Cytopathological effects of banana bunchy top virus (BBTV) and production of infected free banana plants using in vitro culture technique

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Abstract: Banana Bunchy top virus (BBTV) is the most important banana disease worldwide. This study focused on the production of BBTV-free seedlings from infected banana plants. Suckers from the Egyptian plantain Behaira gov., (cv. Grandinane) with distinguished BBTV symptoms were collected. decrease in the number of xylem vessels, severe necrosis of xylem and phloem vessel cells, with blockage of these vessels and the presence of dark-colored foreign materials. Several anatomical changes were observed reflecting the external symptoms on infected plants. Examination of ultrathin sections by Transmission Electron Microscopy revealed changes in the chloroplast, cytoplasm and in nucleus. Cross section of BBTV by microtome technique revealed that Xylem and Phloem cells appear necrotized and blocked with dark stained material. Meristematic apices excised from those suckers were cultured *in vitro* and sub-cultured five times. The presence of BBTV was evaluated by the Double- Antibody Sandwich Enzyme-linked Immunosorbent Assay (DAS-ELISA). The BBTV was detected in all suckers prior to *in vitro* experiment. Results revealed that the regenerated plantlets from meristematic tissues were virus-free. This indicates that *in vitro* culture is a simple and the most effective tool to generate BBTV-free plants.

Keywords: Tissue culture, DAS-ELISA, BBTV, Cytological changes

INTRODUCTION

Banana is an important fruit crop in the world that is cultivated in 8,923,584 hectares, and its annual production is 182,784,070 tons. In Egypt, the total cultivated area is 95 thousand fed., and annual production is 1.29 million tons (FAO, 2021). Viruses are a major concern to banana and plantain production because of their negatively effects on yield and quality and is considered a constraint to the international exchange of *Musa* germplasm. Direct losses are incurred from reduced production, and indirect losses are associated with maintaining plant health, including the production of virus-free planting material. BBTV is among the top 10 viruses worldwide in terms of economic impact. One of the most important crops in the tropical and subtropical regions is the banana, Musa spp. It is grown in more than 130 countries and provides millions of people in these areas with food and income. The most harmful virus that has a catastrophic impact on the output of banana crops is the banana bunchy top virus (BBTV) (Lassois *et al.,* 2013). BBTV was detected by PCR in samples collected from all islands except Kalimantan in Indonesian. Molecular analysis revealed that all BBTV isolates belonged to the Southeast Asian (SEA) subgroup. Based on the DNA-S and DNA-C analysis, the isolates from Sulawesi and Halmahera islands were closely related to those from the Philippines, while the remaining isolates were highly similar to those previously reported from Sumatra, Java, and Bali (Rahayuniati, 2021). Viral diseases are considered one of the most affecting diseases on the productivity of the banana plants due to the losses of crop quality and quantity of the banana fruits, in addition to the difficulty of exchanging the banana seedlings among the countries worldwide (Kumar *et al.,* 2015; Rahayuniati *et al.,* 2021). *Banana bunchy top virus* (BBTV), *cucumber mosaic virus (CMV), and banana streak virus* (BSV) are important banana viruses; there are possible infections frequently with several viruses in the field. Since the viruses are readily transmitted by vegetative propagates, that pose a threat to banana production in banana-growing areas. BBTV genus Babuvirus, family Nanoviridae was discovered in Egypt in 1901. Meristem tip culture and chemotherapy are just a few examples of the many therapeutic in vitro techniques that have been used (Lassois *et al.,* 2013)

In Egypt, *Banana bunchy top virus* (BBTV) is one of the limiting factors in the production of banana crop (Magee, 1940). BBTV was first reported in Fiji Island in 1889 (Magee, 1953). BBTV endangers production in all diseased plantations, destruction occurred, and virus-free plants must be replanted. (Dale, 1987) BBTV is transmitted by the banana aphid (*Pentalonia nigronervosa*) in a persistent manner and also the virus is transmitted through infected plant suckers and other plant components used in banana propagation but is not sap transmissible. Thabet (2000) used double antibody sandwich-ELISA (DAS-ELISA) for detection of *banana bunchy top virus* (BBTV) in different parts (blade, midrib and pseudo-stem) of the infected banana plants and found that the highest concentration of BBTV in the midrib, blade and pseudo-stem respectively in the crude extract. *Banana bunchy top virus* (BBTV) primarily infects *Musa spp.* and causes *banana bunchy top disease* (BBTD), the most serious viral disease in global banana cultivation, according to (Arimbawa *et al*., 2022). Previously double antibody sandwich-ELISA (DAS-ELISA) and Dot-blot immunoassay (DBIA) used as serological methods for detection BBTV in infected samples Hu *et al.,* (1993). El-Dougdoug and El-Shamy (2011) used double antibody sandwich enzyme-linked immunosorbent

