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Molecular detection of banana bunchy top virus and chemotherapy for production of virus-free banana plantlets

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ABSTRACT

One of the most significant banana diseases in Egypt is Banana Bunchy Top Disease, which is brought on by the *Banana Bunchy Top Virus* (BBTV). The creation of BBTV-free plantain seedlings from infected banana plants was the main goal of this investigation. Four separate governorates provided samples (Qalubyia, Giza, Ismailia, and Behira). All collected samples underwent BBTV testing using DAS-ELISA, which revealed that the virus infection was recorded in all inspected governorates in different ratios. The diagnostic host of BBTV was limited, infecting banana, ginger, and bird of paradise using mechanical inoculation, while only banana and ginger were infected using insect inoculation with *P. nigronervosa*. Polymerase chain reaction (PCR)-based detection of an amplicon from BBTV-infected banana tissues (476 bp) The multiple sequence alignment showed that EGY-Behira isolates had a high similarity of 99.7% with isolate BBTV-replicate from India. The phylogenetic tree for the BBTV-Eg Behira isolates is based on the partial nucleotide sequence alignment of the DNA-A genome. BBTV was successfully eradicated from infected plants using a combination of meristem tip culture and thermotherapy at 40 °C for two months. Virazol and Salicylic acid provided the greatest polyphenol oxidase activity (1.64 and 1.78) as well as highly enzymatic activity (2.021 and 1.96 for peroxidase). Significant biochemical changes were represented by a relative reduction in Chl a, Chl b, and carotenoid contents. It was observed that the highest reduction in chlorophyll b was 51.47%, followed by chlorophyll a+b at 51.27, chlorophyll a at 51.17, and carotenoids at -2.65, respectively.

Keywords: *Banana bunchy top virus;* Biochemical; ELISA; PCR; Antiviral activity.

INTRODUCTION

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 (Tripath *et al*., 2016). 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. Molecular analysis revealed that all BBTV isolates belonged to the South East 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 it causes to production as well as the quality of the banana fruits, in addition to the difficulty of exchanging the banana seedlings between the countries worldwide (Kumar *et al.,* 2015; Hamim *et al.,* 2017; Sila *et al.,* 2020; Rahayuniati *et al.,* 2021). Banana bunchy top virus (BBTV) has been registered as one of the world's 100 oldest known disease pathogens Lowe *et al.* (2000). 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 in vegetative propagates, which pose a threat to banana production in banana-growing areas. BBTV genus Babuvirus, family Nanoviridae was discovered in Egypt in 1901. Each DNA component has two conserved regions, the stem-loop common region (CR-SL) and the major common region (CRM) 3. Previous studies 4,5,6 have shown that components 1, 3, 4, 5 ,and 6 encode the replication-associated protein, coat protein, intercellular transport protein, retinoblastoma binding protein, and nuclear shuttle protein, respectively (Horser *et al.,* 2001). BBTV particles have an icosahedral structure and a coat protein that is roughly 20 KDa in size (Harding *et al*., 1993, Nour El-Din *et al*., 2005; Rahayuniati *et al*., 2021) The viral genome was identified as a circular single-strand DNA (ssDNA) (Harding *et al*., 2000; Salama *et al*., 2007; Amin *et al*., 2008) it is a member of the family Nanoviridae and the genus Babuvirus

(Mandal 2010; Vetten *et al*., 2005; Vetten *et al*., 2012). The DNA-1 to DNA-6 BBTV components are made up of several stem-loop common regions, each of which has a length of 69 nucleotides, and six single circular segments of ssDNA with lengths ranging from 1000 to 1100 nucleotides. (Burns *et al.,* 1995; Harding *et al.,* 2000). These segments were given the new names DNA-R, -U3, -S, -M, -C, and -N. The DNA-R segment was coded into two open reading frames, and the remaining segments were each encoded as a separate protein (Burns *et al*., 1995; Beetham *et al*., 1997; Hafner *et al*., 1997). The genes for replication, coat protein, and movement protein have each been demonstrated to be encoded by the three DNA segments DNA-R, -S, and -M, respectively (Burns *et al.,* 1995). Lately, the virus has destroyed 30% of Egypt's banana crop. As a result, the cultivation of virus-free plants is considered to serve as the basis for the treatment of viral infections. Cryotherapy, thermotherapy, meristem tip culture, and chemotherapy are just a few examples of the many therapeutic in vitro techniques that have been used (Hazaa *et al*., 2006; Kabir Shiragi *et al*., 2008; Lassois *et al*., 2013). In vitro, chemotherapy techniques have been used to create virus-free plantlets for important crops. It was necessary to search for a way to get the illness under control to cultivate virus-free varieties of plants such as apple, apricot, peach, tomato, and banana (Paunovic *et al*. 2007; Hazaa *et al*., 2006; Falcioni *et al*., 2014; Paprstein *et al.,* 2013). Salicylic acid and ribavirin (Virazole) were tested among all antiviral agents. This antiviral compound acts on virus synthesis rather than through direct activation of the existing virus. The antiviral compound works directly or indirectly by stopping the synthesis of new virus particles while the existing virus particles are decreased in the course of their ontogeny (Lassois *et al*., 2013).
This research aimed to:

