Marakkal	1.4	102
		Panenchery	2.2	105
		Mannuthy	4.5	180
		Puthur	5.0	205
		Kunnamkulam	5.0	165
		Chalakudy	6.5	150
December-March	Thrissur	Marakkal	0.3	12
		Panenchery	0.6	15
		Mannuthy	1.5	20
		Puthur	2.0	18
		Kunnamkulam	1.5	12
		Chalakudy	0.5	8
April-July	Thrissur	Marakkal	0.8	18
		Panenchery	0.6	23
		Mannuthy	1.9	27
		Puthur	2.2	25
		Kunnamkulam	2.3	21
		Chalakudy	2.5	17

*Average aphid population on 150 plants for one year.

(December-March) were 0.3-2.0% and 8-20% respectively. However, slight increase in aphid population was observed during April-July.

4.1.1 Symptomatology

The initial symptoms were the appearance of a few dark green flecks on the petiole of the youngest emerged leaf. The petiole flecking became more pronounced on all subsequent leaves and these flecks often coalesced to form dark green streaks (Fig.1).

Marginal yellowing of the lamina became evident on the third or fourth leaf. Subsequent leaves showed marginal yellowing and became progressively smaller and narrower. These leaves showed dark green streaking of the midrib and dark green flecks and streaks on the leaf blade.

The leaves of the infected plants became small, rosetted, profusely streaked and possessing highly chlorotic margins slightly rolled upward. Growth of the plant was slow; fruits were rarely developed and if produced, they were not ripened. The plants remained in the field for 2 to 3 years with 3 or 4 generations.

The opening bunches were constricted or 'choked' by the pseudostem and would split instead of emerging in the normal fashion. The bunches were reduced in size resulting in the reduction of the quality and market value of the crop.

Bunchy top was often accompanied by secondary root decay caused by fungi and bacteria and in some cases secondary heart rot occurred especially during the wet season.

4.2 Transmission studies

4.2.1 Transmission through planting material

From the Table 2, it could be seen that BBTV was transmitted through the infected planting material. In all the three seasons, cent per cent transmission was recorded when infected suckers were used.

4.2.2 Mechanical transmission

Results furnished in the Table 3 showed that BBTV could not be transmitted mechanically to banana and to other hosts even when different buffers at different pH were used.

4.2.3 Insect transmission

The insect transmission studies were conducted with nymphs of banana aphids on different test plants as described under materials and methods. It was observed from Table 4 that three months old tissue culture plants were found to be most susceptible to the virus as it could yield maximum per cent of transmission ranging from 20-73.33% with an average of 50.67%. Rhizome buds and 3-4 months old suckers were also found good test plants as the per cent of transmission were 26.67-60.0 and 8.33-66.67 with an average of 47.33 and 38.14 respectively. But 5-6 months old suckers were not good test plants as no transmission was obtained.

With regard to the incubation period in the host minimum incubation period of 17-27 days was obtained in the case of 3 months old tissue culture plants.

Table 2. Transmission through planting material

Seasons	Diseased		Healthy	
	No. of suckers infected / No. of suckers planted	% of infection	No. of suckers infected / No. of suckers planted	% of infection
August-November	75/75	100.00	30/30	0
December-March	27/27	100.0	15/15	0
April-July	40/40	100.0	22/22	0

Table 3. Mechanical transmission of BBTV using different buffers at different pH

Buffers	pH	Per cent tranmission			
		Banana	*Colocasia*	*Canna*	*Hedychium*
0.1M sodium phosphate	7.2	0	0	0	0
0.1M sodium phosphate	8.0	0	0	0	0
01M sodium phosphate + 2-mercapto ethanol	8.0	0	0	0	0
0.01M sodium phosphate	7.2	0	0	0	0
0.01M sodium phosphate	8.0	0	0	0	0
0.01M sodium phosphate + 1% 2-mercapto ethanol	7.2	0	0	0	0
0.01M sodium phosphate + 1% 2-mercapto ethanol	8.0	0	0	0	0
Deionised water	6.5	0	0	0	0
Distilled water	7.0	0	0	0	0

Table 4. Insect transmission of BBTV to different test plants

Experiment No.	2-3 months old tissue culture plants			Rhizome buds			3-4 months old suckers			5-6 months old plants		
	No. of plants infected / No. of plants inoculated	% of transmission	Incubation period (days)	No. of plants infected / No. of plants inoculated	% of transmission	Incubation period (days)	No. of plants infected / No. of plants inoculated	% of transmission	Incubation period (days)	No. of plants infected / No. of plants inoculated	% of transmission	Incubation period (days)
I	0/15	0.00	-	0/15	0.00	-	0/15	0.00	-	0/10	0.0	-
II	3/15	20.00	18-22	6/12	50.00	20-28	8/20	40.00	28-40	0/10	0.0	-
III	6/15	40.00	16-25	8/15	53.33	17-32	1/12	8.33	28	0/10	0.0	-
IV	11/15	73.33	15-26	4/15	26.67	25-34	12/20	60.00	30-35	0/10	0.0	-
V	10/15	66.67	20-32	9/15	60.00	20-36	6/15	40.00	20-32	0/10	0.0	-
VI	8/15	33.33	20-28	7/15	46.67	21-30	10/15	66.67	25-42	0/12	0.0	-
Mean	-	50.67	17-27	-	47.33	21-32	-	38.14	25-36	-	-	-

In case of rhizome buds and 3-4 months old suckers, incubation period varied from 21-32 and 25-36 days respectively.

Among the different test plants used, 3 months old tissue culture plants were found to be good test plants as it could give maximum per cent of transmission and minimum period of incubation in the host (Fig. 2).

Results furnished in Table 5 showed that transmission of BBTV to *Canna, Colocasia* and *Hedychium* did not produce any symptom. Back inoculation from these hosts to the original banana plants could not produce symptoms indicating that these hosts are not collateral hosts of BBTV.

4.3 Virus-vector relationship

4.3.1 Relative efficiency of single nymph and adult on the transmission of BBTV

It is obvious from Table 6 that an adult was an active vector than nymph as a single adult could transmit the virus upto 20%. Maximum per cent of transmission (20.0%) was obtained when 3 months old tissue culture plant was used whereas in case of rhizome bud it was only 13.33%. It was also seen that a single adult was not enough to transmit the virus to 3-4 or 5-6 months old plants. In case of nymphs, no transmission was obtained in any of the test plants showing that a single nymph was not able to transmit the virus.

4.3.2 Effect of number of infective aphids on the transmission of BBTV

From the data presented in Table 7 it is clear that a single nymph of *P. nigronervosa* was not sufficient for transmission of BBTV and a minimum of 20 numbers were required for the successful transmission of the virus. A single adult

Table 5. Insect transmission of BBTV to other host plants

Period	*Canna*		*Colocasia*		*Hedychium*	
	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission
August-November	0/12	0	0/15	0	0/12	0
December-March	0/15	0	0/10	0	0/12	0
April-July	0/15	0	0/12	0	0/10	0

Table 6. Relative efficiency of single nymph and adult on the transmission of BBTV

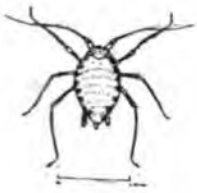
Type of test plants	Nymph		Adult	
	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission
2-3 months old tissue culture plants	0/15	0	3/15	20.00
Rhizome buds	0/15	0	2/15	13.33
3-4 months old suckers	0/15	0	0/15	0
5-6 months old plants	0/15	0	0/12	0

Table 7. Effect of number of infective aphids on the transmission of BBTV

No. of infective aphids	Nymphs		Adults	
	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission
1	0/15	0	3/15	20.00
3	0/15	0	3/15	20.00
5	0/15	0	6/15	40.00
10	1/15	6.67	8/15	53.33
20	9/15	60.00	13/15	86.67
30	11/15	73.33	11/15	73.33
50	15/15	100.00	15/15	100.00
75	15/15	100.00	15/15	100.00
100	15/15	100.00	15/15	100.00

Fig. 1 **Bunchy top affected banana**

Fig. 2 **Aphids on tissue culture plants**



could transmit the disease upto 20.0 per cent. Both nymphs and adults were found efficient vectors when the number increased to 20. Moreover, cent per cent transmission was obtained when 50 nymphs and adults were used.

4.3.3 Effect of Pre-acquisition fasting on transmission of BBTV

Fasting of both nymphs and adults of *P. nigronervosa* prior to acquisition access feeding had no significant effect on the per cent transmission of BBTV. The transmission was reduced considerably when nymphs and adults were given a pre-acquisition fasting of more than 3 h and 6 h prior to acquisition feeding (Table 8).

4.3.4 Effect of acquisition access period on the percentage of transmission of BBTV

Data presented in Table 9 showed that in case of nymphs a period of 15 h was required for the acquisition of virus from the source plant. In the case of adults, only 2 h acquisition was required for the transmission of the virus and 15 h was sufficient for cent per cent transmission. In both the cases, the rate of transmission increased as the acquisition access period was increased.