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assay (DAS-ELISA) using specific polyclonal antibodies for detectioning of *Banana bunchy top virus* (BBTV) and found that the samples were infected with BBTV. Then, the meristem tip (0.3 mm) of infected samples were used in tissue culture technique for eradication BBTV and found that this technique was very effective for BBTV eradication. Genome consists of at least six components of circular single stranded DNA (cssDNA) each of about 1 kb (Xie and Hu, 1995). The cssDNA was initially known as BBTV DNA-1 to -6 but recently were renamed BBTV DNA-R, -U3, -S, -M, -C and –N (Vetten *et al*., 2005). However, once BBTD is established in the field, it is very difficult to eradicate or manage it. Therefore, a more sustainable way of controlling the disease is to develop host plant resistance against the virus and the vector (Jekayinoluwa *et al*., 2020).

The aim of the present study was to isolate and identify the Banana bunchy top virus (BBTV) affecting Banana trees on the bases of symptomatology, study the cytological changes in BBTV infected banana trees and produce virus - free plants through meristem tip culture technique.

MATERIALS AND METHODS

1-Sample collection and virus isolation: From naturally infected banana trees of the cv. Williams and Grandnain in four different governorates (Qalubyia, Giza, Ismailia, and Behira), three distinct types of symptoms were collected. Four hundred samples, including both healthy and infected samples, were collected in 2020 (100 samples for each government). The visual symptoms of banana bunch top disease (bunchy top, yellow margins, and dark green streaks on leaf veins and midribs) were included in the samples that were collected (Fig. 1). As a result, all samples were tested by DAS-ELISA to determine whether BBTV was present. A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to test whether the samples were infected with the *banana bunchy top virus* (BBTV) or the *cucumber mosaic virus* (CMV). At 405 nm, an ELISA reader (Microplate Reader Bio Tek) was used to measure the plate (Clark and Adams, 1977).

Fig. (1): BBTV naturally infected banana trees, (A): William c.v. and (B): Grandnain c.v. showing (A and B) stunting, bunchy top. (C): Yellow at the margins and dark green streaks on leaf veins and midribs.

2- Molecular detection for BBTV: -

2.1. Extraction of total nucleic acid: According to the manufacturer's instructions, the Qiagen Kit (Qiagen Sciences, USA) was used to isolate DNA from leaf samples of infected banana exhibiting typical BBTV symptoms and uninfected banana. Total nucleic acid was extracted from fresh leaf samples of banana infected with the BBTV isolate.

2.2. Detection of BBTV by Polymerase Chain Reaction (PCR): Specific primers vBBTV-1 (5'-GTTCTCCAGCTATTCATCGCC-3') and cBBTV-1 (5'-CATCATCGACGACGAAATGGC-3') of DNA-1 for detection of banana bunchy top virus isolate DNA-1 specific primers, replication associated protein gene was used to amplify approximately 476 bp according to Shamloul *et al*., (1999). PCRs were performed with a total volume of 50 µl, containing 5µl DNA (50 ng/µL), 25µl master mix (OnePCR™ genedirex, Cat. No. MB203-0100), 2.5µl of each forward and reverse primer and 15µl of nuclease-free water. The PCR programme contained 35 cycles: denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C, and finally extension at 72 °C for 5 min. A 1.5% agarose gel was used to visualize amplified products.

3-Cytopathlogical effects: -

3.1. Anatomical changes: It was intended to carry out a comparative anatomical study on the leaves of banana seedling infected with BBTV in addition to healthy seedling (control) to determine the anatomical abnormalities which may occur in the stem of leaves of banana infected with BBTV. Sections of the infected and healthy leaves were made at 15-17 µm thick using rotary microtome then the small sections were taken from leaves then killed and fixed in FAA (10 ml formalin, 5 ml glacical acetic acid and 85 ml ethyl alcohols 70%), washed in 50% ethyl alcohol, dehydrated in a series of ethyl alcohols (70, 90, 95 and 100%), infiltrated in xylene embedded in paraffin wax with a melting point 60-63ºc. Sections were mounted on glass slides and stained with aqueous Safranin (1%) and Fast Green (0.1% in 95% ethanol), as described by Ruzin (1999). Sections were microscopically inspected to detect histological manifestations of noticeable responses resulted from the infection.