1- Identify viruses utilizing symptomatology, ELISA, and polymerase chain reaction methods.
2- Evaluate the efficacy of chemotherapy for eradicating the *banana bunchy top virus* from infected banana plantlets grown in vitro by using ribavirin (Virazole) and salicylic acid.
3- Determination of the oxidative enzymes as a response to chemical treatment
4- Using Meristem Tip culture: for producing BBTV-free banana plants

MATERIALS AND METHODS

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 (Clark and Adams 1977).

Virus detection using Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA):

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).

BBTV Host rang study:

Host range:-

With sap extracted from infected banana leaf tissues ground in cold 0.05 M phosphate buffer pH: 7.0 and insect inoculation using *Pentalonia nigronervosa* from infected banana plants, eight plants of the potential alternative virus hosts species belonging to 8 families (Table 1) were mechanically inoculated. The inoculated plants were maintained in a greenhouse at a temperature of 28 + 2°C with a 14-hour light/ten-hour dark condition, and then daily symptom expression was observed on the inoculated plants.

Table 1. Diagnostic host plants tested against BBTV isolated from naturally infected banana cv.

No.	English name	Scientific name	Families
1	Banana	*Musa*	Musaceae
2	Red Ginger	*Alpinia purpurata*	Zingiberales
3	Canna	*Canna indica*	Cannaceae
4	Bird of paradise	*Strelitzia reginae*	Strelitziaceae
5	Alocasia	*Alocasia brisbanensis*	Araceae
6	Heliconia	*Heliconia*	Heliconiaceae
7	Orchidantha	*Orchidantha maxillarioides*	Lowiaceae
8	Costaceae	*Tapeinochilos ananassae*	Costaceae

Aphid transmission:

These investigations used micro-propagated banana plants (cv. William and Grandnaine), all of which were derived from a specific source and were confirmed to be free of BBTV by DAS-ELISA. Ain Shams University's Plant Protection Department graciously provided groups of 20 *Pentalonia nigronervosa* aphids, which were fed for 24 hours. Subsequently spread to healthy banana plants from the infected BBTV plants (5 adult insects per plant). Following 24 hrs, a systemic insecticide (Malathion 57% EC) was sprayed on the inoculated plants to kill the aphids. The banana bunchy top disease's visual symptoms were observed on the control and inoculated plants for two months while DAS-ELISA testing was conducted (Thabet, 2000).

Molecular detection for BBTV virus in Banana trees:

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 naturally infected banana trees exhibiting typical BBTV symptoms and uninfected banana. Total nucleic acid was extracted from fresh leaves samples of banana infected with BBTV isolate.

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 about 476 bp according to PCR reactions were done with a total volume of 50 µl, containing 5µl DNA (50 ng/µL), 25µl master mix (OnePCR™ genedirex, Cat. No. MB203-0100), 2.5µl from each forward and reverse primers and 15µl of nuclease-free water. PCR program containing 35 cycles of denaturation for 1 min at 95 ° c., annealing for 1 min at 55 ºC., extension for 1 min at 72 ºc., and finally the extension at 72 °C step for 5 min at 72 ^{o}c Under ultraviolet (UV) light, a 1.5% agarose gel was used to visualize amplified products (Shamloul *et al.,* 1999).