4.3.5 Effect of inoculation access period on the percentage of transmission of BBTV

In case of nymphs, a minimum period of 6 h was required for successful inoculation of the virus. But the rate of transmission was only 20.0 per cent. In case of adults, 1 h inoculation feeding was sufficient for the transmission of 13.33 per cent. In both the cases, transmission percentage rose progressively with corresponding increase of inoculation access (Table 10).

Table 8. Effect of pre-acquisition fasting on transmission of BBTV

Pre-acquisition fasting period	Nymphs		Adults	
	No. of plants infected / No. of plants inoculated	% of transmission	No of plants infected / No. of plants inoculated	% of transmission
No fasting	15/15	100.00	15/15	100.00
10 min	13/15	86.67	15/15	100.00
30 min	10/15	66.67	12/15	80.00
1 h	12/15	80.00	14/15	93.33
3 h	7/15	46.67	12/15	80.00
5 h	5/15	33.33	11/15	73.33
6 h	3/15	20.00	7/15	46.67
12 h	2/15	13.33	4/15	26.67

Table 9. Effect of acquisition access period of the percentage of transmission of BBTV

Acquisition access period	Nymphs		Adults	
	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission
10 min	0/15	0.0	0/10	0.0
30 min	0/10	0.0	0/10	0.0
1 h	0/10	0.0	0/10	0.0
2 h	0/10	0.0	3/10	30.00
3 h	0/10	0.0	6/15	40.00
6 h	0/15	0.0	8/15	53.33
12 h	0/15	0.0	12/15	80.00
15 h	2/15	13.33	15/15	100.00
18 h	8/15	53.33	15/15	100.00
24 h	15/15	100.00	15/15	100.00

Table 10. Effect of inoculation access period on the percentage of transmission of BBTV

Inoculation access period	Nymphs		Adults	
	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission
10 min	0/15	0.0	0/15	0.00
30 min	0/10	0.0	0/10	0.00
1 h	0/10	0.0	2/15	13.33
3 h	0/10	0.0	5/15	33.33
6 h	3/15	20.00	8/15	53.33
12 h	7/15	46.67	11/15	73.33
24 h	15/15	100.00	15/15	100.00
48 h	15/15	100.00	15/15	100.00

4.3.6 Incubation period of virus in the vector

From Table 11 it was found that incubation period of the virus in the vector varied with nymphs and adults. In nymphs, the incubation period was found to be 6 h in which 20.0 per cent transmission was obtained. In case of adults, the incubation period was found to be 1 h which could yield only 13.33 per cent transmission. In both the cases, cent per cent transmission was obtained after an incubation period of 24 h.

4.4 Purification of BBTV

Purification of BBTV from diseased plants was successfully completed as per the procedure of Thomas and Dietzgen (1991). In this method, the final purification steps involved sucrose gradient centrifugation, fractionation of gradients and concentration of virus by centrifugation.

BBTV was purified from systemically infected banana tissues 17-27 days after aphid inoculation and also from field infected plants. The average yield of the virus was more (1.62 mg/kg tissue) when purified from the inoculated tissue culture plants than from the field (1.10 mg/kg tissue). When BBTV was purified from aphid inoculated 3-4 months old suckers and from rhizome buds, the yield of the virus was 1.24 mg/kg tissue and 1.34 mg/kg tissue respectively (Table 12 and Fig. 3).

It is evident from Tables 13, 14, 15 and 16 that the concentration of BBTV from different portions of infected banana viz., midrib, petiole, leafsheath and rhizome were 1.66, 1.61, 1.08 and 0.59 mg/kg tissue respectively. With regard to influence of the period of sample collection on the concentration of BBTV, the

Table 11. Incubation period of virus in the vector

Incubation period	Nymphs		Adults	
	No. of plants infected / No. of plants inoculated	% of transmission	No. of plants infected / No. of plants inoculated	% of transmission
10 min	0/15	0.0	0/15	0.00
30 min	0/10	0.0	0/10	0.00
1 h	0/10	0.0	2/15	13.33
3 h	0/10	0.0	5/15	33.33
6 h	3/15	20.00	8/15	53.33
9 h	4/15	26.67	10/15	66.67
12 h	7/15	46.67	11/15	73.33
24 h	15/15	100.00	15/15	100.00

Table 12. U.V. absorption of purified preparations of BBTV from different sources of banana during August-November

Source of BBTV Aphid inoculated Nendran	Dilution of purified BBTV	Absorption at 260 nm	 280 nm	A_{260}/A_{280}	Concentration of virus mg/kg tissue
Tissue culture plants	1:10	0.583	0.405	1.44	1.62
3-4 months old suckers	1:10	0.447	0.319	1.40	1.24
Rhizome buds	1:10	0.482	0.339	1.42	1.34
Field infected Nendran	1:10	0.395	0.279	1.42	1.10

Table 13. U.V. absorption of purified preparations of midrib from BBTV infected banana

Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
	260 nm	280 nm		
1:10	0.623	0.441	1.41	1.73
1:50	0.115	0.082	1.40	1.60
1:100	0.058	0.042	1.38	1.61
1:500	0.012	0.008	1.50	1.67
1:1000	0.006	0.004	1.50	1.67
Mean	-	-	1.44	1.66

Table 14. U.V. absorption of purified preparations of petiole from BBTV infected banana

Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
	260 nm	280 nm		
1:10	0.583	0.405	1.44	1.62
1:50	0.113	0.080	1.41	1.57
1:100	0.059	0.042	1.41	1.64
1:500	0.011	0.008	1.38	1.53
1:1000	0.006	0.004	1.50	1.67
Mean	-	-	1.43	1.61

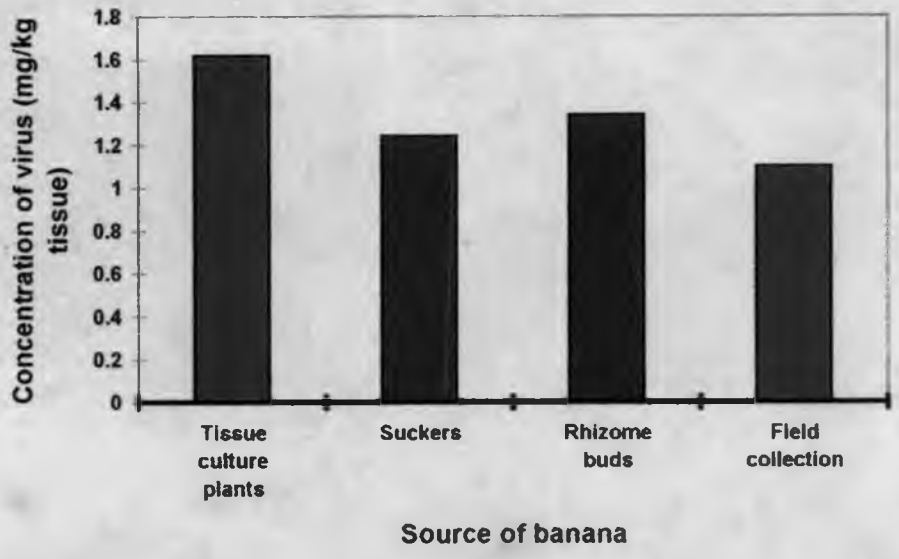
Table 15. U.V. absorption of purified preparations of leafsheath from BBTV infected banana

Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
	260 nm	280 nm		
1:10	0.384	0.268	1.43	1.07
1:50	0.080	0.057	1.40	1.11
1:100	0.036	0.025	1.44	1.00
1:500	0.008	0.006	1.33	1.11
1:1000	0.004	0.003	1.33	1.11
Mean	-	-	1.39	1.08

Table 16. U.V. absorption of purified preparations of rhizome from BBTV infected banana

Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
	260 nm	280 nm		
1:10	0.198	0.141	1.40	0.55
1:50	0.045	0.032	1.41	0.63
1:100	0.019	0.014	1.36	0.53
1:500	0.005	0.003	1.60	0.69
1:1000	0.002	0.0018	1.50	0.56
Mean	-	-	1.45	0.59

Fig. 3
Concentration of purified BBTV in different sources of banana



highest concentration (1.73 mg/kg tissue) was obtained during August-November and lowest concentration (0.22 mg/kg tissue) was during December-March (Tables 17, 18, 19 and Fig. 4).

Purification and extraction of BBTV from infected leaves from different positions were done and the concentration was determined. From Table 20, it is clear that among the leaves, the three leaves of the top position (crowded portion) ranked the highest concentration of virus (1.65 mg/kg tissue) followed by middle portion (1.11 mg/kg tissue) and bottom portion (0.37 mg/kg tissue) respectively (Fig. 5).

