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Screening of banana bunchy top diseased plants: A way to control its spreading

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Abstract

Banana bunchy top disease (BBTD) is a nano-viral banana disease, which has been reducing both vegetative and reproductive growth of banana. It is spreading with the passage of time but minimize-able, if detected earlier through ELISA and PCR. For the concerned matter, an economic protocol for ELISA has been established for both time and chemicals. During this study, twenty BBTV infected and non-infected (on the basis of morphological symptoms) samples [Dwarf Cavendish banana cv. Sindhri banana or Basrai (AAA)] were collected from old (~10 years) and new (less than 4 years) eighteen different banana farms. Each was cultured under *in-vitro* conditions. After 3-sub-culturings, they were grown in the wire-house. When the age of the growing plants was reached to almost 3-months than presence of BBTV was detected through ELISA and PCR. They were remained infected even after micro-propagation. The BBTV severity was observed to be variable from farm to farm, which was four to six-folds higher in old banana farms than new farms. The causal source of variation may be the cultivation of infected banana nursery from the developed (old) to new farms. The infection was observed similar in each even after their micro-propagation. One possibility is present to control the spreading of this pathogen; by using BBTV free banana nursery could be helpful.

Introduction

Banana bunchy top virus (BBTV) of genus *Babuvirus* (family *Nanoviridae*) has been considered as serious and economically destructive pathogen of banana (*Musa* spp.) crop, when (1920s) the banana growing industry (in Australia) was almost 100% destroyed by this disease. *Banana bunchy top disease* (BBTD) is epidemic in Asian (Philippines, Indonesia, Japan, China, Taiwan Vietnam) as well as in South Pacific (Fiji, Australia, Burundi, Tonga, Egypt, India, Hawaii, Pakistan, Myanmar) regions of the world, while not in Southern-Africa, Central and South America (Furuya et al., 2004 & 2005). According to Panhwar (1991), BBTD was recorded first time in Pakistan during 1989 at Sakro-Keti Bunder, District Thatta (Sindh province). This disease has reduced rapidly banana cultivation area as well as its production. In 1978, the area was 60,000 ha with 17.6 tons per hectare yield, while during 1999-2000 it reduced to 26,000 ha (22,400 ha in Sindh).

The causal pathogen is phloem-tissue specific causing various cytopathological effects. Dash like streaks with yellowing of leaves is first morphological symptom of BBTV infected plants. With the progression of disease, leaves become narrow than plants are stunted with bunched appearance of leaves at the top. Plant is unable to inflorescence, finally results into plant death because of its direct cytological influence on plant growth (Wanitchakorn et al. 2000; Natsuaki and Furuya, 2007).

The BBTV has been transmitting in banana fields through inoculums by banana aphid (*Pentalonia nigronervosa* Coq.) and vegetative planting material but not through mechanical inoculation. Today, controls for BBTV are not available, while the use of pathogen free planting material may be of valuable for growing such crop (Arce-Montoya et al., 2006; Jouira et al., 1998; Adelberg et al., 1999; Azeqour et al., 2002; Tyagi et al., 2007).

Meanwhile, plant health certification scheme is required for BBTV indexing. A number of serological and molecular tools have been developed for early plant virus detection. BBTV is also detectable through TAS-ELISA (triple antibody sandwich, enzyme-linked immunosorbent assay) as by Njukenga et al. (2002&2005) as well as by polymerase chain reaction. Through the application of these economic markers, screening of BBTV is possible.

In this manuscript, the farmers are confessed to make their fields virus free by screening BBTV infected banana plants through ELISA and/ PCR. Both of these markers has been established in our as well as may be in other institutes. They are available for banana growers at very cheapest costs. This work may be helpful for others in screening of BBTV infected banana plants either from field or its nursery.