3.2. Ultra-thin sections: Infected banana leaves tissues were examined using thin sections by transmission electron microscope. The infected leaves were cut into small pieces about 1-2 mm., fixed in 2% Glutralhyde in 0.1 M Na-Cacodylate buffer, pH 7.2 and subjected to a vacuum for 1-4 min every 15 min for 2 hours on ice. Prior to vacuum treatment, floating samples were poked under the buffer surface with pointed metal pokers. Rinsing took place in 0.1 M Na-Cacodylate buffer, pH 7.2 for 45 min., with buffer changes at 15 buffer surface with pointed metal pokers. Rinsing took place in 0.1 M Na-Cacodylate buffer, pH 7.2 for 45 min., with buffer changes at 15 and 30 min. Further fixation in 1% Osmium Tetraoxide in Na-Cacodylate buffer, pH 7.2, under intermittent vacuum and poking, took place for 1.5 hours (Osmont and Freeling, 2001). The samples were rinsed in the Na-Cacodylate buffer then, dehydrated through an ethanol series in buffer (35 - 50 - 70 - 80 - 95 - 100%) for 60 min. and then infiltrate with resin. Propylene: resin (no accelerator) 2:1and 1:1 for 1hr; Propylene: resin (no accelerator)

1:2 for 1 hr ; Pure resin (no accelerator) for 1 hr; Pure resin (no accelerator) overnight; Pure resin+accelerator for 2 hr and Embed samples into molds 60°C oven. Semi-thin sections were prepared on glass slides through cutting at 1 µm using the ultra-microtome. Semi-thin sections were cut and stained with Toludine blue for 5 min. and examined by light microscopy model Olympus UC 30 BX 53 Both semi thin and ultrathin -sections were cut using ultra-microtome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mish). Ultra-thin sections were stained with double stain (Uranyl acetate 2% 10 min., followed by Lead citrate for 5 min and examined by transmission electron microscope (Cairo univ.) JEOL (JEM–1400) at the candidate magnification. Electron micrographs were captured by CCD camera model AMT, optronics camera with 1632 x 1632-pixel format as side mount configuration.

3.3.Meristem Tip culture: Suckers of the two cultivars were collected from plants with visual symptoms of BBTV. A total of 20 infected suckers were collected and tested using DAS-ELISA and were confirmed as positives. The DAS-ELISA method used involved BBTV extraction from the leaves, incubation and addition of monoclonal antibody and antibody coupled to alkaline phosphatase B in the presence of positive and negative BBTV controls. All the processes were conducted in the laboratory of the virus and phytoplasma department, serological lab, agricultural research center. As a starting point, the meristem tip used to produce BBTV free plants. Meristem was isolated from banana cv. Grandnaine suckers infected with the disease were grown under field conditions when they were around four months old. The explants were cleaned with flowing water from the faucet. Afterwards, 70% ethanol was used for one minute of surface sterilization in a laminar airflow cabinet, followed by 10% sodium hypochlorite for 20 min. After that, sterile distilled water was used to wash the explants three to four times. Direct cultivation of the meristematic tissue took place in a culture tube containing 50 ml of MS media (Murashige and Skoog, 1962) supplemented 4.4 g MS, 30 g L−1 sucrose, 6 g L-1 agar, and 0.1 mg L 3- α - naphthalene acetic acid (NAA) at pH 5.7 at a density of 10 shoots per culture jar. Following cultivation, cultures were incubated for 16 hours every day in a culture room with 40 µmole m-2 s-1 cool white, fluorescent light, a temperature of 27±2ºC, and optimal light (1-10 K Lux). Every month, in vitro shoots, were subcultured, increasing the number of shoots.

In vitro cultures of infected plants were established on standard media with mineral salts (Murashige and Skoog, 1962) (Figure 4). This medium was enriched with 30 g/l of sucrose, 2 g/l of gelrite, nicotic acid (0.5 mg/l), pyridoxine (0.4 mg/l), thiamine (0.5 mg) and 2 mg/glycine and supplemented with a 10µM 6-benzylaminopurine (BAP) and 1µM of indole acetic acid (IAA) according to Banerjee et al. (1985, 1986) and Vuylsteke (1989) (Figure 3). Each cultivar was sub-cultured 5 times at one-month intervals. The *in vitro* plants were regenerated and acclimatized in the screenhouse for two months until the plantlets reached a size of 20 cm and then tested twice for BBTV by DAS-ELISA.