Sequence analysis:

Based on DNA sequencing, the viral isolate was identified. DNA-1 replication-associated protein gene-specific primers were utilized in the PCR amplification of a purified DNA amplicon using a Geneaid PCR purification kit according to the manual protocol. The multiple sequence alignment tool Clustal Omega was used to create the phylogenetic trees. The sequence was performed using an ABI 3730xl DNA Sequencer at the facilities for gene analysis, Analysis Company, & Color Lab (Egypt). The multiple sequence alignment tool Clustal Omega was used to create the phylogenetic trees (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Viral Elimination Strategies for BBTV:

Chemotherapy Treatments:

In vitro BBTV-free banana plantlets were created using two different antiviral compounds. 5 mm long meristem tips were excised and cultivated in basal media that had salicylic acid and virazole added at different concentrations (0, 10, 20, 30, and 40 mg/l). Using sterile 0.2 m filters, the chemical compound stock solutions were filtered. During rooting, these substances were added to half-strength MS media without any plant growth regulators. The cultures were then incubated as previously indicated. Every week, the growth index was determined. To detect BBTV, DAS-ELISA was performed when the roots began to appear in the medium (after 25–30 days).

Meristem Tip culture:

As a starting point, the meristem used to. It 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 as a result.

Plant regeneration from in vitro multiplied buds:

In vitro proliferated shoots were cut into separate sections and placed on culture media with varying concentrations of 0.1 mg L^{3-} naphthalene acetic acid (NAA). to distinguish the shoots for the formation of the roots

Determination of Peroxidase (POD):

Peroxidase activity in a 4 ml light path cuvette to measure the oxidation in the presence of hydrogen peroxide and the enzyme at intervals of 30 sec. The reaction mixture contained 8 µmoles of hydrogen peroxidase, 60 µmoles of guaiacol, and 60 µmoles of sodium acetate buffer in a volume of 3 ml. PH 5.6 and peroxidase concentrations which produced a linear response over 3 min. A unit of peroxidase activity is the amount of enzyme that causes one optical density (OD) change per minute. The reaction is initiated by adding the enzyme and mixing (Ghazi, 1976).

Determination of polyphenol oxidase (PPO):

Polyphenol oxidase (PPO) activity was assessed using a 2401 PC UV- Vis recording spectrophotometer (Central Lab., for Biotechnology) to measure the initial rate of quinine production, which would be indicated by an increase in absorbance at 420 nm (Coseteng and Lee, 1987). PPO activity was assessed in triplicate measurements, with one unit of enzyme activity being defined as the amount of enzyme that generated a change in absorbance of 0.001/min. 2.95 ml of a 20 nM catechol solution in 0.1 M phosphate buffer composed the sample cuvette 0.05 ml of the enzyme solution at PH 6.0. The amount of substrate solution in the blank sample was just 3 ml.

Determination of photosynthetic pigments:

According to Wettstein, (1957) chlorophyll a, b, and carotenoid were extracted and estimated. To neutralize organic acids in the fresh leaf homogenate, fresh leaf samples (0.5g) were homogenized in a mortar with 85% acetone, washed dried sand, and a little amount of CaCO3 (0.1g). After that, a powdered glass funnel was used to filter the homogenate. Acetone was used to wash the residue several times until the filtrate was colorless. A spectrophotometer was used to measure the optical density of this extract at 440 nm for carotenoids and 662, 644, and 662 nm for chlorophyll, respectively.

RESULTS

Virus isolation and propagation:

All banana samples were examined for the presence of the cucumber mosaic virus and banana bunchy top virus (BBTV) (CMV) using (DAS-ELISA). The leaves of naturally infected cv. Grandnain banana trees from the four governorates had a bunchy top, yellow margins, and dark green streaks on the veins and midribs (Fig. 1). The results clearly showed that BBTV is the major limiting virus in these germplasms. BBTV (67.2%) and CMV (32.8%) occurrences as well as viral symptoms were confirmed in the Grandnain cultivar (despite all examined cultivars having been grown under the same conditions for a lengthy period) (Table 2). El-Behira had the highest infection percentage rate (18%), while Ismailia had the lowest percentage (8.25%).

Fig. 1. Banana trees with natural sources BBTV infections of the Grandnain cultivar show stunting, bunchy top, yellow at the margins, and dark green streaks on the veins and midribs.

