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NUCLEIC ACID HYBRIDIZATIONS AS A RADIOACTIVE TOOL FOR RAPID DETECTION OF BANANA BUNCHY TOP VIRUS

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ABSTRACT

Banana bunchy top disease (BBTD) caused by banana bunchy top virus (BBTV) was radioactively detected by nucleic acid hybridization techniques. Results showed that, ^{32}P-labelled insert of pBT338 was hybridized with nucleic acid extracts from BBTV-infected plants from Egypt and Australia but not with those from CMV-infected plants from Egypt. Results revealed that BBTV was greatly detected in midrib, roots, meristem, corm, leaves and pseudostem respectively. BBTV was also detected in symptomless young plants prepared from diseased plant materials grown under tissue culture conditions but was not present in those performed from healthy plant materials. The sensitivity of dot blot and Southern blot hybridizations for the detection of BBTV was also performed for the detection of BBTV.

Key words: Banana bunchy top disease, Banana bunchy top virus, radioactive detection, Southern and dot blot hybridizations.

INTRODUCTION

It is well known that banana bunchy top disease (BBTD) is the most economically important virus disease for bananas (*Musa* spp.) and has been reported in many parts of the world (Dale, 1987, Sadik *et al*., 1999, Nour El-Din *et al*., 2005, Allam *et al*., 2008, Amin *et al*., 2008, Oben *et al*., 2009, Kumar *et al*., 2011 and Lokossou *et al*., 2012). Statinton *et al*., (2012) reported that BBTV (Family Nanoviridae, Genus Babu-virus) is a multi component, single stranded DNA virus, causes widespread banana crop losses throughout tropical Africa and Australia.

Between 1923 and 1927 bunchy top nearly destroyed the NSW banana industry, reduced the yield to less than half of the peak of 1922 production (Eastwood, 1946). Jones (1935) revealed that the rate of infections of BBTV in Alexandria, Egypt reached up to 60% and the virus reduced the quality of banana fruits.

El-Afifi (1984) reported that the rate of infection with BBTV in the field of Egypt was 31.3, 19.2, 17.4 and 3.6% for Hindi, Basri, Magarbi and Sindhi cvs. respectively. Allam *et al.,* (1986) found that the rate of infectious BBTV was 17.5, 13 and 2% in El-Monofeia, El-Kalyobia and Kena governorates respectively in Egypt. Allam *et al.,* (1988) reported that the banana plants infected with BBTV did produce neither yield nor good quality fruits.

Thomas and Dietzgen (1991) purified 18 to 20 nm isometric virus-like particles (VLPs) from infected plants, containing 1kb ssDNA. It's infectious and transmission

ability was confirmed further using a probe cloned from ssDNA (Harding, *et al.*, 1991), detection and replication of this ssDNA was also noted in banana aphid (*P. Nigro-nervosa*) (Hafner, 1992 and Abdel-Salam *et al*., 2012).

In Australia, Dietzgen and Thomas (1991) isolated isometric virus particles, 18-19 nm in diameter from field samples of Hawaii, Indonesia, and Togo. They also found that these particles contained a coat protein of M_r about 20000 and DNA of about 1 kb. Isometric VLPs from both BBTV of Hawaii and Togo had a buoyant density of 1.30 g/cm^3 in caesium sulphate (Cs_2SO_4). Similar isometric particles with a diameter of 18 to 20 nm and a density of 1.28 to 1.30 g/ml were also purified by Harding *et al*., (1991).

Abdel-Hamid *et al*., (2003) found that polymerase chain reaction (PCR) and chemiluminescence were helpful for the accurate detection of BBTV. Among the six different DNA concentrations extracted from the virus infected banana plant, 125 ng DNA was found to be the most effective. This DNA concentration was used for the detection of the BBTV in 3 parts of the apical meristems obtained from virus infected plants micropropagated *via* tissue culture. Results showed that 10, 50 and 80 % of the upper, middle and bottom parts were infected, respectively. In case of chemiluminescence detection, the virus was easily detected in either DNA extracted from BBTV-infected or symptomless banana plants up to 10^{-3} dilution using a cold DNA probe labeled with digoxygenin. In the light of the above findings, this study was proposed for using the nucleic acid hybridizations as a rapid and sensitive radioactive tool for BBTV detection.