RESULTS

1-Virus isolation: Three types of different symptoms were collected from banana trees naturally infected *with M. acuminate*. cv. Grandnaine and William from four different governorates (Qalubia, Giza, Behira and Ismailia), respectively. Symptoms include visual symptoms of *banana bunchy top disease* (bunchy top, yellow at the margins, dark green streaks on leaf veins and midribs).

2-PCR Amplification of BBTV DNA Components: BBTV was detected using primers specific to the coat protein coding sequences and replicas pair coding sequences of healthy and infected banana plants, and total DNA was successfully isolated and used as a template for direct PCR. The coat protein gene amplicon was 476 bp in the symptomatic Banana sample (Fig. 2). In a PCR combination that included DNA from healthy samples, the identical primer pair failed to produce an amplicon. From infected tissues, an important DNA fragment of the predicted size, 476 bp, was amplified (Lane 2). With sample uninfected banana leaves (Lane 1), there was no amplification; Lane N is the negative control.

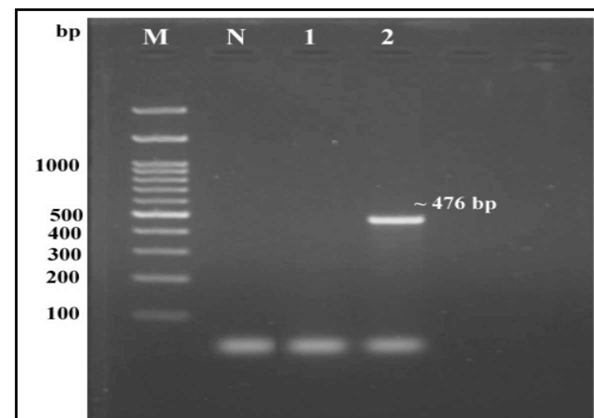

Fig. (2): 1.5% Agarose gel electrophoresis analysis of BBTV amplified product for DNA-1 component using vBBTV-1- and cBBTV-1-specific primers. M: 100 bp DNA marker, Lane N: negative control, lane 1: healthy banana plant and lane 2: PCR product from BBTV DNA-1 component infected banana plant.

3-Cytological changes: -

3-1-Anatomical changes: The anatomical abnormalities which may occur in the leaves of banana seedlings infected with BBTV were determined using microtome sections. The obtained results revealed that the cells of pith, xylem, phloem, and vascular bundle were normal in the healthy control. While in the infected leaves, the cells of xylem were compacted. The number of xylem vessels was clearly reduced. Xylem and Phloem cells appear necrotized and blocked with dark stained material. The cells surrounding the vascular bundles and the cambium appear hypertrophic with undulated walls (Fig. 3)

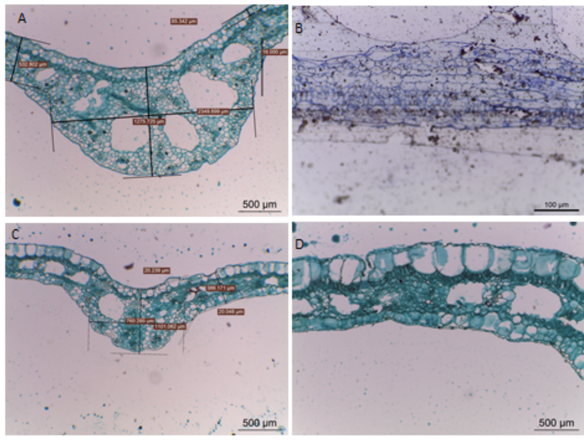

Fig. (3): Cross section on leaves of healthy and infected banana seedling showing normal pith, xylem, phloem and vascular bundle in the healthy cells (A & C) and reduced and necrotized xylem, blocked Phloem cells in infected cells (B & D). (X40).

3.2. Ultrathin section: Ultrathin sections were examined using transmission electron microscopy. Investigations reveal that mesophyll cells of BBTV-infected banana leaves showed laminated associated with vesicles, as well as degradation of chloroplast. The changes in the different tissues and cells organelles of BBTV- infected banana leaves were illustrated in (Figs. 4-7). The number and organization of chloroplasts were different in cells of infected tissues. The chloroplasts exhibited several degrees of deformation and lyses. The nucleus of the infected cell also was affected as observed to be misshapen.