Table 2. Occurrence of BBTV and CMV viruses in infected banana samples using DAS-ELISA.

Governorate	No. of samples	No. of infected samples	Infected ratio % for total infected	Virus type		Infected ratio %	
				BBTV (W.cv)	CMV (G.cv)	BBTV	CMV
Giza (ARC)	100	42	10.5	28	14	66.7	33.3
Qualubyia	100	36	9	22	14	61.1	38.9
El-Behira	100	72	18	53	19	73.6	26.4
Ismailia	100	33	8.25	20	13	60.6	39.4
Total	**400**	**183**	**45.75**	**123**	**60**	**67.2**	**32.8**

Host range:

Mechanical inoculation:

The results showed that BBTV's diagnostic hosts both mechanical and insect inoculations were limited. All ten banana plants, three ginger plants, and three bird of paradise plants were mechanically inoculated with BBTV (Table 3). Some mechanically inoculated plants showed no symptoms. All ten banana plants and three ginger plants were BBTV-infected following insect inoculation.

Table 3. The reaction of host range of BBTV isolate by Mechanical inoculation.

Host plants	Symptoms	No: of infected plants
***Musa acuminate*. Grandnaine**	-Yellowing at the leaf margins. -Dark green streaks on leaf veins and midribs	10/10
Canna indica	No symptoms	0/10
Alpinia purpurata (ginger),	Yellowing at the leaf margins	3/10
Alocasia brisbanensis	No symptoms	0/10
Strelitzia reginae **(Bird of paradise)**	yellow streaks	3/10

Aphid transmission:

The insect inoculated banana plants Williama and Grandnain cvs. Showed symptoms of yellowing at the leaf margins and dark green streaks on leaf veins and midribs after 30 days post inoculation by the Aphis *Pentalonia nigronervosa* insect. Data in Table (4) revealed the result of susceptible inoculated plants to BBTV. In *Alpinia purpurata* (ginger), yellowing at the margins of leaves has appeared (Fig. 2). No symptoms were observed on other inoculated plants. DAS-ELISA technique against specific BBTV antiserum and gave +ve results was used to detect the virus inoculated plants and showed *Musa acuminate* .cv. Grandnaine and *Alpinia purpurata* were infected.

Table 4. The reaction of host range of BBTV isolate by insect inoculation

Host plants	Symptoms	No: of infected plants	DAS-ELISA
Musa acuminate. **Williams** **Grandnaine**	-Yellowing at the leaf margins. -Dark green streaks on leaf veins and midribs	10/10	+
Canna indica	-	0/10	-
Alpinia purp **Murata** **(ginger)**	Yellowing at the leaf margins	4/10	+
Alocasia brisbanensis	-	0/10	+
Strelitzia reginae	-	0/10	+

Fig. 2. Infected leaf of banana with BBTV (yellowing at the margins and dark green streaks on a leaf vein

PCR Amplification of BBTV-DNA Components:

BBTV was detected in a symptomatic banana sample by field visual check and PCR testing. Using primers specific to the coat protein coding sequences and replicate pair coding sequences of healthy, infected, and healthy banana plants, 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. 3). In a PCR combination that included DNA from healthy samples obtained in the field, 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 the sample of uninfected banana leaves (Lane 1), there was no amplification; Lane N is the negative control.

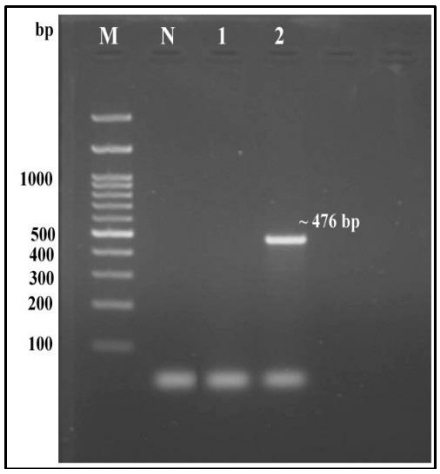

Fig. 3. 1.5% Agarose gel electrophoresis analysis of the amplified product of BBTV DNA-1 component using vBBTV-1 and cBBTV-1 specific primers. M: 100 bp DNA marker, Lane N is the negative control; lane 1: is uninfected banana plant and lane 2: PCR product from BBTV DNA-1 component infected banana plant.