Materials and methods

Plant materials

On the basis of morphological symptoms, almost same aged plants were selected. Of course, BBTV infected and non-infected (20 of each) young plants (~1.5-2.0 ft in height) of mature banana [Dwarf Cavendish cv., Sindhri banana or Basrai (AAA)] were collected from eighteen different banana farms located along National Highway in Hala and Nawabshah Districts on the basis of apparent BBTV symptoms. They were named as Hala-west, left to highway; Matyari-east, left to highway I; Matyari-Baudero; Nawabshah-west; Hala-Pir-Jhando ; Hala-east, left to highway; Hala-west, right to highway; Hala-west, right to highway; Australian cobe; Matyari-east, left to highway II; Matyari-east, left to highway III; Sukrund-Noshero; Hala-Australian-west, right to highway; Sukrund-NawabShah-Link; Sukrund-east, left to bypass; Sukrund Magsigot; Hala-east, left to highway II; Matyari-east, right to highway I; Sukrund-bypass 1 and Sukrund-bypass II. The collected plants were assayed through ELISA and PCR before their culturing under *in-vitro* conditions (Haq and Dahot, 2007) as well as after their micro-propagation (3-sub-cultures), when they were 3-months old plants (infected and non-infected) propagating (*ex-vitro*) in the wire-house. The experimental region is located at distance almost 60km from banana fields. The multiplying plants were secured from banana aphids by using *imidacloprid* (Provado®)@ 60ppm (Robson and Wright, 2007).

Enzyme Linked Immuno-sorbent Assay (ELISA)

The viral proteins were extracted (Wu and Su, 1990b) by taking 0.5g pieces of fresh midrib of leaf (at center) in plastic bags (6"x10"), than 5 ml viral protein extraction buffer [PBST {PBS (136.89mM NaCl, 1.47mM KH_2PO_4, 7.31mM Na_2HPO_4, 2.683mM KCl, 3.076mM NaN_3) + 5ml Tween-20} + 2% PVP + pH 7.4] was added. It was grinded by rolling glass rod over plastic bag thoroughly for 3 min. The mixture was poured into vials (10ml capacity) than placed at 4°C for 15 min, upper cleared supernatant was used as a sample, leaf derbies were settled down.

The new micro-titer plate was labeled (Fig 1) and 100µl diluted [(1µl:200µl coating buffer (15mM Na_2CO_3, 34.88mM $NaHCO_3$, 30.07mM NaN_3)] coating antibody (IgG) was pipette for two times after once in each well. The plate was incubated at 4°C for over-night than washed (3x3 times) with washing buffer (PBST + pH 7.4). The excess liquid was rinsed out from wells by hitting or jerking micro-titer plate (upside downward) on paper towels.

The 100µl of each sample (including positive and negative controls) were poured in the designated wells for once than again (duplicated). Similarly, 100µl extraction buffer was also added, than micro-titer plate was covered with lid and incubated for 2hrs at 37°C. Again micro-titer plate was washed (3-4x3 times) with washing buffer than 100µl EC_I or enzyme conjugate [4.50ml conjugate buffer stock (2% PVP, 0.5ml Tween-20, 0.2% BSA, pH 7.4) + 25µl phosphotase (A & B)] was dispensed into each well than reaction was incubated at 4°C for overnight. The plate was washed (3x3 times) with washing buffer and 100µl PNP or substrate buffer (0.492mM $MgCl_2.6H_2O$, 3.076mM NaN_3, 97.0ml Diethanolamine, pH 9.6-9.8) was poured in each well. The reaction was incubated at room temperature. After 60 min, reaction was fixed by adding 50µl NaOH (3M).

Developed color in ELISA was observed and its OD was read at 405nm through ELISA reader. The threshold value for BBTV was calculated by applying following mathematical formula;

[Threshold value = (Average -ve control value x 2) – Average +ve control value]

The OD of a sample was equal to or above to threshold value than sample was considered +ve for BBTV.