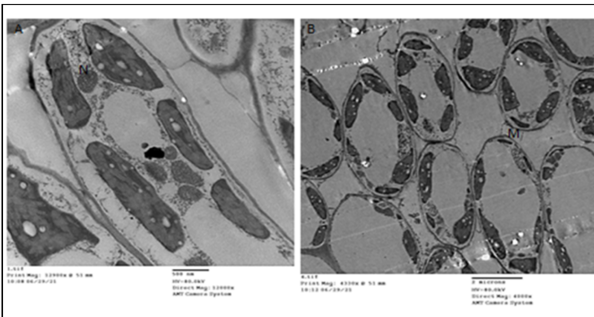

Fig. (4): Ultrathin section of healthy banana leaves showing normal chloroplasts and nucleolus in the cells. (A&B).

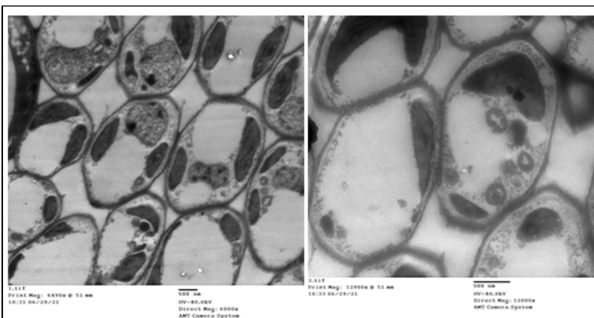

Fig. (5): Ultrathin section of infected banana leaves showing deformation, lyses of chloroplasts, degradation of chloroplast, Grana of thylakoids and their disconnecting lamella.

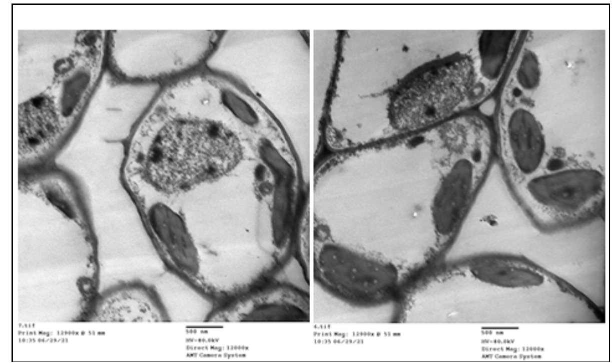

Fig. (6): Ultrathin section of infected banana leaves showing misshapen of nucleus. Parenchyma tissue revealed thin and degradation cell wall and intracellular.

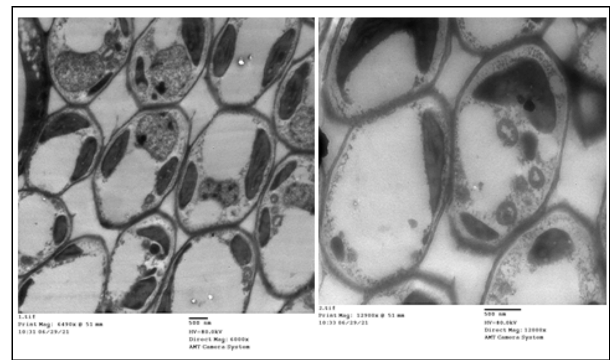

Fig. (7): Ultrathin section of infected Palisade tissue revealed infected irregular, Elongated cells, big intracellular and small vacuole, and nucleus degradation.

3-2. Meristem tip culture: Plantlets regenerated from successfully meristem tips approximately 3 mm long were tested by visual inspection and ELISA for BBTV, and the absorbance values of meristem tips were close to those of the negative control, then the plantlets were transferred to greenhouse for acclimatization. (Figs. 8 and 9).

Fig. (8): Elimination of BBTV by tissue culture technique.

Fig. (9): Banana plantlets free of BBTV produced by tissue culture technique.