The light scattering zone of virus was not observed in sucrose density gradient tubes at any distance. The zone containing virus particles was collected by puncturing the bottom of the tube with a syringe and collected each gradient in eppendorf tubes. They were then made to 1 ml each by the addition of 0.07 M sodium phosphate buffer pH 7.2 and read at 260 nm and 280 nm in a U.V. monitor. In the purification procedure, chloroform and butanol was used in the initial clarification process.

In density gradient centrifugation about 1 cm zone (absorbance values at 200-340 nm) was obtained at the middle of the tube, 2.1-3.1 cm from the bottom which was found infectious on banana plants tested.

4.4.1 Infectivity assays of BBTV

Purified BBTV was successfully transmitted to tissue culture plants of nendran (6/15) by aphids after feeding through parafilm membrance for 24 h.

Table 17. U.V. absorption of purified preparations of BBTV from different portions of banana during August-November

Aphid inoculated tissue culture plants	Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
		260 nm	280 nm		
Midrib	1:10	0.623	0.441	1.41	1.73
Petiole	1:10	0.583	0.405	1.44	1.62
Leafsheath	1:10	0.384	0.268	1.43	1.07
Rhizome	1:10	0.186	0.133	1.40	0.52

Table 18. U.V. absorption of purified preparations of BBTV from different portions of banana during December-March

Aphid inoculated tissue culture plants	Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
		260 nm	280 nm		
Midrib	1:10	0.078	0.056	1.39	0.22
Petiole	1:10	0.059	0.041	1.44	0.16
Leafsheath	1:10	0.032	0.023	1.39	0.09
Rhizome	1:10	0.021	0.015	1.40	0.06

Table 19. U.V. absorption of purified preparations of BBTV from different portions of banana during April-July

Aphid inoculated tissue culture plants	Dilution of purified virus	Absorption at		A_{260}/A_{280}	Concentration of virus mg/kg tissue
		260 nm	280 nm		
Midrib	1:10	0.175	0.125	1.40	0.49
Petiole	1:10	0.146	0.103	1.42	0.41
Leafsheath	1:10	0.102	0.070	1.46	0.28
Rhizome	1:10	0.080	0.056	1.43	0.22

Table 20. BBTV titre in individual banana leaves (August-November)

Position of leaves	Dilution of BBTV	Absorption at 260 nm	Absorption at 280 nm	A_{260}/A_{280}	Concentration of virus mg/kg tissue
Top					
1	1:10	0.636	0.448	1.42	1.77
2	1:10	0.585	0.418	1.40	1.63
3	1:10	0.559	0.394	1.42	1.55
Middle					
1	1:10	0.447	0.324	1.38	1.24
2	1:10	0.396	0.285	1.39	1.10
3	1:10	0.361	0.254	1.42	1.00
Bottom					
1	1:10	0.172	0.122	1.41	0.48
2	1:10	0.120	0.092	1.40	0.33
3	1:10	0.103	0.074	1.39	0.29

Fig. 4
Concentration of purified BBTV in different portions of banana during different seasons

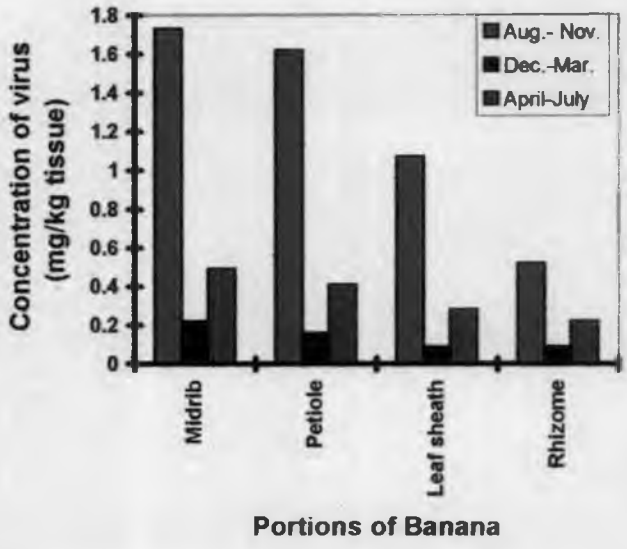
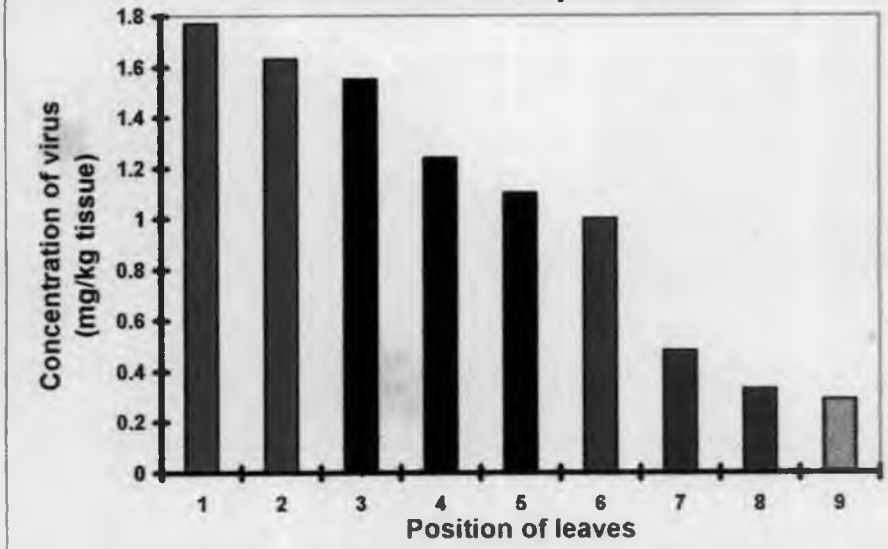


Fig. 5
Concentration of purified BBTV in leaves of different positions



4.4.2 Determination of concentration of purified virus

The final 1 ml purified virus from different portions of banana was serially diluted, 1:10, 1:50, 1:100, 1:500 and 1:1000 for spectrophotometric observation. The U.V. absorption was measured from 200-340 nm at 10 nm intervals for 1:10 dilution. They had a maximum U.V. absorption at 200 nm and minimum at 340 nm and at 260 nm, it showed another peak in U.V. absorption (Fig. 6). For other dilutions the absorption at 260 nm and 280 nm was measured. The purified preparations had A_{260}/A_{280} ratio ranging from 1.33 to 1.46.

4.4.3 Particle morphology of BBTV using electron microscopy

Purified BBTV preparation was negatively stained with 1% (v/v) ammonium molybdate pH 5.8 and 2% uranyl acetate, the virus isolates showed isometric particles of 18-22 nm size which were found more stable in 2% uranyl acetate. Electron-microscopy also revealed that the final product was clean and most of the isometric particles were well preserved. The virus particles appeared hexagonal or circular ones. Upon closer examination, coat protein subunits and capsid cores of the virus particles were also visible (Fig. 7).

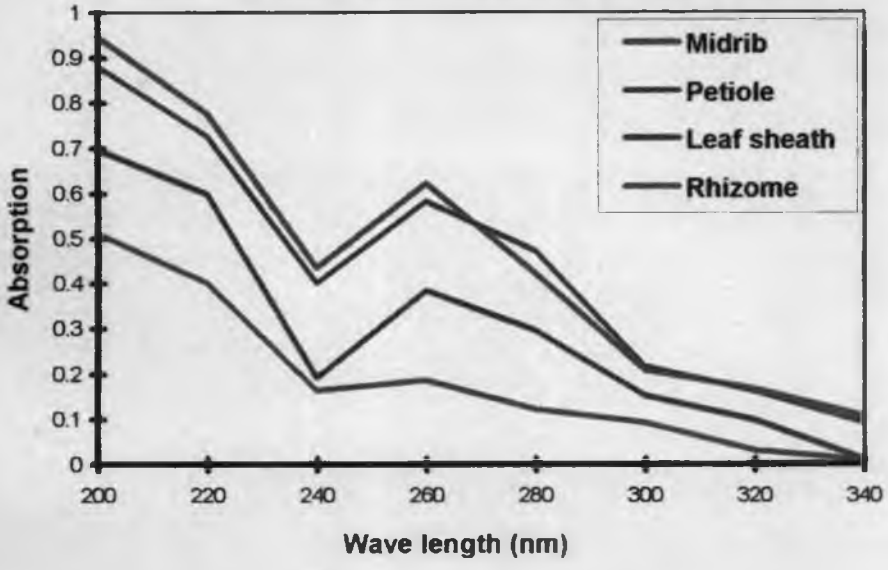
4.5 Estimation of nucleic acids

Nucleic acids of both RNA and DNA were estimated from different parts of diseased and healthy banana as per the method of Schneider (1945) and Ogur and Rosen (1950). RNA in PCA extract was estimated by orcinol reaction at 660 nm and DNA by diphenylamine method at 595 nm. From Table 21, it is clear that RNA was more in healthy plants whereas DNA was more in diseased plants.