Sequence analysis and Phylogenetic tree:

A nucleotide sequence resulting from amplified fragment sequencing was compared to Gen Bank. The analysis showed that the partial sequence was the replicase gene from the banana bunchy top virus. Using BLAST, the sequences of the purified PCR product were compared with the one-way sequences of six different BBTV isolates from different regions that were available in Gen Bank databases (Table 5). Using DNAMAN sequence analysis software, the resulting sequence data were assembled. The nucleotide sequence was submitted to Gen Bank with the accession number MT876529 as a BBTV Egy Behira isolate.

For BBTV isolates, the phylogenetic tree (Fig. 5) with a high bootstrap value showed two main clades. BBTV Behira isolates form a monophyletic group with BTTV isolates. The Indian isolates with accession numbers GU125415, LC468138, and AY222303, as well as the Egyptian strain with accession number BBTV-DNA-1, were all clustered into one clade. Table 5: The Accession numbers, description and the Homology matrix with different *Banana Bunchy Top Virus* (BBTV) isolates available in Gen Bank

Isolate name	Accession No.	Country	Homology matrix	References
EGY-Bahair (the current isolate)	MT876529	Egypt	100%	This study
BBTVisolate West Bengal replicase gene	GU125415	India	99.7%	Selvarajan *et al*., 2010
BBTV-DNA-1 Egy	LC468138	Egypt	99.7%	Abdel Razek Fatma *et al*., 2022
BBTV replicase gene	AY222303	India	98.5%	Ghosh *et al*.,2003
BBTVMY01	AB252639	Myanmar India	98.5%	Furuya and Natsuaki 2006
BBTV isolate IL3-98	GU085265	India	98.3%	Selvarajan *et al*., 2010

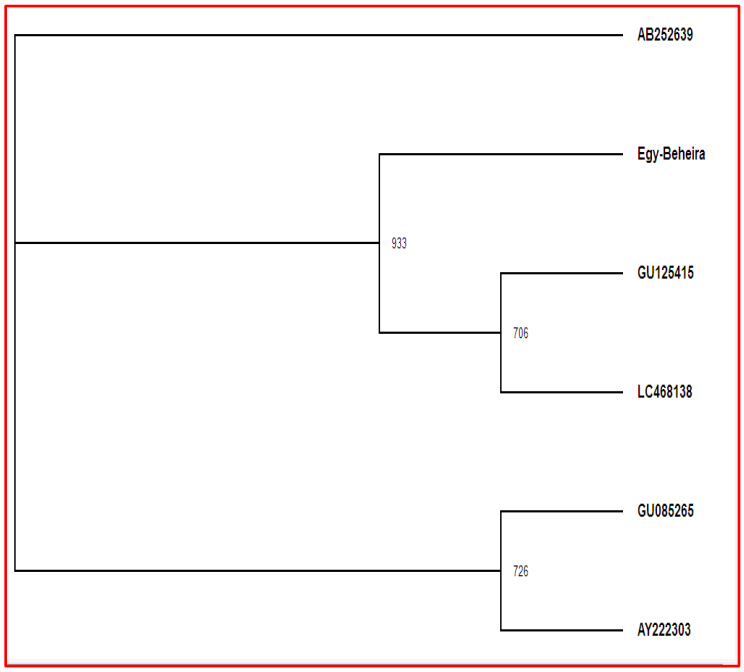

Fig.4. The phylogenetic tree for the BBTV- Eg Behira isolates based on the partial nucleotide sequence alignment of DNA-A genome with BBTV-isolates available in Gen Bank using DNAMAN software.

The efficiency of some treatments on BBTV elimination:

Meristem tip culture technique:

The absorbance values of plant meristem tips were near to negative control when plantlets generated from meristem tips of plants treated at 40°C were tested to determine the presence or absence of BBTV by visual and absorbance values at ELISA. Thermotherapy at 40 ° C. for 60 days and meristem-tip culture were employed to successfully eradicate the virus (Fig. 5). In this case, it was found that certain cultures generated from main meristem tips were 100% free from BBTV.

Fig. 5. Efficiency evaluation of chemotherapy on the banana bunchy top virus (BBTV).