DISCUSSION

Plant diseases caused by viruses cause substantial losses worldwide but are particularly important in the tropics and regions The first requirement for the control of such disease is the identification of the Banana Bunchy Top virus or other viruses. Banana bunchy top disease (BBTD) is the most economical disease affecting banana plants and causes severe disease, and infected plants usually do not produce any fruits or economically valuable fruits. In this study, work was conducted to investigate, detect, and identify the occurrence of BBTV in some banana samples collected under Egyptian conditions. Moreover, the distinctive characteristics of BBTV and their effect on the banana plant host (main host) were also studied, and *banana bunchy top virus* (BBTV) was identified. Investigating such observations revealed a *Banana Bunchy Top Virus*-like infection in the area. These results are in agreement with that of several authors (Thomas and Dietzgen, 1991; Espino et al., 1993; Thabet, 2000; Rezk, 2001; Yasmin et al., 2001; Hooks et al., 2009; Nelson, 2004; Selvarajan et al., 2011; Watanabe et al., 2013). dark streaks on the shoot; yellowing and dark green streaks. The representative samples were collected and tested by serological and molecular assays. Preliminary results confirmed the presence of the *Banana Bunchy Top Virus*, which motivated the initiation of this study. BBTV is an emerging viral pathogen that is highly virulent, very aggressive, and fast spreading, belongs to the genus Babuvirus and causes significant yield losses to banana plants and their fruit quality (Mansour et al., 2013). Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used for serological detection of BBTV in doubtful samples collected from different governorates. The technique was performed according to Clark and Adams (1977). DAS-ELISA succeeded in detecting BBTV in 10 Behira governorate samples (40%), one Giza governorate sample (5%) and no Qalubia governorate samples (0%). DAS-ELISA was previously used to detect BBTV. Several investigations (Lestari and Hidayat, 2020; Rahayuniati *et al*., 2021) have established the validity of PCR as a BBTV detection technique. Hapsari *et al*. (2023) confirmed that 10 Indonesian banana accessions had been infected with the banana Bunchy top virus (BBTV), this study used a PCR assay with a primer from the BBTV coat protein (CP) gene. Cytological changes induced by viruses were studied in detail by examining semi-thin and ultra-thin sections under an electron microscope in host-virus combinations, specific or broad changes in ultra-structure of the cellular constituents, which represent the signature of the virus groups or even individual viruses. Moreover, in infected cells the number of chloroplasts was reduced than healthy ones. This may be due to the degradation of the chloroplasts. This finding reflects the symptoms of bunchy top on BBTV-infected leaves. Lassois et al. (2013) reported that meristem culture was the approach that most efficiently and rapidly removed phloem-associated viruses. For this approach to function, the meristematic dome of the plant must be isolated. This process requires isolating the meristematic dome of the plant under aseptic circumstances and nurturing it in the appropriate nutritive media to produce new plants free of viruses.

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تأثيرالتغيرات السيتولوجية لفيروس تورد القمة في الموز علي نباتات الموز وانتاج نباتات موز خالية من الفيروس باستخدام تقنية زراعة الانسجة

نادي أحمد حامد - أحمد شوقي جمال الدين - عبدالعزيز علي سلام

معهد بحوث امراض النباتات - قسم بحوث الفيروس والفيتوبلازما – مركز البحوث الزراعية - جيزة مصر
جامعة قناة السويس – كلية الزراعه - قسم النبات الزراعي - الاسماعيلية – مصر

يعد فيروس تورد القمة في الموز (BBTV) من أهم أمراض الموز في العالم. ركزت هذه الدراسة على إنتاج شتلات خالية من BBTV من نباتات الموز المصابة. باستخدام كورمات من الموز المصري محافظة البحيرة (cv. Grandinane) يظهر عليها اعراض اصابة مثل انخفاض ملحوظ في عدد أوعية الخشب ونخر شديد في خلايا الخشب وأوعية اللحاء مع انسداد هذه الأوعية ووجود مواد غريبة داكنة اللون. وقد لوحظت العديد من التغيرات التشريحية التي تعكس الأعراض الخارجية على النباتات المصابة. كشف الفحص بواسطة المجهر الإلكتروني النافذ عن تغيرات في البلاستيدات الخضراء والسيتوبلازم والنواة. بواسطة تقنية الميكروتوم للمقاطع العرضية لـ BBTV، تظهر خلايا الخشب واللحاء منخرية ومصبوغة بمادة داكنة اللون. تم استزراع القمم المريستيمية المستأصلة من هذه الكورمات في المعمل وتم نقلها خمس مرات. تم الكشف وجود BBTV بواسطة إختبار (DAS-ELISA). تم اكتشاف BBTV في جميع الكورمات قبل الزراعة في المعمل. وكانت النباتات المجددة من الأنسجة المرستيمية خالية من الفيروسات. يشير هذا إلى أن زراعة الانسجة هي أداة بسيطة وأكثر فعالية لإنتاج نباتات خالية من فيروس تورد القمة في الموزBBTV.