Table 21. Estimation of nucleic acids

Source of materials	RNA at 660 nm		DNA at 595 nm	
	Healthy	Diseased	Healthy	Diseased
Midrib	0.636	0.531	0.439	0.633
Petiole	0.704	0.419	0.382	0.657
Leafsheath	0.443	0.305	0.251	0.332

Fig. 6
Ultraviolet absorption spectrum of purified BBTV from different portions of banana



4.6 Isolation of DNA from plants

DNA was isolated from diseased and healthy Nendran banana as per the procedure of Hattori *et al.* (1987). Agarose gel electrophoresis showed that DNA obtained was seen moving towards the aniodic end of the gel indicating that it is of low molecular weight DNA but no such band was obtained in healthy banana (Fig. 8).

4.7 Extraction of nucleic acid of BBTV

Nucleic acid of BBTV was extracted as per the method of Harding *et al.* (1991) and Thomas and Dietzgen (1991). Nucleic acid preparations were incubated at 37°C for 1 h with (i) DNase (0.2 µg/µl, BM) in 50 mM Tris HCl, 10 mM $MgCl_2$ pH 8.0 (ii) RNase (20 µg/µl, BM) in water and (iii) S1 nuclease 50 units/ml in 0.033 M sodium acetate pH 4.5 containing 0.3 M NaCl and 0.001 M $ZnSO_4$ for 30 min.

4.8 Gel electrophoresis of nucleic acid of BBTV

When the nucleic acids extracted from the purified BBTV were analysed by agarose gel electrophoresis, three bands of nucleic acids of about 21 kb, 2.8 kb and 2.4 kb were observed when compared with nucleic acids of uninfected tissues and DNA marker. These nucleic acids were found sensitive to DNase I and S1 nuclease but not to RNase A, while the respective control nucleic acids were digested by the appropriate enzyme, further confirming that BBTV contain ssDNA (Fig. 9).

Fig. 7

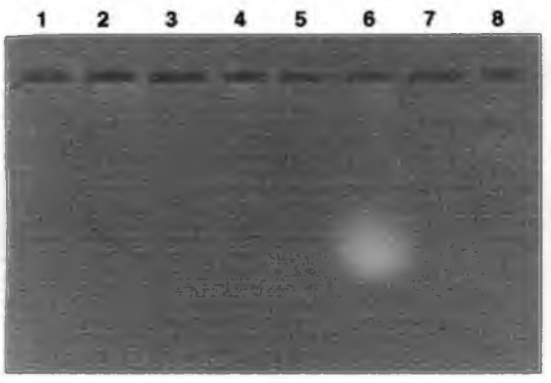
Electron micrograph showing BBTV particles in purified preparation.

[X 1,00,000]

[X 1,68,000]

Fig. 8

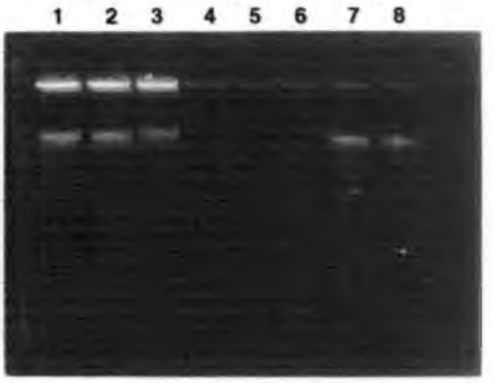
DNA isolated from BBTV affected plant



2 - DNA from healthy plant
4 - DNase treated nucleic acid of diseased plant
6 - RNase treated nucleic acid of diseased plant

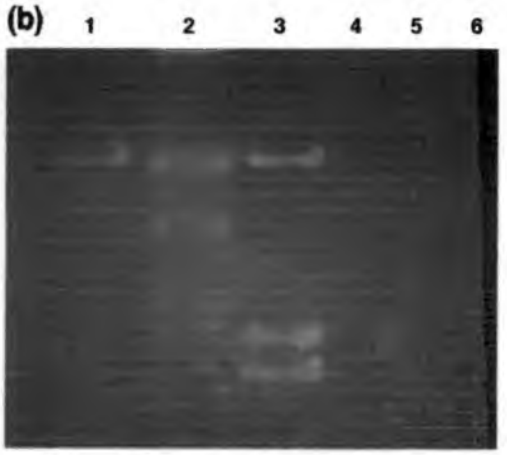
Fig. 9

Analysis of BBTV nucleic acid (a)



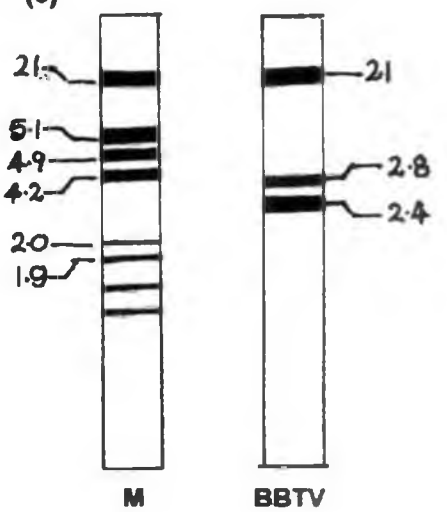
Lanes 1 - 8 DNA isolated from partially purified virus
1 - 3 BBTV from midrib portions
4 - 6 from healthy portions
7 - 8 BBTV from petiole portions

(b)



Lane 1 BBTV DNA before density gradient centrifugation
2 DNA digested with Hind III EcoR I
3 BBTV DNA treated with RNase A
4 BBTV DNA treated with DNase I
5 BBTV DNA treated with S1 nuclease

(c)



Molecular weight in Kb

4.9 Characterisation of proteins by SDS-PAGE method

Polyacrylamide gel electrophoresis of BBTV coat protein in sodium dodecyl sulphate revealed that BBTV, after sucrose density gradient consists of a major protein component of molecular weight 21,000. The BBTV viral protein showed a single major band with Rf value between that of ß lactoglobulin (Mr 18400) and α-chymotrypsinogen (Mr 25700) and the calculated molecular weight of BBTV protein corresponds to 21,000 (Fig. 10).

SDS-PAGE was also conducted with midribs, petiole, leafsheath and rhizome portions of diseased and healthy plants. Protein bands were seen more on the diseased plant parts thus coming to the findings that infected tissues contain extra cellular proteins in the midrib portion when compared to healthy ones. The position of the bands were photographed and traced on transparency sheets and difference between them were noted. A graph was plotted with distance migrated on X axis and log of molecular weights of the marker on Y axis. From the graph the molecular weight of unknown proteins were calculated.

4.10 Estimation of proteins

Protein content in various samples of diseased and healthy plants was estimated by Lowry's method and the results are given in Table 22. It is evident from the Table that the percentage of protein in diseased portions such as midrib, leaf lamina, leafsheath and rhizome were found high (0.265, 0.235, 0.164 and 0.056) when compared to healthy parts (0.154, 0.175, 0.085 and 0.044).

Table 22. Estimation of protein from different portions of banana

Source of materials	Absorbance at 660 nm			
	Leaf	Midrib	Leafsheath	Rhizome
Healthy	0.154	0.175	0.085	0.044
Diseased	0.265	0.235	0.164	0.056

4.11 Serology

4.11.1 Production of antiserum against BBTV

Antiserum was successfully produced in rabbit against BBTV by giving three intramuscular injections at weekly intervals with the purified or partially purified virus with an equal volume of Freund's adjuvant. For the first injection Freund's complete adjuvant was used and in subsequent injections Freund's incomplete adjuvant was added with the purified virus. Blood taken 7 days after the last injection was used as antiserum.

4.11.1.1 Determination of antibody titre

Antisera produced against purified preparations of BBTV reacted with purified virus of BBTV to give titres of 1/128 in agar gel diffusion tests and 1/512 in ring interface tube precipitin tests (Table 23). The concentration of antigen used in antibody titration was approximately 1 mg/ml. The precipitin bands were observed after 12 h of incubation at room temperature in agar gel diffusion test. But in ring interface precipitin test, the precipitin ring was obtained 2-3 h after incubation at room temperature.

4.11.2 Antigen detection

4.11.2.1 Chloroplast agglutination test

This test was found succesful for virus detection from crude sap of banana infected with BBTV which was visible in transmitted light. Partialy purified preparations of BBTV at 10^{-3} dilution, the sap aggregated together forming a precipitate after mixing with the antisera on slight warming. But with purified virus no agglutination was obtained.

Table 23. Determination of antiserum titre of BBTV.