***In vitro* screening of chemotherapy activities of Virazol and Salicylic acid on BBTV:**

The effect of Virazol and Salicylic acid on BBTV infection was evaluated in this current work using banana plants. These chemicals were added to a half-strength MS medium without any plant growth regulators during the rooting stage. DAS-ELISA was carried out when the roots appeared in the medium (25–30 days) for the presence of BBTV. The obtained results revealed that Virazol and Salicylic acid gave a high effect on virus multiplication in the infected plants according to the results of ELISA in Table (6).

Table 6. Effect of Ribavirin (Virazole) and salicylic acid treatments on BBTV

Chemotherapy	DAS-ELISA Treatments mg/l				
	0	10	20	30	40
Virazol	++	++	++	+	-
Salicylic acid	++	++	++	+	-

Detection of resistance in treated plants:

Oxidative enzymes:

Results in Table (7) demonstrate an increase in polyphenol oxidase and peroxidase activity in banana trees with BBTV infected when treated with Virazol and salicylic acid as compared to untreated banana trees (healthy control).

Non-treated infected plants had a 1.70 higher polyphenol oxidase activity than healthy plants (0.864). The maximum polyphenol oxidase activity for the diseased plants treated with various Virazol concentrations was 1.64, followed by Salicylic acid (1.78).

Meanwhile, peroxidase activity in non-treated infected plants was 1.94 compared with healthy ones (1.53). The peroxidase activity in the infected plants treated with different concentrations of Virazol was 2.021, followed by salicylic acid (1.96) respectively

Table 7. Peroxidase and polyphenol oxidase activity in BBTV in banana infected plants treated with acquired resistance inducers.

Enzyme	Healthy	+BBTV	% (±)	Virazol.	% (±)	Salicylic acid	% (±)
Polyphenoloxidase	**0.864**	**1.70**	**(+)49.1**	**1.64**	**(+) 46**	**1.78**	**(+)29.75**
Peroxidase	**1.53**	**1.94**	**(+)21.13**	**2.021**	**24.25**	**1.96**	**(+) 21.93**

% (±): relative changes

Phytochemicals change in BBTV-infected banana trees:

Chlorophyll contents:

Data represented in **Table (8)** indicate that the infected banana leaves with BBTV showed significant biochemical changes represented in a relative reduction in Chl a, Chl. b and carotenoid contents. It was observed that the highest reduction in Chlorophyll b, 51.47% followed by chlorophyll a+b 51.27, chlorophyll a, 51.17, and Carotenoids -2.65 respectively when compared with the healthy control.

Table (8): Effect of BBTV infection on Photosynthetic pigments and carotenes in Banana leaves.

Sample	Carotenoids	Chl. A	Chl. B	Chl. (a+b)	Total pigments
Healthy control	2.989[a] ±0.0001	2.358[a] ±0.004	1.433[a] ±0.012	3.276 [a] ±0.006	11.963 [a] ±0.006
Infected leaves (BBTV)	2.756[c] ±0.003	2.312[c] ±0.002	1.299 [b] ±0.001	4.79 [b] ±0.002	6.98 [b] ±0.005
Relative changes (%)	**51.17**	**51.47**	**51.27**	**-2.65**	**41.65**

* Means ± SE (Standard Error). Means within columns with the same letter are not significantly different using Tukey's HSD test at $p < 0.01$. (Each value resulted from 10 plants).

DISCUSSION

Banana Bunchy Top Virus (BBTV) is invading disease in Egypt, infecting the most important economic crops in the region. The recent development of symptoms in cultivated banana was reported by a few farmers who believed it was a nutrient deficiency or water irrigation problem. Investigating such observations revealed a *Banana Bunchy Top Virus*-like infection in the area. These results are in agreement with that of several authors (Sastry *et al.,* 1980; 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). While Rao (1980) reported that (BBTV) was isolated using *Myzus persicae*, *Aphis gossypii*, and *Aphis spiraecola* from infected banana plants to healthy banana plants, and the obtained symptoms were bunchy top, dark streaks on the shoot; yellowing and dark green streaks 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)

In this study, PCR and sequencing analysis was employed to identify the BBTV responsible for the observed symptoms on banana trees. The amplified sequences were identical to each other and matched the previously reported BBTV ribosomal RNA gene from Gen Bank, and were different from other reported species infecting banana trees. Detection of BBTV in infected banana samples using polymerase chain reaction (PCR) with specific primers showed the amplifying of BBTV at 476bp. A similar result was mentioned by (Shamloul *et al.,* 1999; Rezk, 2001).

