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REDUCTION IN BANANA BUNCHY TOP VIRUS INFUTION RATE BY USING *STREPTOMYCES CHIBAENSIS* FILTRATE

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ABSTRACT

Banana bunchy top virus (BBTV) is one of the most important viral agent affecting banana production worldwide. The present study aimed at performing a trial to control BBTV biologically by using Streptomyces *chibaensis*, via induction of the plant resistance either as a cell-free filtrate or spore suspension before and after virus inoculation. Various morphological and physiological measurements were estimated. Also enzyme-linked immunosrobent assay, e.i., ELISA and polymerase chain reaction, e.i., PCR were employed in virus indexing among banana plants under study. The viral infection in plants with pre-inoculated through the cell-free filtrate was disappeared, while the other treatments didn't acquire any resistance against BBTV. The cell-free filtrate of S. *chibaensis* having antagonistic agent(s) as a secondary product(s) that inhibit BBTV infection, or this filtrate is inducing the plant resistance. Two filtrate may contain chitinase enzyme that could be in developing resistance against BBTV either through two inhibition if biting of aphids (*Pentalonia nigronervosa*) to banana. As it is a ruler for BBTV.

INTRODUCTION

The banana is the fourth most important crop after rice, wheat and maize (Piertersen and Thomas, 2001). It is widely grown in the tropics and subtropics in all types of agricultural systems. It serves in many developing countries as a stable food or the cornerstone of the country's economy (Anonymous, 2001).

Viruses as systemic pathogens cause serious damage in crops propagated *via* vegetative materials (Kubiriba and Tushemerirwe, 2001). There are some viruses known to infect bananas, *Banana bunchy top* virus (BBTV) genus *Babuvirus*, is the most serious (Furuya *et al.*, 2005).

The first symptoms of BBTV consist of darker green streaks on the lower portion of the midrib, and later on the secondary veins of the leaf. When fruit is produced, some of the hands may have distorted and twisted fruit. Leaves are usually short, stiff, erect and more narrow than normal that give the rosette shape (Smith *et al.*, 1998 and Rao *et al.*, 2002).

No cultivar has so far been identified which is resistant to BBTV. Chemical control of the vector is the most commonly used method to control this disease. However, it is expensive and poses serious health and environmental hazards (Kavino *et al.*, 2007).

Tissue culture and genetic engineering also play an important role in BBTD control (Gleba *et al.*, 2004). Another method of control is the trial of found a biological control agent that may be used against banana aphid in order to reduce the

aphid number and dispersal in banana plantations. This was done by using entomopathogenic fungi (Young and Wright, 2005) but this method was too hard to be applied because the aphid was found to be hidden in the leaf sheath just adjacent to the pseudostem.

The new approach of controlling of a plant disease now is by inducing resistance, this was done by the action of plant growth promoting rhizobacteria (PGPR), the plant immunity induced as a result of these bacteria is referred as induced systemic resistance (ISR) against fungal (Liu *et al.*, 1995), viral (Maurhofer *et al.*, 1998) and insect disease (Zehnder *et al.*, 1997).

Streptomyces sp. filamentous gram positive rhizobcteria was found to be very active that produces a wide range of biologically active substances including enzymes and antibiotics (Petinate *et al.*, 1999). Cell-free suspension of *Streptomyces rochei* was succeeded to inhibit *Tobacco mosaic virus* (TMV) from leaves of *Datura metel* (Mansour *et al.*, 1988), while Ghaly *et al.* (2005) found that *S. sparsogenes* was able to produce clindamycine which act as an antiviral.

This study is a trial to focus on the ability of the PGPR using *S. chibaensis* as a safe and cheap method not only to reduce and control BBTV infection but also to improve the growth of banana plants.

MATERIALS AND METHODS

Plant materials

Virus-free, 4 weeks old banana plants were supplied by Pico Modern Agriculture Co. Dokki, Giza, Egypt, the clones were cv. Williams, a representative of AAA clones (De Ascensao *et al.*, 2000). These healthy suckers were grown in pots containing sand and peat in equal ratio and were maintained in a greenhouse at 30-35^{o}C, in Botany Department, Women's College, Ain Shams University. 100 ml/plant of full strength Hoagland nutrient solution (Yasmin *et al.*, 2001) was added to each plant at two weeks interval. Banana plants of three months age were used in the experiment.