Dilutions of antiserum	Double diffusion test	Ring interface precipitin test
Undiluted	+	+
1/2 dilution	+	+
1/4 dilution	+	+
1/8 dilution	+	+
1/16 dilution	+	+
1/32 dilution	+	+
1/64 dilution	+	+
1/128 dilution	+	+
1/256 dilution	-	+
1/512 dilution	-	+
1/1024 dilution	-	-
1.2048 dilution	-	-
1/4096 dilution	-	-

+ visible bands
- No bands
Purified BBTV at a concentration of 1 mg/ml was used in the tests

Agglutination was not obtained with normal rabbit serum and purified virus preparation (Fig. 11).

4.11.2.2 Agar gel diffusion test

Agar gel diffusion test was not successful in detecting BBTV in the crude sap of infected banana, because of too low concentration of virus in the sap. But with purified virus preparations, clear precipitin bands were seen after 12 h incubation at 37°C or at room temperature. When purified antigen were placed in alternate wells against BBTV antiserum in the centre well, the bands obtained in the form of 'curved line' or 'arc' near to the BBTV antigen showed the presence of viral antigen and was absent in the place of control (Fig. 12). Double diffusion test was also found not successful in detecting purified BBTV in serial dilutions ranging from 10^{-1} to 10^{-3} using the antiserum. However, with 10^{-1} dilution of purified virus, very faint/light bands were obtained with homologous antiserum.

4.12 ELISA

In the present study, Indirect ELISA detected BBTV antigens in crude extracts from infected banana. BBTV was also detected from apparently healthy looking banana collected from the field. From Table 24 it is clear that BBTV antigens were detected to a maximum of 10^{-3} dilution. The reaction was always significantly distinguishable from that of the healthy samples (Fig. 13 and 14).

Table 24. Reaction of BBTV with polyclonal antibodies in I-ELISA

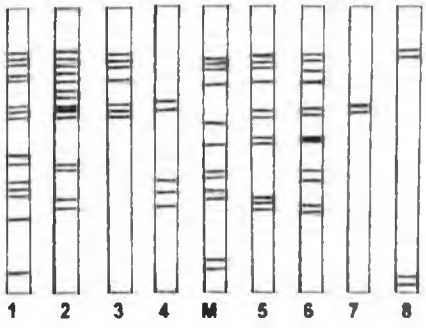
Source of sample	A_{405}				
	Sample dilution				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Diseased plants					
Midrib	1.267	0.881	0.623	0.22	0.09
Petiole	1.027	0.721	0.416	0.18	0.08
Leafsheath	1.016	0.625	0.384	0.21	0.09
Rhizome	0.956	0.362	0.198	0.20	0.07
Apparently healthy banana	0.881	0.312	0.176	0.15	0.03
Healthy plants					
Midrib	0.022	-	-	-	-
Petiole	0.016	-	-	-	-
Leafsheath	0.004	-	-	-	-
Rhizome	0.002	-	-	-	-
Buffer	0.012	-	-	-	-

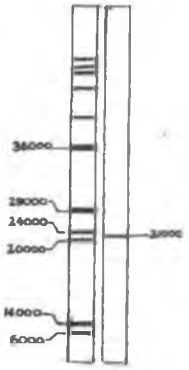
Test samples extracted in PBS-T-PVP

A_{405} values are the means of 8 wells

Fig. 10

Analysis of protein associated with BBTV





BBTV COAT PROTEIN

M - Marker

1 - Midrib
2 - Petiole
3 - Leafsheath
4 - Rhizome
〉 BBTD

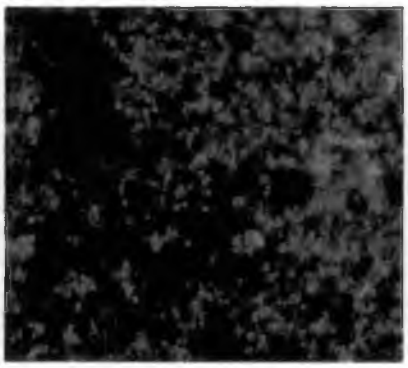
5 - Midrib
6 - Petiole
7 - Leafsheath
8 - Rhizome
〉 Healthy

9 - Partially Purified BBTV 10 - Purified BBTV 11 - Purified healthy sap

Fig. 11

Chloroplast agglutination test for antigen detection

Infected sap + BBTV antiserum



Infected sap + BBTV antiserum [10x]

Purified BBTV + BBTV antiserum

Fig. 12

Agar gel diffusion test for antibody titre

A - purified BBTV antigen
1 - 6 - BBTV antiserum

As - BBTV antiserum
1 and 3 - Sap from infected banana
2 Sap from healthy banana
4 and 6 - purified BBTV antigen
5 Buffer.

A - BBTV antiserum
1 Healthy plant sap
2 Purified BBTV antigen
3 Buffer
4 Sap from infected banana

Fig. 13

ELISA for detection of BBTV

(Midrib)

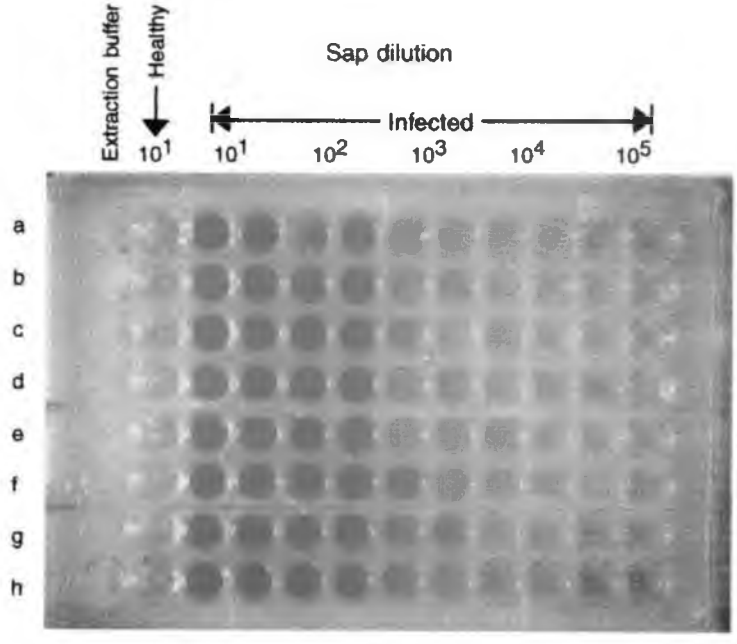
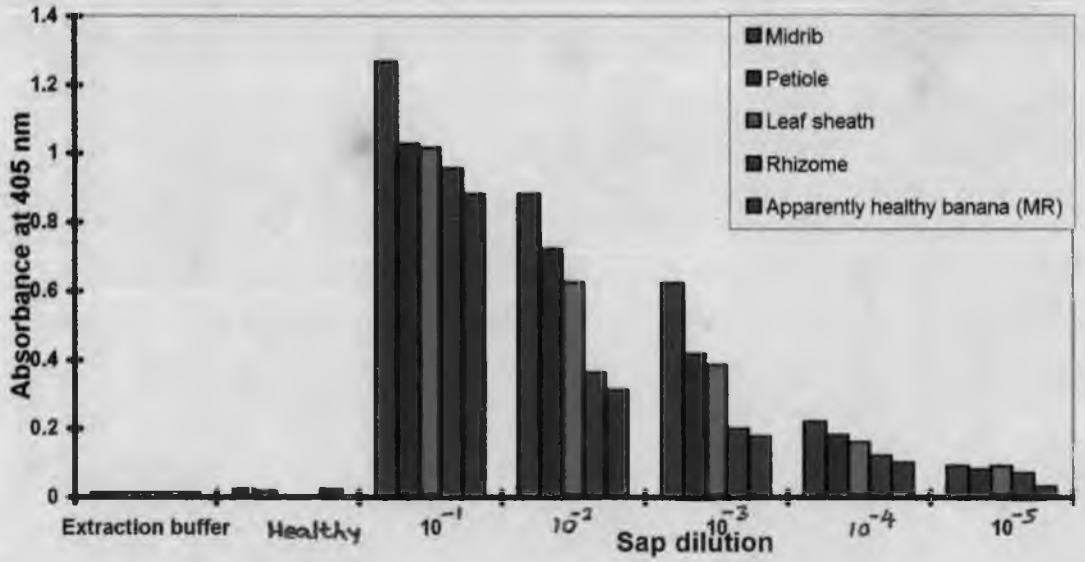


Fig. 14
Reaction of BBTV with polyclonal antibodies in Indirect ELISA



Discussion

DISCUSSION

Banana bunchy top disease (BBTD) caused by banana bunchy top virus (BBTV) is the most economically important disease of banana. The disease was first reported from Fiji as early as 1889 (Stover, 1972). This disease was introduced into South India from erstwhile Ceylon by about 1940. But in Kerala the disease became conspicuous in 1943. The disease is prevalent in our country for the last several years as a serious threat to banana cultivation where the crop is grown commercially.