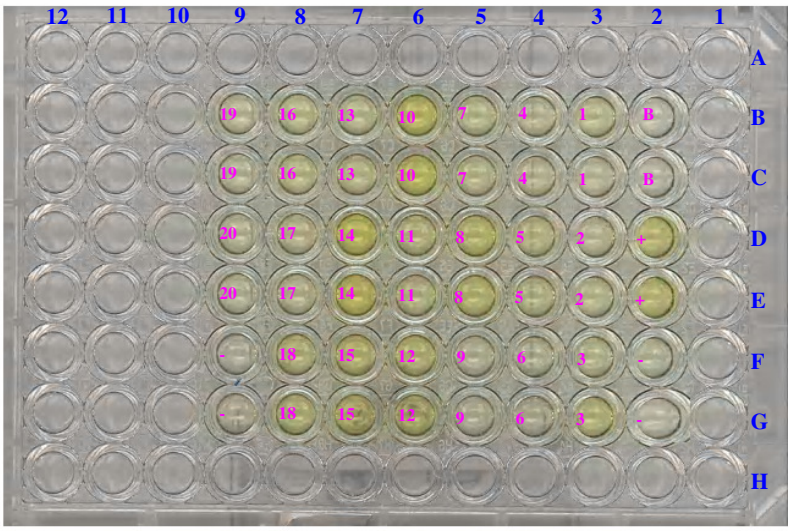

Fig 1. Microtiter plate with ELISA for BBTV detection in the field collected banana samples from Hala and Nawabshah Districts. A number of samples were assayed i.e. B: Blank; +: Positive control (known antigen); -: -ve control (*in-vitro* propagated healthy banana plant); 1: Hala-west, left to highway; 2: Matyari-east, left to highway I; 3: Matyari-Baudero; 4: Nawabshah-west; 5: Hala-PirJhando; 6: Hala-east, left to highway; 7: Hala-west, right to highway; 8: Hala-west, right to highway; 9: Australian cobe; 10: Matyari-east, left to highway II; 11: Matyari-east, left to highway III; 12: Sukrund-Noshero; 13: Hala-Australian-west, right to highway; 14: Sukrund-NawabShah-Link; 15; Sukrund-east, left to bypass; 16: Sukrund Magsigot; 17: Hala-east, left to highway II; 18: Matyari-east, right to highway I; 19: Sukrund-bypass 1 and 20: Sukrund-bypass II.

Polymerase chain reaction (PCR)

It is also necessary for the confirmation of ELISA results so viral DNA was extracted as Haq et al., (2009). Briefly, leaf midrib (0.300mg) was washed in washing buffer [100 mM Tris-Cl (pH 8.0), with 5mM EDTA (pH 8.0), 0.35 M sorbitol and 1% 2-mercaptoethanol]. It was centrifuged at 3,000 rpm for 2 min and settled material was homogenized in extraction buffer [1M Tris-HCl (pH 8.0), 5M NaCl, 2%CTAB (hexa-decyltrimethyl-ammonium bromide), 50mM EDTA (pH 8.0), 1% PVP (polyvinylpyrrolidone) and 0.2% 2-mercaptoethanol (added just before use)] and incubated at 65°C for 1h. After clarification with 0.5 ml solution of phenol:chloroform:isoamyle alcohol (25:24:1 v/v), DNA was precipitated with 80% isopropanol. The 25µl reaction was contained, 2.5µl PCR buffer (10x), 1.25µl $MgCl_2$ (25mM), 1µl dNTPs mixture (2.5mM); 1µl of each primer (20µmol l^{-1}) i.e. forward primer (F) 5'-CAGGCGCACACCTTGAGAAACGAAAGGGA A-3' and reverse primer (R) 5'-GGAAGAAGCCTCTCATCTGCTTCAGAGAGC -3'), 0.5µl Taq DNA polymerase (Fermentase, Germany, 2 units/ µl) and 0.3 µl of sample DNA (50 ng). The PCR profile was set as; Template DNA denaturation at 94ºC for 5 min, than followed by 30 cycles at 94°C for 45 sec (denaturation), 62ºC for 1 min (annealing), 72°C for 1 min (extension) and final one cycle of 72°C for 10 min (reaction completion) and finally stand by at 10°C. Amplified DNA was fractionated through 1.2% gel [0.1x TAE buffer (50x TAE= 1M Tris-Cl, 57.1ml Glacial acetic acid, 68.44mM EDTA (pH 8.0) pH 8.18-8.29].