Virus source

Infected banana plants showing typical BBTV symptoms were collected from two different locations in El-Qalubia Governorate in Egypt. The presence of the virus was confirmed serologically by using indirect enzyme-linked immunosorbent assay (I-ELISA) using PAbs specific to BBTV as described by Wu and Su (1990a).

Propagation and maintenance of the banana aphid

The banana aphid (*P. nigronervosa* Coq) was collected from banana field in El-Qalubia Governorate in Egypt. By the aid of a fine paintbrush the insects were shifted on healthy plants and allowed to feed for two weeks to get red of any viral particles that may be acquired. Then aphids were transferred to BBTV-infected banana plants separately in cages for acquisition period of 10 days. Then ten viruliferous adults were shifted to each test plant and let for inoculation period of 24 hrs, followed by killing by spraying with a systemic insecticide (Malathion 1000 ppm) as recommended by Wu and Su (1990b). The plants were followed and observed for symptoms development.

Source of *Streptomyces* isolate:

The *S. chibaensis* was locally isolated and characterized by Hewedy (2003). It was cultivated on a starch nitrate liquid medium (Shirling and Gottlieb, 1966) for 6 days on a rotary shaker at 150 rpm, at 3^{o}C. The cell-free filtrate was prepared as described by Ghaly *et al.* (2005).

For the spore suspension preparation, the organism was cultivated on starch nitrate agar medium (Jetiyanon and Kloepper, 2002) for 4-6 days. It was

formed with sterile saline solution (0.9% NaCl). The concentration was adjusted at approximately 10^8 CFU /ml, 100 ml of the suspension was applied / the soil of each pot.

Pre- and post-inoculation treatments

S. chibaensis cell free-filtrate (F) and the spore suspension (s) was inoculated to the pots 10 days either before (groups Fpre & Spre) or after the infection transmission (groups Fpost & Spost). Control plants were inoculated with BBTV (infected control group IC), distilled water (healthy control group HC), cell-free filtrate (group FHC) and spore suspension (group SHC). The symptoms were observed and recorded after 45 days of inoculation.

Morphological measurements

These measurements included shoot length, number of leaves (included healthy and unhealthy leaves), leaf area and leaf thickness. The leaf area of the third leaf was calculated through the weighing method (Cornelissen *et al*., 2003). The third leaf thickness (µm) was determined microscopically using stage micrometer (Baby *et al.*, 2004).

Physiological measurements

In this experiment the amount of the photosynthetic pigments (chlorophyll A, chlorophyll B, and the carotinoids content) for each plant under was estimated according to the method of Metzner *et al.* (1965).

Protein analysis

BBTV tested plants and their corresponding healthy plants were used as a source of protein samples. Total soluble proteins were extracted from 1 g of each sample as described by Sambrook *et al.* (1989). The extracted proteins were suspended in loading buffer (Laemmli 1970) and subjected to electrophoresis for 2 hours at 100 volt followed by staining the gel with Coomassie Brilliant Blue dye and destaining using the destain solution (Sambrook *et al.*, 1989). Molecular weight of the protein was estimated from molecular weight standard (Mw from 28 to 116 KDa). The SDS-PAGE protein profils was analyzed using the Computer Protein Gel Image Analysis Software.

Disease resistance

The disease resistance was determined in the plants under study using I-ELISA as described by Wu and Su (1990a), PCR detection and symptom development after 45 days from virus inoculation.

DNA extraction

The total genomic DNA was isolated by using Doyle and Doyle (1990) Procedure of banana tested plants.