Many workers have studied the biochemical and transmission aspects of BBTV. However, information on purification and characterization of BBTV isolate from India is scanty. Hence the present investigation was undertaken with a view to throw light on purification of BBTV, identification of nucleic acid of the virus, electron microscopy of the purified virus preparation for particle size determination and production of antisera for the presymptomatic detection of virus present in the planting materials of banana, in addition to the studies on virus-vector relationship.

Survey conducted to study the BBTD incidence and aphid population in banana growing areas of Thrissur district of Kerala revealed that the percentage of disease incidence and aphid population varied with seasons. Maximum disease incidence (6.5%) was observed in August-November as the aphid population was also high (205 nos.) because of low temperature and low wind velocity during this period. Moreover, August-September is the planting period of Nendran in Kerala so that during this period (August-November) the plants will be in the seedling stage which is more vulnerable to the infection. Decrease in disease incidence during December-July is due to the low aphid population during that period and also due to the age of the plant. Aphid population is drastically reduced in dry warm climate

when daily maximum temperature often exceeds 30°C (Basu and Giri, 1993). Low aphid population in Kerala may be due to high temperature in March to May and heavy rainfall during June-July. Singh *et al.* (1981) also found that the incidence of aphid borne potato viruses like leaf roll, PVY, PVA, PVM depended on vector population.

Studies on symptomatology are essential for the early detection of virus. The symptoms noticed were same as described in literature (Magee, 1940; Pathak, 1976; Frison and Putter, 1990 and Iskra, 1990). BBTV infected plants exhibited symptoms like marginal yellowing of the leaf lamina, dark green flecks/streaks on the petiole, reduction in size of the leaf lamina, rosetted appearance of leaves at the apex, stunting of the plant, secondary root decay and secondary heart rot. The infected plants remained in the field for 2 to 3 years by 3 or 4 generations.

Drew *et al.* (1989) opined that symptomatology is unreliable as the sole means of virus identification. They further added that BBTV infected micropropagated plantlets can be symptomless for over one month after planting out and mild strains of BBTV also exist. Thus, total reliance on the symptomatology as the sole means of virus identification may not be dependable in all the cases.

Transmission studies conducted through the planting materials indicated that infected suckers are the primary source of inoculum for BBTV. Allen (1978a) and Jose (1981) also reported transmission of BBTV through infected suckers.

Mechanical transmission conducted on banana and to other reported hosts such as *Canna*, *Colocasia* and *Hedychium* with different phosphate buffers at different pH revealed that BBTV is not mechanically transmissible. This supports the earlier findings of Ross (1964) and Matthews (1982). The failure to infect

banana by crude sap could be attributed to considerable amounts of latex and phenolic compounds in banana which might interfere with virus infectivity and thereby prevent the transmission of virus from these plants to test plants (Yarwood, 1953; Hollings, 1957; Berg, 1962; Dale, 1987 and Wu and Su, 1990). It is also known that viruses located in phloem or xylem vessels are not sap transmissible (Basu and Giri, 1993).

Insect transmission studies conducted with *P. nigronervosa* on various test plants revealed that tissue culture plants were more susceptible to BBTV recording 50.67 per cent transmission while rhizome buds and 3-4 months old suckers yielded 47.33 per cent and 38.14 per cent respectively. Moreover, shortest incubation period of 17-27 days was also observed in the case of tissue culture plants. Wu and Su (1990) also found that tissue culture plants were ideal for BBTV transmission studies as the incubation was shortest in this. Non-transmission of BBTV to 5-6 months old plants indicated that susceptibility of plants decreased with age of plants. Wu and Su (1990) also reported that the transmission rate of BBTV was directly correlated with the number of viruliferous aphids feeding on the plants and is inversely correlated with the age of the host plantlets. Su *et al.* (1993) also reported that symptom development of BBTV was affected by plant age and plant height. Non-appearance of symptoms on inoculation of BBTV on *Canna*, *Colocasia* and *Hedychium* and on back inoculation to the original host showed that these hosts were neither symptomless carriers nor collateral hosts of BBTV. However, Ram and Summanwar (1984) reported *Colocasia esculenta* L. Schott. to be the reservoir of bunchy top of banana. Su *et al.* (1993) observed *Canna indica* Linn. and *Hedychium coronarium* Koenig as intermediate hosts of BBTV transmitted through *P. nigronervosa* var. *Caladii* v.d. Goot.

As the studies on virus-vector relationship is necessary for the better understanding of the virus, the first approach was on the basic aspects of transmission. The efficiency of a single nymph and adult was determined on the basis of the percentage of transmission as evidenced by their ability to transmit the virus to different test plants. In the comparative tests it was found that a single nymph was not sufficient for transmission of virus irrespective of different test plants whereas a single adult could transmit the virus to a few plants of 3 months old tissue culture plants and rhizome buds, but not in 3-6 months old plants. These findings are supported by the earlier observations made by Thomas and Dietzgen (1991) and Xie and Hu (1995) who have isolated virus from a single aphid but could not trace any of the BBTV particles from an individual nymph.

In the investigations on the effect of number of viruliferous nymphs and adults of *P. nigronervosa* on BBTV transmission, it was found that a single nymph could not transmit the virus and minimum of 20 nymphs were required for successful transmission of BBTV whereas a single adult was sufficient to transmit the disease. However, both were found efficient vectors as the number of insects per plant was increased. The plausible explanation for this is that every vector individuals exposed to the source will not necessarily be infective. This result confirmed the earlier investigations of Thomas and Dietzgen (1991) as they could detect BBTV scarcely among nymphs from an infective colony. However, earlier Magee (1940) had observed that the nymphs of *P. nigronervosa* were more efficient vectors of BBTV than adult aphids.

Attempt on the effect of pre-acquisition fasting of nymphs and adults of *P. nigronervosa* revealed that fasting had no effect on increasing transmission of the virus as no marked difference could be noticed between fasted and non-fasted vectors. Moreover, the transmission rate was reduced considerably as the pre-

acquisition fasting was increased. Watson and Roberts (1939) reported that only non-persistent relationship demands the pre-acquisition fasting for effective transmission. Being a persistent relationship, it is presumed that BBTV did not require any fasting before acquisition. Pre-acquisition fasting is known to have beneficial effect on acquisition of non-persistent aphid-borne viruses, but not in case of persistent and semi-persistent ones. An interesting exception, though in other group of vectors, may be referred to in this connection. Varma (1952) found beneficial effect of pre-acquisition fasting on acquisition of bhindi yellow vein mosaic virus, which is known to be transmitted by its whitefly vector *Bemisia tabaci* Gen. in a persistent manner.

During the studies on acquisition access period, it was found that nymphs required minimum 15 h to acquire the virus from the source plant where it was only 2 h in the case of adults. Percentage of transmission increased with an increase in the length of acquisition period. Stover (1972) reported 17 h as the shortest acquisition feeding period in the case of nymphs and observed that the efficiency of vector increased with an increase in the acquisition time. The present findings appear to be complementary to these observations.

In the experiment on inoculation threshold the minimum period required to transmit the virus to healthy plants was 6 h in the case of nymphs and 1 h in the case of adults after an acquisition access of 24 h. Percentage of transmission increased with increasing the inoculation feeding period. Sylvester (1956) reported that there was a positive correlation between duration of the inoculation feeding and successful transmission.

As incubation period of virus in the vector is an important aspect in the virus-vector relationship, investigation was carried out in this aspect also which

revealed that incubation period of the virus in nymphs and adults were 6 h and 1 h respectively and cent per cent transmission was obtained after an incubation period of 24 h. Duffus (1971) and Elnagar and Murant (1978) reported that the latent period of the BBTV was normally between 12 and 24 h.

Banana contains considerable amount of latex and phenolics which interfere with virus extraction and purification (Dale, 1987). Pulverization of banana tissues frozen in liquid N before extraction greatly reduced such interference and appeared to be one of the main factors contributing to the successful extraction and purification of BBTV. Extraction of the pulverized tissues with potassium phosphate buffer containing sodium sulphite (Na_2SO_3) reduced the amount of copurified host material to a large extent. For easy extraction and reextraction of the fibres, acid-washed sand was used which did not affect the concentration of the virus. This was further accomplished by clarification with chloroform:butanol, stirring and incubating viral extracts at 4°C followed by differential centrifugation further removing the host contaminants which prevented the detection of BBTV by U.V. scanning and electron microscopic observations. In the present study, chloroform and butanol were found to be good in the initial clarification process since they preserved the isometric particles better than ether and ethanol. The method of purification adopted in the present study was found to be useful for the purification of BBTV from different portions of banana also.

The concentration of virus obtained was more from the aphid inoculated 3 months old tissue culture plants than that of 3-6 months old suckers since the tissue culture plants were found more susceptible to bunchy top disease. Virus concentration showed great variation in different portions of banana and also in different seasons. Among the different portions purified, high concentration of virus was obtained in midrib because of low phenolic content and high nutrient content.