MATERIALS AND METHODS

Detection of Egyptian BBTV isolate: For this purpose, samples of midribs infected with BBTV and CMV infected leaves from Egypt were collected and transferred to Australia in 1991. Egyptian-BBTV isolate was detected using Southern blot hybridization as described by Maniatis *et al.*, (1982).

Extraction of BBTV-nucleic acid: 0.2 gram from health and diseased banana tissue was taken, cut into small pieces, placed into a sterile 1.5 eppendrof tube and frozen using liquid nitrogen. The frozen tissue was ground into a five powder, 500 µl of 1% SDS containing 0.5% sodium phosphate buffer was added and vortexed for 1 minute. To the tube 500µl of phenol / chloroform were added, vortexed vigorously for at least 5 minutes, centrifuged for 10 minutes at room temperature (RT). Supernatant was transferred to a fresh tube and equal volume of chloroform was added and spun for 5 minutes at RT. To the aqueous phase 2 volumes of 100% ethanol and 0.1 volume of sodium acetate (3 M, pH 5.4) were added, vortexed and stored at 70^{o}C for 1 hour. The tube was then centrifuged for 15 minutes at 4^{o}C and the pellets were washed with 70% ethanol and dried under vacuum for 10-20 minutes. The pellets were finally resuspended in 50 µl of sterile TE buffer (pH8.0) and the concentration was measured using spectrophotometer.

Gel electrophoresis analysis: 1% TAE agarose gel was prepared and 1µl of DNA from health, diseased and pBT338 insert as positive control was loaded along with the DNA marker VI. The gel was run at 65 V for 1.5 hour, stained with ethidium bromide solution for 10-15 minutes. The bands were checked by visualising the gel under the UV light illuminator.

Southern transfer of DNA from agarose to Hybond-N membrane: The gel was transferred to a glass baking plate and the DNA was denatured by soaking in 1.5 M NaCl and 0.5 M NaOH for 1 hour at RT and then neutralized by soaking the gel in 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 1 hour at RT. A tray with perspex black covered in blotting was set up as shown in figure-3 for southren transfer of DNA from agarose gel to Hybond-N membrane. The dish was filled with 10xSSC till to the top of the support. After nearly 14 to 16 hours, towels and the 3MM paper above the gel were removed. The dehydrated gel and membrane were turned over and then laid on a dry sheet of 3MM paper. The gel was restained again with ethidium bromide and visualised under the UV light illuminator to confirm all DNA transfer from gel to membrane. The membrane was soaked in 6xSSC at RT for 5 minutes and then excess fluid was drained out and dried at RT on a sheet of 3MM paper. The DNA was fixed by drying the membrane at 80^{o}C under vacuum for 1 hour. Then, the membrane was stored at RT under vacuum between sheets of 3MM paper until use.

Labelling of pBT338 insert with ^{32}P: An oligo-labelling Kit (Bresatec) was used to prepare ^{32}P-labelled hybridization probe as described by Harding *et al*., (1991). The labelled insert was stored at -20^{o}C until use.

Hybridization, washing and exposing autoradiograph: The back membrane was wets using 6xSSC and immersed in the 6xSSC for 2 minutes. A piece of nylon mesh about the same size as the membrane rolled up together and placed them in a hybridization bottle. To the bottle 0.2 ml of prehybridization (pre-hyb) fluid pre-warmed at 65^{o}C for 1 hour was added and incubated at 65^{o}C for at least 3 hours. The pre-hyb solution was discarded and the solutions (50µl/Cm2) as well as the denatured labelled insert (boiling for 5 minutes) were added. After incubation for 14 to 16 hours, membrane was removed and immediately washed with a low stringency wash (2xSSPE, 1%SDS) for three times for 15 minutes at 65^{o}C, dried at RT on a sheet of Whatman 3MM paper. The membrane was wrapped in Saran Wrap and exposed to X-ray film to obtain an autoradiographic image and then the X-ray was developed after 6 hours.

Detection of BBTV in different parts of banana plant: Three plants of cv. Cavendish banana plant grown under greenhouse conditions and infected with BBTV were used. Nucleic acid extracts from roots, corm, pseudostem, midrib, leaves and meristem were prepared as described before according to Harding *et al.*, (1991). Six dilutions of gDNA in d.H_2O, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 were prepared. For Southern blot hybridization, nucleic acid extracts separated by electrophoresis in 1% TAE agarose gel, stained with ethidium bromide and then blotted onto Hybond-N membrane using 10XSSC as a transfer buffer (Maniatis *et al.*, 1982). For dot blot analysis, nucleic acid extracts were denatured by adding 5 µl 2N NaOH. Aliquots (50 µl) were then spotted onto Hybond-N probe membrane using a Bio-Dot apparatus (Bio-Rad) according to Harding *et al.*, (1991). All membranes were hybridized with ^{32}P-labelled pBT338 insert as described before. The X-ray films were developed after 6 hours, overnight (O/N), 24 hours and 7 days. Unlabelled insert was also used as positive control.