Statistic analysis

The significance of the BBTV severity in the infected banana samples was checked in comparison to control reactions by applying the ANOVA (analysis of variance). It was computed by using a *COSTAT* computer package (*CoHort* Software, Berkeley, USA).

Results

The 3rd leaf from top to bottom was collected from 3-months old infected and non-infected (in figure included only one for reference) banana plants propagating in the wire-house after their micro-

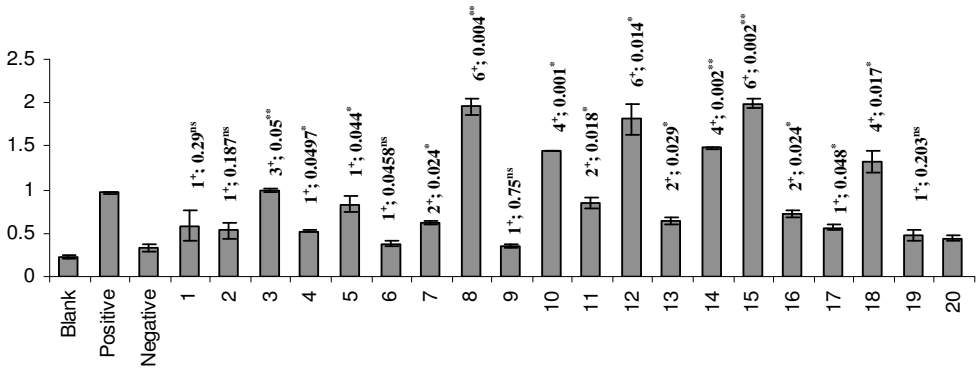

Fig 2. The severity of BBTV in the banana samples collected from Hala and Nawabshah Districts (i.e.1-20 as in Fig 1). The calculated BBTV threshold value was 0.3015, while signs 1^+, 2^+, 3^+, 4^+, 5^+ and 6^+ represents the severity of BBTV i.e. ≤ threshold value, 2, 3, 4, 5 and 6 times increased folds of the threshold values respectively. While others are the ANOVA values with ns and *, which are for the non-significance and significance of the data field collected samples in comparison to control plants at $P<0.05$.

propagation (3 sub-cultures). They were dissected and subjected to BBTV detection separately. Firstly, ELISA was conducted and observed that all the samples were observed to be infected with BBTV, in comparison to controls i.e. blank (ddH_2O), negative (-ve/ *in-vitro* developed banana plant) and positive (known BBTV antigen), as shown in Figure 1. Similar results were also observed when collected from the field initially. General appearance of color density in wells of micro-titer plate and its reading from ELISA reader, it was observed that the samples 8, 10, 12, 14, 15 were severely infected with BBTV than others. Regarding to threshold value, it was almost four to six folds (these were those samples, which were collected from ~ 10 years old farms) more than threshold value for BBTV infection in the samples from ≥4 years old farms i.e. 3, 4, 5, 7, 13,16, 17 and 18. Some samples which were collected from ~2 years old farms i.e. 1, 2, 6, 9, 19 and 20 were infected but non-significantly (Fig 2).
Non-significant results from ELISA were creating confusion. In these samples, there possibility that viral protein level may be very low or infected with more than one virus. Such confusions were removed, when the presence of BBTV was confirmed by PCR for each case (Fig 3). Overall, it was concluded that samples collected from various farms were infected with BBTV. But infection rate was variable among farm to farm. Such variations may be depending upon the age of farm, from where its nursery was taken as well as usual improper management of banana crop in old farms by farmers. The infected plants were observed to be infected again even after their *in-vitro* micro-propagation for 3 sub-cultures.

Discussion

The outbreak of plant viruses has been increased tremendously to an explosive level from last two decades because of direct and indirect involvement of human, during improper transportation of plant material as well as viral vectors has also successfully transmitted them through out the world. Managing of a viral disease is dependent on its correct identification, which has been considered as most important step. Currently there are certain techniques, which have been used for virus screening (Zaitlin and Palukaitis, 2000; Yamane et al., 2008; Saidi and Warade, 2008). These are serological and nucleic acid based techniques (Webster et al., 2004). In present study, a protocol for ELISA has been established for BBTV detection by using BBTV specific antibody. The composition of buffers, which has been used during ELISA, is very important for getting its results efficiently. Simply, if ratios of chemicals in the buffers are altered than fast and accurate results may be possible.