The virus concentration was found to be low in the leaf sheath due to the expanded cell structure and high water content. The availability of high water content in leaf sheath was one among the factors contributing to the low concentration of the virus. In rhizome portion, starch and high phenolics interfere with virus multiplication and hence the concentration was reduced. Within the leaves, the youngest three leaves showed higher concentration of the virus, since the top most portion is the active growing part with low phenolics and high protein content. The more the protein content present in the youngest leaves, the more will be the virus concentration. Seasonal difference was also noticed in virus infection. Higher incidence of BBTV and maximum aphid population were observed during August-November. It was during this period, the virus concentration was also found high. From these findings it is clear that the midrib portion of the top most infected leaves (3 leaves) are ideal for extraction and purification of the virus. Similar findings were also reported by Drew *et al.* (1989); Wu and Su (1990) and Thomas and Dietzgen (1991).

The purified preparations of BBTV could be successfully transmitted to banana by the banana aphid which were fed on the purified virus through parafilm membrane. Similar findings could be obtained by Mathew (1988) also who studied the transmission with whiteflies in cassava. The present results showed that *P. nigronervosa* is capable of acquiring and transmitting BBTV even from purified preparations.

Electron microscopic studies of the purified preparations of BBTV showed isometric particles of 18-22 nm size. This also revealed that the purified preparations of the isometric particles were well preserved and clear. Electron microscopic studies on BBTV conducted by Kang (1984); Dale *et al.* (1986); Iskra *et al.* (1989); Su and Wu (1989); Wu and Su (1990a); Harding *et al.* (1991) and Thomas and Dietzgen (1991) also yielded similar observations.

The serial dilution of the purified virus (10^{-1}) had a maximum U.V. absorption at 220 nm and minimum at 320 nm and it showed a peak at 260 nm. In further dilution of 1/50 and above, the absorbance values were considerably reduced showing a great decline in the concentration of the virus. The A_{260}/A_{280} ratio of the virus (BBTV) ranges from 1.33 to 1.42 and average yield of the virus found to be 1.73 mg/kg tissue assuming an extinction coefficient of 3.6. Wu and Su (1990) reported that BBTV had a maximum absorbance at 257 nm, minimum absorbance at 240 nm and A_{260}/A_{280} ratio of 1.46. Thomas and Dietzgen (1991) reported that A_{260}/A_{280} ratio of purified preparations of BBTV was 1.33. These findings were also in consonance with that of the present results.

In the nucleic acid estimation studies, the type of nucleic acid was determined by diphenylamine reaction for DNA and orcinol reaction for RNA. From the results it is clear that RNA was more in healthy plants whereas DNA was more in diseased ones. The reduction in RNA content in diseased plants might be due to these reasons. In diseased plants, one of the early virus specific proteins produced inhibited the cell RNA synthesis and produced nucleotides and amino acids available for the synthesis of viral components. The increase in the DNA content of the diseased plant might be due to the virus concentration. Thus, it is evident from the findings that the BBTV nucleic acid is of DNA.

DNA was isolated from diseased as well as healthy plants and the results of the study revealed that DNA obtained from diseased plant was of low molecular weight when compared with that from healthy plants and DNA marker. The DNA of healthy plant was retained in the loading well itself showing that the plant DNA is of high molecular weight. Similar findings were also reported by Harding *et al.* (1991) and Thomas and Dietzgen (1991).

Agarose gel electrophoresis of nucleic acid from sucrose gradient BBTV consisted of 20 kb, 2.8 kb and 2.4 kb DNA respectively. These bands were found sensitive to digestion by DNase I and S1 nuclease but not to Rnase A confirming the nucleic acid of BBTV as ssDNA. No corresponding nucleic acid band was obtained in total nucleic acid isolated from healthy banana confirming that the plant DNA is of high molecular weight DNA. In this study the method suggested by Thomas and Dietzgen (1991) was followed and isolated ssDNA of 18-22 nm diameter. The findings obtained were similar to the reports of Harding *et al.* (1991) and Thomas and Dietzgen (1991) with regard to the size and type of nucleic acid. Particle properties of BBTV generally agree with those reported from Taiwan (Su and Wu, 1989; Wu and Su, 1990a). However, a discripancy concerning the type of nucleic acid was found. Wu and Su (1990a) described ssRNA of Mr 2.0 x 10^6 associated with purified particles, which is consistent with the characteristics of a luteovirus despite other conflicting particle properties. However, Harding *et al.* (1991) and Thomas and Dietzgen (1991) described the possibility of ssDNA virus of 18-20 nm diameter constituted the genome of BBTV. The physical and chemical properties of the purified virus in this study was also inconsistent with the classification in the luteovirus group. The BBTV particles shared several properties with the SCSV virus, which were described to have ssDNA of 17-19 nm diameter and transmitted by aphids in the persistent manner and mechanically not transmissible.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (PAGE-SDS) of BBTV revealed that the virus had a major protein of molecular weight 21,000. The coat protein subunits of luteoviruses so far investigated were all of molecular weight 24,000. Wu and Su (1990) reported that BBTV possessed a single protein subunit of molecular weight 21,000 and they also reported that the BBTV viral protein showed a single major band with a Rf value between that of

ß lactoglobulin and α-chymotrypsinogen. Harding *et al.* (1991) reported of protein of molecular weight 20,100 and Thomas and Dietzgen (1991) detected a major protein of molecular weight 20,500 from the purified preparations of BBTV particles. The present findings are also similar to those of the above reports.

SDS-PAGE analysis of different parts of infected banana and healthy plants were conducted and observed more number of protein bands in diseased parts than in healthy samples.

Protein estimation studies also showed that the protein content of infected samples were more when compared to healthy sample. The increase in protein content might not be due to an increase in virus concentration alone but large amount of other non-viral proteins might also be produced during active virus synthesis. The present findings endorse the observations of Nair and Wilson (1970), who reported that the leaves of bunchytop infected plants contained higher percentage of total sugars, amino acids and tannin along with the presence of aspartic acid, alanine, methionine and isoleucine.

In India, this is the first successful attempt for the purification of BBTV, the nucleic acid identification and protein determination of the virus.

Antisera was successfully produced against BBTV with the partially purified/purified virus preparations. The BBTV antiserum thus produced had a titre of 1/128 and 1/512 in double diffusion and in ring interface tube precipitin tests. Detection of BBTV in crude sap of infected banana by chloroplast agglutination test was found successful because of large quantities of chloroplasts in crude sap. However, agglutination was not obtained with purified virus preparations probably due to the complete removal of chloroplasts in the purified preparations.

In double diffusion tests with crude sap and homologous antiserum precipitin band was not obtained due to low concentration of virus in the crude sap. The variation in the occurrence of precipitin bands/lines with purified virus preparations and their homologous antiserum was presumable due to low/high concentration of virus in the extract. But with purified preparations of BBTV used for the production of antiserum, the reaction was well clear in agar gel diffusion test. Purified BBTV placed one after the other with healthy preparation in alternate wells against their homologous antiserum in the centre well, a precipitin line in the form of 'arc' near the BBTV antigen well indicates that the antibody is moving faster than antigen and when a straight line is formed in between the two wells it indicated that both the reactants move at the same diffusion rate (Basu and Giri, 1993). They also reported that the double diffusion test is 2-5 times more sensitive than liquid or tube precipitation test. The present findings were also similar to that of Rajagopalan (1980) who had developed the serum containing polyclonal antibodies and tested the antigenic properties of BBTV by tube precipitation test and gel diffusion methods. He also reported that these methods involved large quantity of antiserum.

Enzyme linked immunosorbent assay (I-ELISA) was used for the detection of BBTV in crude extracts and in partially purified virus preparations from infected banana using BBTV antiserum/purified immunoglobulin thus obtained. ELISA could detect BBTV antigen at 10^{-3} dilution of crude extracts from infected host plants. ELISA absorbance values were found higher in extracts from midribs of infected plants followed by petiole, leafsheath and rhizome when compared with healthy extracts. The present findings on purification also yielded higher concentration of virus from the midrib portion of the top three leaves. These observations clearly established that virus is more concentrated on the midrib portion, thus implying that the midrib portion of the top leaves would be the best

part for conducting ELISA test. This finding is similar to that of Wu and Su (1990b) who reported that the BBTV is restricted to the phloem tissue in infected plants and observed higher ELISA absorbance values (1.5-5.0 times) from midribs than from leaf lamina. Thomas and Dietzgen (1991) also reported higher absorbance values at A_40_5 (1.3-4-6 fold) from midrib portion than from leaf lamina.

Wu (1987) produced monoclonal antibodies for the detection of BBTV from viruliferous aphids and phloem parenchyma cells of BBTV infected plants. Wu and Su (1990) reported that monoclonal antibodies produced were able to detect BBTV in crude extracts from BBTV infected plants and not from healthy sap.