Detection of BBTV in plant materials obtained from tissue culture: The experiment used the application of ^{32}P hybridization for detection of BBTV in plant materials with no apparent symptoms prepared from health and BBTV-infected

meristems by using tissue culture techniques. For this purpose, twenty plants were used and nucleic acid extracts were prepared as mentioned before. Ten µl of crude extracts from each plant was used for Southern blot and dot blot hybridizations in the presence of pBT338 as a positive control. The X-ray films were developed after 3 and 21 hours.

RESULTS AND DISCUSSION

Nucleic acid hybridization as a radio-active diagnostic tool using ^{32}P was first applied for detection of Epstein-virus in animal cells (Brandsma and Miller, 1980). Until recently, ^{32}P was the pre-dominantly reported group used for labelling of nucleic acids in many phyto-pathogens such as plant viruses (Harrison *et al.*, 1983, Maule *et al.*, 1983, Boulton *et al.*, 1986, Harding *et al.*, 1991, Harding *et al*., 2000, Khalil *et al*., 2007) and viroids (Owens and Diener, 1981, 1984 and Diener, 1987).

The aim of this present study was the use of ^{32}P-labelled pBT338-insert for the detection of Egyptian-BBTV isolate in different parts of plants with apparent bunchy top symptoms and symptomless obtained from tissue culture.

Detection of Egyptian BBTV isolate: Southern blot analysis of nucleic acid extracts from midribs of BBTV and CMV-infected banana plants collected from Egypt, and the health plants from Australia were used for the detection of BBTV using the pBT338 insert labelled with ^{32}P according to Harding *et al.*, (1991). Results in Figures 1 and 2 showed that the labelled insert of pBT338 specifically hybridized with a Mr band about 1033bp in nucleic acid extracts from both BBTV-infected plants collected from Egypt and Australia but not with banana plants infected with CMV from Egypt and healthy plants of Australia. Similar results were observed by Harding *et al.* (1991). Thomas and Dietzgen (1991) found that, BBTV isolates from Australia, Taiwan, People's Republic of China, Tongo, Western Samoa and Hawaii were serologically related, which suggests a common aetiology for the BBTD.

Nour El-Din *et al*., (2011) investigated component 6 (DNA-6) of BBTV encoding a protein of 17.39 KDa. The full length of BBTV DNA-6 was amplified *via* PCR, cloned into the pJET1.2 and sequenced. Results showed that the size of DNA-6 was 1087 nucleotides (nts) with one open reading frame (ORF) and its molecular length was 465nt. The nucleotide sequences of DNA-6 of the Egyptian isolate (Egy6m) were compared with eleven overseas isolates (three isolates from China, two from Pakistan, one from Taiwan, four from India and one from Australia). Results showed that the isolates were matched with the first cluster called South Pacific group which includes isolates from Egypt, Indian, Australian and Pakistan.

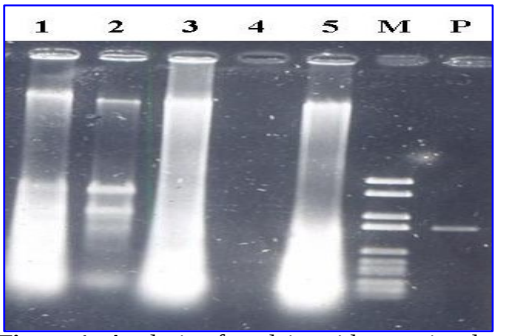

Figure-1: Analysis of nucleic acids associated with BBTV. The nucleic acid extracts were separated in 1% TAE agarose and stained with ethidium bromide. Lanes, 1, 2, 3 and 5 nucleic acid extracts from Australia-BBTV infected plant, healthy plant, Egyptian-CMV infected plant and Egyptian-BBTV infected plant, respectively. Lane M DNA marker VI and lane P, insert of pBT338 as a positive control.