Oligonucleotide primers for PCR were derived from the published sequence of BBTV DNA-1 (Furuya *et al*., 2005) as follows primer FRF01 (5\ GGA AGA AGC CTC TCA TCT GCT TCA GAG A 3\) and the primer RRF01 (5\ TTC CCA GGC GCA CAC CTT GAG AAA CGA AAG 3\). The reaction mix was performed as followed: 200 ng of the extracted DNA (template), 1x PCR-buffer containing $MgSO_4$ (Biron), 200 µM dNTPs, 0.4 pmol 5\ primer, 0.4 pmol 3\ primer, 0.05 U *Taq* DNA polymerase and then the volume was adjusted to 50 µl with double d.H_2O. The program of the thermal cycler was 3 min at 94°C, 1min at 94°C, 1min at 55°C, 2 min at 72°C for 35 cycles and 7 min at 72°C. The PCR DNA product was electrophoresis in 1.2 % agarose gels and stained with ethidium bromide (Sambrook *et al.*, 1989) and visualized by illumination with UV transilluminator (Pharmacia, Sweden) with UV light and then photographed.

The disease resistance was calculated according to the suggested formula of Rao *et al*. (2002): Disease resistant (%) = No. of healthy plants/No. of tested plants X 100 / (%).

RESULTS AND DISCUSSION

Morphological characters

Microbe inoculated plants showed a significant improvement in growth characters when compared with control. Among the different treatments, plants treated with cell free filtrate either pre (Fpre) or post (Fpost) infection showed better growth parameters than those treated with the spore suspension (Spre & Spost respectively). Characteristics such as pseudostem height, numbers of leaves, and leaf area showed high variations among the treatments. The untreated plants (IC) recorded with the lowest morphological characters. The highest growth parameters were recorded for group Fpre and its control FHC which record no incidence of the virus. While in the remaining groups such as group Fpost it was noticed that in spite of being infected (positive for virus) it was found to have morphological characters that don't significantly changed from the healthy control (HC), (**Tables 1**).

Table-1: Effect of type and time of treatment on morphological characteristics after 45 days of virus inoculation.

Treatments	Pseudostem height (cm)	Number of leaves			Leaf area in (cm^2)
		Total	Healthy	Non-healthy	
FHC	24.88^{a}	8.0^{a}	8.0^{a}	0.0^{b}	212.2^{a}
F pre	22.30^{a}	8.0^{ab}	8.0^{a}	0.0^{b}	192.5^{ab}
F post	17.78^{d}	8.0^{a}	4.0^{d}	4.0^{a}	121.80^{c}
SHC	20.10^{b}	7.0^{bc}	7.0^{b}	0.0^{b}	154.4^{bc}
S pre	17.96^{c}	8.0^{a}	5.0^{d}	3.0^{a}	117.2^{c}
S post	15.12^{d}	7.0^{bc}	3.0^{cd}	4.0^{a}	90.20^{d}
HC	18.38^{c}	6.0^{c}	6.0^{b}	0.0^{b}	157.1^{b}
IC	11.34^{e}	7.0^{bc}	3.0^{c}	4.0^{a}	68.0^{d}

Values are the means of five replicates ± standard division. Means in a column followed by same superscript letters are not significantly different.

Similar results obtained by Raupach *et al.* (1996) who affirmed that seed-treatment with *Pseudomonas fluorescens* strain 89B-27 and *Serratia marcescens* strain 90-166 had reduced the number of *Cucumber mosaic virus* (CMV) infected plants and delayed the development of symptoms in cucumber and tomato. Comparable results were obtained by Mello *et al.* (2000) in pineapple, Jaizme-Vega *et al.* (2004) and Kavino *et al.* (2007) in banana. In contrast to these results, Hazaa *et al.* (2006) found that on treating BBTV infected banana micropropagated shoot tip with virazol and salicylic acid at concentration 10 mg/L enhanced the growth, but decrease the percentage of virus-free plants, while on increasing the concentration up to 30 mg/L of two antiviral decrease the development of shoot micropropagated plantlets and had a deleterious effect on the regeneration and growth of the propagated shoots and increase of virus free plantlets.