A polyclonal antiserum to BBTV was prepared by Thomas and Dietzgen (1991) and was used for ELISA. They also reported that the detection limit for BBTV in the sap of 'Cavendish' banana was 1/128 when polyclonal Ig was used for coating and MAb 3D12 as enzyme conjugate in DAS ELISA. However, the limits were only 1/32 and 1/16 when MAb 3D12 and polyclonal Ig respectively were used for both coating and enzyme conjugates.

Mariappan and Mathikumar (1992) reported that the direct antigen coating (DAC) method has been found quite suitable in the detection of virus at a very high dilution of 1:1000. The OD value at 405 nm for the dilutions of 10^{-3} for antigen and 1:500 for antiserum was 0.412 for infected outer most sheath covering the rhizome as against 0.004 to 0.007 in healthy. They also reported that the samples containing virus inoculum even at a very low concentration was detectable by this method from the outer most sheath covering the rhizome.

From these findings, it is evident that the ELISA method is highly sensitive than the other methods and is helpful in identifying the virus infected plants even when the virus concentration was very low especially in recently infected plants which are devoid of visual symptoms of the bunchy top disease of banana.

Summary

SUMMARY

Banana is the major fruit crop of Kerala commonly affected by the infection of banana bunchy top disease. The disease is easily spread through infected suckers, which are the usual planting materials and secondary spread is through banana aphid *Pentalonia nigronervosa*. Though field level quarantine measures may check the spread of the viral disease, rapid and convenient methods for the detection and identification of virus in the suckers as well as micropropagated plants have not been developed.

In this background a study was designed and carried out to purify the banana bunchy top virus, to identify the nucleic acid of the virus and to produce antisera for developing a serological technique for the presymptomatic detection of the virus infection in the planting materials of banana. Electron microscopy of the purified virus preparation was also done to determine the particle morphology of the virus.

The studies included transmission of BBTV to healthy banana plants and to reported collateral hosts of banana viz., *Canna*, *Colocasia* and *Hedychium* through banana aphids as well as by mechanical inoculation. Purification of the virus from different source plants and from different plant parts (midrib, petiole, leaf sheath and rhizome) in different seasons was conducted as suggested by Thomas and Dietzgen (1991). Antiserum of BBTV was produced in Newzealand White rabbit by giving injections with purified virus preparation emulsified with Freund's adjuvant. Blood taken 7 days after last injection was used as antiserum for serological tests.

Serological techniques such as tube precipitation, agar gel diffusion, chloroplast agglutination and ELISA were conducted for the detection of virus

specific antigens in different parts of the plant (midrib, petiole, leaf sheath and rhizome). Nucleic acid was extracted from both healthy and diseased samples and were compared with DNA standard. Virus preparation was negatively stained with 2% Uranyl acetate to make observations on particle morphology.

The major findings of the study were as follows:

1. The survey conducted on the BBTD incidence in Thrissur district of Kerala revealed that the disease incidence and aphid population varied with seasons, the maximum incidence of BBTD and aphid population being during August-November and the minimum during December-March.

2. The typical symptoms of bunching of leaves stunted growth, profusely streaked leaves with chlorotic margins were observed often accompanied by secondary root decay caused by fungi and bacteria.

3. The mechanical transmission studies clearly indicated that BBTVwas not mechanically transmissible by using crude sap as well as by purified virus preparation.

4. Insect transmission studies conducted with *P. nigronervosa* revealed that the aphids successfully transmit the virus to the healthy banana and the tissue culture plants were found to be the most susceptible plants (50.67%) followed by rhizome buds (47.33%). The minimum incubation period was 17-27 days in tissue culture plants and 21-32 days in rhizome buds.

5. Aphid transmission studies in *Canna, Colocasia* and *Hedychium* did not produce any symptoms of BBTD and back inoculation to banana plants gave

negative results indicating that these plants are neither collateral hosts nor symptomless carriers of BBTV.

6. Virus-vector relationship studies revealed that BBTV was transmitted in a persistent manner by *P. nigronervosa*. A single adult was able to transmit the virus while a single nymph was not sufficient to transmit the disease. But in groups, the adults and nymphs acted as efficient vectors in carrying the disease.

7. The study indicated that the pre-acquisition fasting had no effect on increasing the transmission efficiency of the aphid. The percentage of transmission of BBTV increased with the increase in acquisition access period for both adults and nymphs of *P. nigronervosa*.

8. A minimum inoculation access period of 1 h. for the adult and 6 h. for the nymph of banana aphid was required for the successful transmission of BBTV.

9. Hundred per cent transmission was obtained after an incubation period of 24 h. for both adults and nymphs of *P. nigronervosa*.

10. BBTV was successfully purified from aphid inoculated tissue culture plants of Nendran. The average yield of the virus from tissue culture plants was 1.62 mg/kg tissue followed by rhizome buds (1.34 mg/kg tissue).

11. Purification from different portions of banana during different seasons yielded high concentration of virus from midribs of leaves (1.66 mg/kg tissue) during August-November.

12. Among the leaves of different positions, maximum concentration of virus was obtained in the top three leaves (1.65 mg/kg tissue).

13. Spectrophotometric studies with 1:10 dilution of purified preparations of BBTV showed a peak absorbance at 260 nm. A_{260}/A_{280} ratio of purified preparation of BBTV ranged from 1.39 to 1.44.

14. Purified preparation of BBTV was successfully transmitted to banana by *P. nigronervosa* through membrane feeding.

15. Electron microscopy of purified BBTV preparation revealed isometric particles of 18-22 nm.

16. Nucleic acid estimation studies revealed that RNA was more in healthy plants while DNA was more in diseased plants.

17. It was further revealed that the DNA obtained from diseased plant was of low molecular weight when compared to the DNA from healthy banana.

18. Nucleic acids extracted from both healthy and infected samples were compared. The bands obtained were sensitive to DNase I and S1 nuclease but not to RNase A, confirming the nucleic acid of BBTV as ssDNA.

19. SDS-PAGE analysis of BBTV coat protein revealed that it contained a major protein component of Mr 21000.

20. SDS-PAGE analysis of protein of diseased and healthy samples revealed that the infected tissues contained extracellular proteins in the midrib portion when compared to healthy ones.

21. Protein content was found higher in diseased plants when compared to healthy plants.

22. Antiserum was produced in rabbits and showed a titre of 1/128 in agar gel diffusion test and 1/512 in ring interface tube precipitin test.

23. Using BBTV antiserum, chloroplast agglutination test was found successful in detecting BBTV in crude sap of infected plants, while the agar gel diffusion could not.

24. Agar gel diffusion test was found successful to detect BBTV in purified preparations.

25. ELISA test conducted with BBTV antiserum gave positive reactions and was found to be a highly sensitive method for the detection of the virus.

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PURIFICATION AND SEROLOGY OF BANANA BUNCHY TOP VIRUS

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ABSTRACT OF THE THESIS

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ABSTRACT

Banana is one of the major fruit crop in Kerala and is often affected by the bunchytop disease caused by banana bunchytop virus. The disease is easily spread through infected suckers, which are used as the planting materials. Secondary spread is also seen through banana aphid, *Pentalonia nigronervosa*. Though field level quarantine measures may check the spread of the disease, rapid and convenient methods for the detection and identification of the virus in the suckers as well as in micropropagated plants have not been developed.

In this background a study was designed and carried out to purify the BBTV, to produce antisera for developing a serological technique for the pre-symptomatic detection of virus in the planting materials of banana. Studies were also conducted to identify the type of nucleic acid of the virus and its morphology by direct electron microscopy.

The study revealed that the disease incidence was maximum during August-November. The virus was not mechanically transmitted and tissue culture plants were the most susceptible planting materials for aphid transmission.

Basic studies of virus-vector relationship were also conducted and the adult aphids were found to be effective vectors. In purification studies, among the different portions of banana plants used, the midribs of younger leaves yielded high concentration of the virus. Tissue culture plants yielded more virus concentration than other planting materials.

Electron microscopy of the purified BBTV preparation revealed isometric particles of 18-22 nm size.

Nucleic acids extracted from both healthy and infected samples were compared. The bands obtained were sensitive to DNase 1 and S1 nuclease but not to RNase A, confirming the nucleic acid of BBTV as ssDNA.

SDS-PAGE analysis of BBTV coat protein revealed that it contained a major protein component of Mr 21000 with Rf value between that of β lactoglobulin (Mr 18400) and α chymotrypsinogen (Mr 25700).

Antiserum of BBTV was produced in the rabbit and used for detection of virus specific antigens in different parts of the plant (midrib, petiole, leafsheath and rhizome) by chloroplast agglutination, agar gel diffusion, tube precipitation and ELISA. Among these methods ELISA was found to be highly sensitive for identification of the virus.