According to ELISA, banana farms are differentially infected with BBTV, evenly propagated through in-vitro multiplication or collected from the filed (Thomas et al., 1995). Its severity has been reached at a highly significant ($p>0.005$) level in those farms, which were propagating from year to years (Zaitlin and Palukaitis, 2000; Triques et al., 2008). Interestingly, new farms were also observed to be infected, its main reason may be young banana suckers (nursery) were already infected. In non-significant samples, there is possibility that one plant may be infected with more than one virus. Under such conditions, both ELISA and PCR are very useful

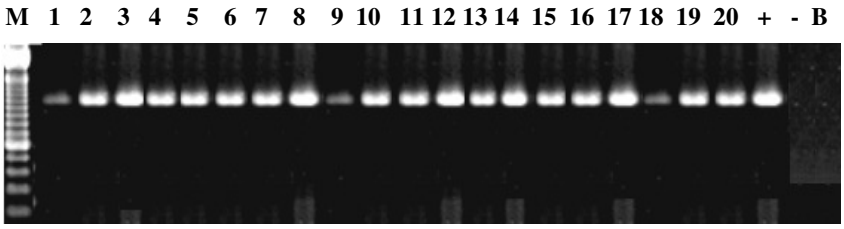

Fig 3. PCR-based detection of BBTV by using the C1 gene specific primers than followed to the defragmentation of PCR product through agarose gel electrophoresis. A band of 1.1 kb (arrowed) in sized was amplified in BBTV infected field collected samples (i.e 1-20 as in Fig 1) as well as in the known BBTV genome as positive control (lane +), while not in healthy banana used as a negative plant control (lane -) and in blank (lane B) for water control. The 100-bp DNA ladder (lane M) was also included for marking the size of amplified fragment.

markers because of its efficiency, accuracy and inexpensive for screening a huge number of plants for a single virus by using generally one well of the micro-titer plate or one PCR tube per plant sample, while for multiple viruses alternatively a single plant can be simultaneously tested on a single plate by using different viral antigen specific antibodies coated wells either in the duplicate or triplicate manner for a specific reproducibility. However, PCR should be conducted separately for each case for the prevention of primers dimerisation.

Like as world widely, banana crop in Pakistan is also infected with BBTV. Areas of the Sindh province i.e Mityari, Hala and Nawabshah Districts are the main banana producing regions of the country. There most of the farmers are un-educated and have named this disease as banana cancer. Conventionally, their method for detection of diseased banana plants totally base on its morphological appearance. The farmers are unknown for actual situation; infection stage is end of plant life. If young plantlets are propagating from the infected pants may be apparently healthy but are infected. Meanwhile, farmers are cultivating such young suckers as a nursery of banana unconsciously. This is a main way that BBTV has been spreading through out the Sindh province. Presently, most of the farmers have been replacing their banana fields with other crops. Now the limited agricultural area is under banana cultivation. Today, its production is not fulfilling the required banana fruit quantity for domestics.

The BBTV is not a curable or eradicable banana disease even through *in-vitro* propagation of banana. Its spreading is limitable by growing onlyscreened banana plants. Generally, banana nursery is multiplied from the parent suckers. At the time of banana cultivation, framers should sure banana nursery is BBTV free or not. Only visual symptoms are not authentic for BBTV presence or absence as its initial infection may not be visible can appear laterally. The detection of such disease is possible through ELISA or PCR. For the removal of this disease, farmers should *in-vitro* propagated nursery (Roels et al., 2005) and arrange some viral diagnostic tests after every 3-4 years. Today the developed constrained for the banana crop in the country is demanding from the local Government that should help them by providing such diagnostic facilities. Otherwise this crop has already reached at a very critical point, may be eliminated from this region.

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