Photosynthetic pigments content

As in case of the morphological characteristics of the plants, the photosynthetic pigments were affected by the application of S. *chibaensis* either in the form of cell free filtrate or as a spore suspension. The photosynthetic pigments content was increased in all treatments but overall enhancement was greatest in case of cell free filtrate application prior to virus transmission (Fpre). Group Fpost came in the second class in whish the media filtrate was applied after the infection transmission. While groups Spre and Spost showed the lowest values compared to controls as shown in Table 2.

Table-2. ffect of type and time of treatments on photosynthetic pigments µg/g after 45 days from virus inoculation**.**

Treatments	Photosynthetic pigments		
	Chla	Chlb	Cart .
FHC	125.83[a]	212.32[a]	336.80[a]
F pre	122.67[a]	205.03[a]	321.84[a]
F post	100.03[b]	171.59[b]	217.32[b]
SHC	114.55[a]	192.36[a]	319.78[a]
S pre	83.62[b]	139.28[b]	186.82[b]
S post	53.22[c]	91.04[b]	147.10[c]
HC	128.21[a]	215.72[a]	327.80[a]
IC	29.96[d]	53.74[d]	90.78[d]

Values are the means of five replicates ± standard deviations. Means in a column followed by same superscript letters are not significantly different.

This result was in harmony with Ghaly *et al.* (2005) who stated that treatment the cucumber plants with *S. sparsogenes* and *S. albovianceus* increased the photosynthetic pigments content and retard the chlorosis symptoms caused by ZYMV than the untreated plants. Nafie (2003) also found the same results when *Lupines termis* seedlings were treated with the spore suspension of S. *chibaensis* prior to infection with *Fusarium* oxysporum. Furthermore, Kavino *et al.* (2007) on examining the effect of the rhizobacteria on the resistance of banana plants against BBTV they found the same increase in the photosynthetic pigments than the untreated plants. The above results were obtained after 45 days of viral inoculation.

Protein analysis

In a trial to explain the above results, the protein banding patterns of all plants under this research were examined by applying SDS-PAGE. It was found that a band of 30 KDa was observed in gradually decreased OD in lanes of FHC, F pre, SHC, HC, S pre, F post, S post and IC, respectively (Fig-1). This protein is believed to be a protein that may belonged to the chitinase family as assumed by Santos *et al.* (2004). So the disappearance of disease symptoms in plants representing group (F pre) may be due to the high concentrations of chitinase enzyme that found in them as one of PR proteins.

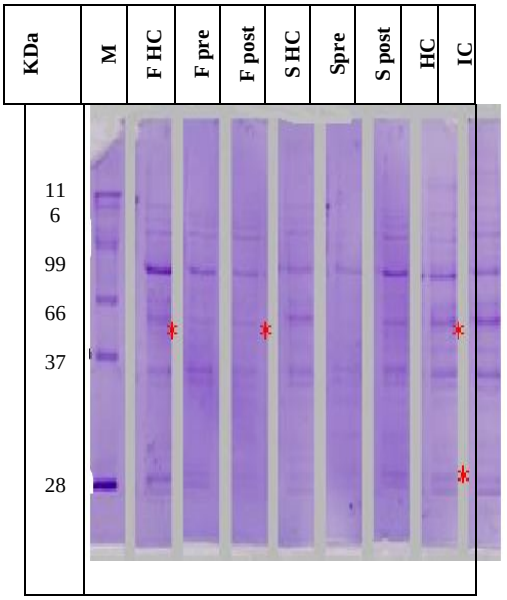

Fig-1. Protein-banding pattern of treated and untreated banana plants and their controls. Lane M: protein markers, F pre: cell free filtrate (pre), S pre: spore suspension (pre), F post: cell free filtrate (post), S post: spore suspension (post), IC: infected control. HC: healthy control, FHC: healthy plants treated with the cell free filtrate and SHC: healthy plants treated with the spore suspension.