PCR, sequencing which is a specific and sensitive nucleic-acid-based method for the detection of plant viruses (Ichiki *et al*., 2013), was performed to diagnose viruses. The tissue sample leaves of the selected Banana plants tested positive for BBTV by PCR, with the expected size of 476 bp. fragment of the DNA-1 specific primers, replication-associated protein (Mansour *et al*., 2013) was amplified, sequenced, and confirmed in terms of the presence of the virus in the study area. The main purpose of the molecular studies was to confirm the presence of BBTV in the study area and the makeup of Egypt isolates of BBTV and their percentage identity and phylogenetic relationship with other isolates reported worldwide. (Rahayuniati *et al*., 2021)

DNA component 1 of the fragment of the DNA-1 specific primers, replication-associated protein (476bp) was sequenced and analyzed. Data showed that the identity percentage of the Egyptian isolate group of BBTV component- with DNA 1 of BBTV isolates West Bengal replicase gene Indian and BBTV-DNA-1 Egy DNA Egypt isolates group was 99.7% (Selvarajan *et al*., 2010; Abdel Razek Fatma *et al*., 2022) and with Myanmar India isolate was 98.5% (Furuya and Natsuaki 2006). On the other hand, reported that the identity of component-1 DNA of India isolate of BBTV was 98.3% with BBTV isolate IL3-98 (Selvarajan *et al*., 2010), but Mukwa *et al.* (2016) mentioned that the identity of BBTV component-1 of Belgium isolate was 98 % (Mathiyazhagan *et al.,* 2011).

Using of tissue culture technique to produce banana seedlings gave banana an improved efficiency compared to other crops. High virus eradication efficiency was achieved by chemotherapy combined with the culturing of 0.5 mm long meristem-tips. In this case, all cultures regenerated from main-shoot tips and axillary

shoot tips were free of BBTV. All regenerated plants were tested for the presence of BBTV by DAS-ELISA and PCR. Virus titers were evaluated by their A405 values in ELISA. Results indicated that chemotherapy had significant effects on titers of these viruses in tips of *in vivo* and in *vitro*-cultured plants.

Applying some compounds (Virazol and Salicylic acid) resulted in changes in the activity of peroxidase and polyphenol oxidase which were estimated in healthy and infected banana trees, as well as infected plants treated with some compounds in an attempt for virus elimination. The infected plants showed higher enzymatic activity compared with healthy ones. The infected plants treated with Virazol and Salicylic acid compounds were found to be unable to induce the highest polyphenol oxidase activity in the infected plants. The Virazol compound gave the highest activity followed by Salicylic acid compared with the healthy and inoculated control. Concerning peroxidase activity, its rates increased in infected banana when compared with the healthy ones.

Peroxidases have been found to play an important role in the regulation of plant cell elongation, phenolic compounds oxidation, polysaccharide cross-linking, indol acetic acid oxidation, cross-linking of extension monomers and mediate the final step in the biosynthesis of lignin and other oxidative phenols. Peroxidases and polyphenol oxidase activities were greater in the plants treated with Virazol and Salicylic acid, compared with control plants. Polyphenoloxidases can be induced through the octadecanoid defense signal pathway and it oxidizes phenolic compounds to quinines, and the enzyme itself is inhibitory to viruses by inactivating the RNA of the virus. Enhanced polyphenol oxidase activities against disease and insect pests have been reported in several beneficial plant-microbe interactions (Harish *et al*., 2009).

CONCLUSIONS

The results of this study demonstrate the occurrence of Banana BBTV in Egypt and show the percentage of the disease in the studied area; an alarm call to start searching for a treatment and introduce management programmers. Production of banana-free seedlings by tissue culture technique gives an optical method to eradicate BBTV and other pathogens. The study also shows that banana cultivars differ in their susceptibility/tolerance to BBTV infection and suggests using tolerant cultivars to grow at the edges and borders of banana farms to dilute the disease before spreading. Physiological studies and additional molecular data are required to understand the transmission of the disease and its interactions in vectors and host plants to find a solution and stop the spread of the banana BBTV disease in the region.

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