This assumption comes in agreement with Botha *et al.* (1998) who stated that the

physiological role of chitinases in the general metabolism of plant cells has not been documented. Indeed, a general role is difficult to envisage, because its substrate, chitin, does not occur in higher plants. It has been postulated that plants produce chitinase in order to protect themselves from chitin-containing parasites. They also found that chitinase may also be involved in the defense of wheat against Russian wheat aphid (RWA), as substantial levels of chitinase protein were present in resistant wheat faster, and to a higher level than in susceptible plants. Similar observation was established by Khattab (2007) in cabbage plant in which the resistant plants processed high concentrations of chitinase than the infested ones attacked by the phloem-sucking aphid, *Brevicoryne brassicae* L.

In the same direction, Harish *et al*. (2004) found that the plants treated with endophytic bacteria seem more resistant to the BBTV and the aphid vector more than the untreated ones and the activity of defense related proteins in plants, such as chitinase increase. Further Gohel *et al*. (2005) affirmed that a great deal of interest has been generated on chitinase because of its applications in the biocontrol of plant pathogenic fungi, molting process of insects and mosquito control.Additionally Maurhofer *et al*. (1998), Silva *et al*. (2004), Van Loon and Bakker (2005) and Kavino *et al*. (2007) declared that inoculation with plant growth promoting rhizobacteria (PGPR) lead to increase in pathogenesis related proteins (PRs) such as chitinase enzyme.

Interestingly, the band of 62.7 KDa (that appeared in protein banding pattern of cell free filtrate, (Fig-2) was observed in lanes (F pre10), (F pre), (F post) and (FHC), Fig. 1 in which the plants were treated with cell free filtrate of S. *chibaensis* only.

This band may be responsible for trehalase enzyme which is responsible for the degradation of trehalose which is a main blood sugar of insects and an important energy source for insect tissues. This trehalose is synthesized mainly by fat body and released rapidly into the hemolymph without its storage within the fat body. To utilize the trehalose, other insect tissues should have trehalase enzyme which hydrolyzes 1 mole of trehalose to 2 moles of glucose that is finally used in the glycolysis of tissue cells. Therefore, disturbance of the homeostasis of trehalose in insect hemolymph lead to an insecticidal activities against these insects. Many Streptomyces spp. were examined in their abilities in trehalase production and used as a microbial pesticide in the form of trehazolin (Ando *et al.*, 1995 and Sato *et al*., 1997).

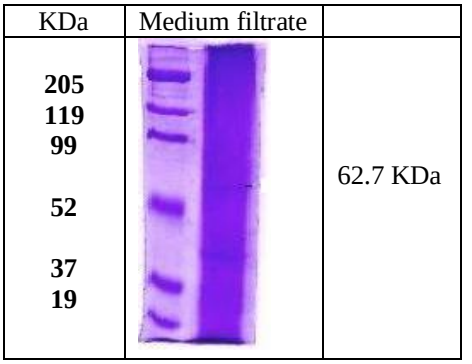

Fig-2: Protein-banding pattern of the cell free medium filtrate.

A decrease in the number of polypeptides in groups (S pre), (F post) and (S post) -that possessed the disease symptoms and reached maximum in the infected control (IC) - was observed. This may be due to suppression effect of virus in protein synthesis or assimilation as speculated by El-Dougdoug *et al*. (2002) and Khattab (2007).

Disease resistance: The disease resistance in the groups under study was illustrated in Table 3 and Fig-3. It was found that group Fpre had no infected plants while the virus was found in the remaining groups in different values.

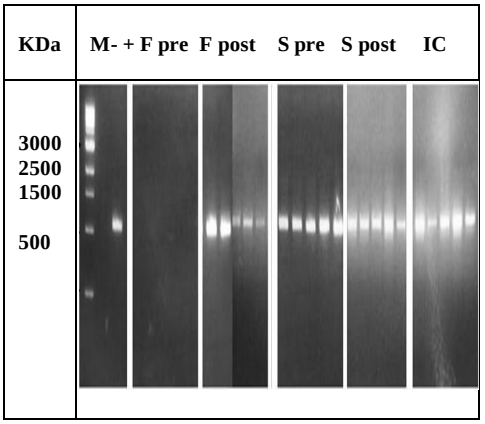

Fig-3. Agarose gel electrophoresis of the amplified BBTV DNA-1 from the plants under study 5 plants/each treatment. F pre: plants pre treated with cell free media filtrate, S pre: plants pre treated with the spore suspension, F post: plants post treated with cell free media filtrate, S post: plants post treated with the spore suspension and IC: BBTV-infected control. M: 1Kb marker. Note: a number of five plants for each treatment were tested.

Table-3: Percentage of BBTV resistant plants in response to the treatment with *S. chibaensis* in the form of cell-free filtrate or spore suspension either pre or post virus inoculation in reference to ELISA, PCR and symptoms development 45 days post viral inoculation.

Treatments	% of positive plants		Symptoms	Resistance (%)
	ELISA	PCR		
F pre	00	000	-	100
F post	20	100	++	80
S pre	60	100	+++	40
S post	80	100	++++	20
IC	80	100	++++	20

F pre: cell free filtrate (pre), S pre: spore suspension (pre), F post: cell free filtrate (post), S post: spore suspension (post), IC: BBTV-infected control. (+): indicated the degree of severity, (-): no symptoms

In the view of results of Fig. 3, on performing PCR for treated plants, it was found that all plants under investigation possessed the viral DNA, as they gave positive results in compare with an infected control and a healthy control, except for group Fpre, which their plants (5 plants) showed negative results. This may be due to the difference in sensitivity level between the serological method, e.i., ELISA which depends on the measurements of the viral coat protein so it in turn depending on the viral load, and the molecular detection, e.i., PCR which depends on the presence or absence of the viral DNA which in turn become more sensitive as speculated by Sadik *et al.* (1999) who declared that PCR technique play an important role in detection of BBTV during purification steps regardless the concentration of the virus. Additionally, Thomas (2000) stated that the most significant recent advance in virus indexing has been the advent of the PCR as it is a very sensitive assay. Similar speculation was stated by Selvarajan *et al.* (2004), who declared that DAS-ELISA didn't detect the viral isolates, while PCR method detected all the isolates except for one.

The disappearance of the viral infection in group Fpre in which the plants were pre inoculated with the cell free filtrate came in agreement with Ghaly *et al.* (2005) who found a 100 % inhibitory effect of *Streptomyces sparsogenes* against *Zucchini yellow mosaic virus* (ZYMV). In comparison with other workers, Yassin and Galal, (1998) reported that filtrate of some *Streptomyces* gave antiviral activities against *Tobacco necrosis virus* (TNV).

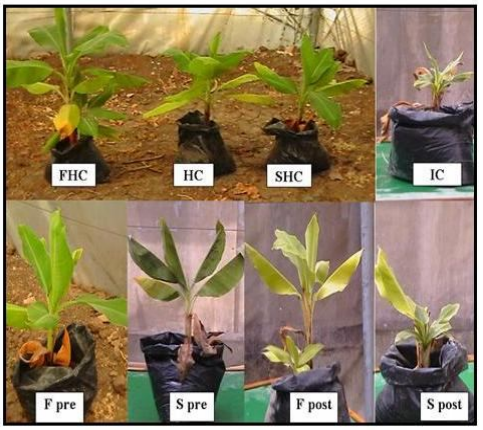

Fig-4: The plants of different treatments and their control. F pre: cell free filtrate (pre), S pre: spore suspension (pre), F post: cell free filtrate (post), S post: spore suspension (post), IC: infected control, HC: healthy control, FHC: healthy plants treated with the cell free filtrate and SHC: healthy plants treated with the spore suspension.

On combining these results with the results obtained in SDS- PAGE analysis suggested that the resistance observed in this study may be induced against the banana aphid (the vector) rather than the virus. It will know that BBTV is persistently transmitted by its aphid vector *Pentalonia nigronervosa* in a circulative non-propagative manner (Franz *et al.*, 1999). The beneficial effects of PGPR include direct plant growth promotion, biological control and inducing systemic resistance in host plants. The reduction in symptoms pf BBTV disease is an indication of an induced systemic resistance response to treatment with S. *chibaensis*. It can conclude that using S. *chibaensis* as pre-conditioning for resistance can provide an effective, economical and practical way of plant protection. Results are illustrated in Figure -4.

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