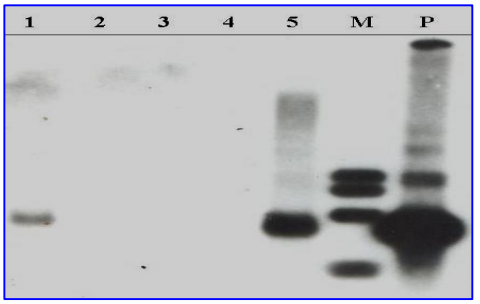

Figure-2: Southern blot hybridization of nucleic acid extracts from healthy plant (lane, 2), Egyptian-BBTV infected plant (lane, 5), Egyptian-CMV infected plant (lane, 3), Australian-BBTV infected plant (lane, 1) and the insert of pBT338 as a positive control (lane P). Lane M, DNA marker VI. Nucleic acids were analysed on a 1% agarose gel, blotted onto Hybond-N probe membrane and hybridized with ^{32}P-labelled insert of pBT338.

Detection of BBTV in different parts of banana plant: The sensitivity of Southern blot and dot blot hybridization using the insert of pBT338 labelled with ^{32}P studied for the detection of BBTV in different parts of banana infected plants grown under the glasshouse conditions. Nucleic acid extracts from roots, corm, pseudostem, midrib, leaves and meristem from 3 cv. Cavendish banana plants were prepared. Six dilutions: 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 were used for dot blot hybridization according to Harding *et al.*, (1991).

Results in Figures 3, 4 and 5 revealed that the labelled insert of pBT338 hybridized with nucleic acid extracts from all the different parts of banana infected plants but not any hybridization was found with those from health plants. The visibility of bands in Southern and dot blot hybridizations depended on the concentration of nucleic acid and exposure time of radiographs. Results could be concluded as follows: **a**. Dot blot hybridization is more sensitive than Southern blot hybridization, where, the dots are completely visible after O/N exposure time (Figure-5) but not the Southern blot hybridization (Figure- 4). **b**. The hybridization of pBT338 with nucleic acid extracts from midrib, roots, meristems are higher than those from corm, leaves and pseudostem respectively (Figures 4 and 5). **c**. Nucleic acids in 1/16 dilution could be detected after O/N exposure time in Southern blot hybridization. On the other hand, both 1/16, 1/32 and 1/64 dilutions are detected after 6 hours in dot blot hybridization. **d**. For the lowest concentration of nucleic acid in dilution (1/256) a visible band appears after 7 days for Southern blot hybridization, but the dot blot of the same dilution is completely visible after 24 hours. The sensitivity of dot blot may be due to blotting and trapping most of nucleic acid in the spots onto the Hybond-N probe membrane. The lowest efficiency of detection in Southern blot hybridization is due to incomplete DNA transfer from agarose to the Hybond-N probe membrane.

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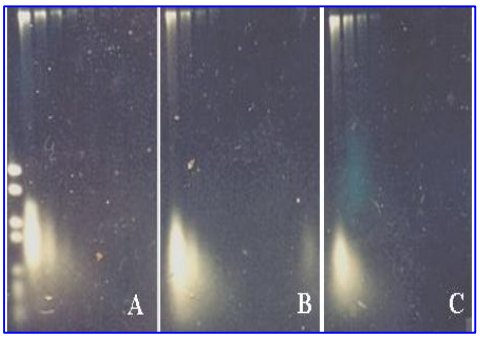

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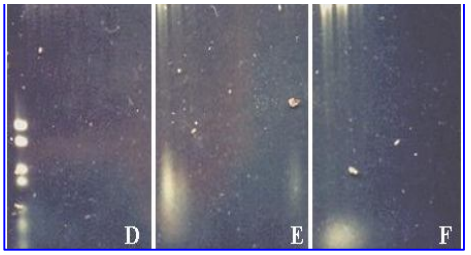

Figure-3: Analysis of nucleic acids in different parts of banana plant. Lanes 1 and 26, DNA marker VI. Lanes 8, 15, 22, 24, 33, 40, 47 and 49 blanks. Lanes 25 and 50, the insert of pBT338 as positive control. Lanes from; (2-7), (9-14), (16-21), (27-32), (34-39) and (41-47), dilutions (1/8, 1/16, 1/32, 1/64, 1/128 and 1/256) of nucleic acids from BBTV-infected roots, corm, midrib, pseudostem, leaf and meristem respectively. Nucleic acids were separated by 1% TAE agarose and stained with ethidium bromide.

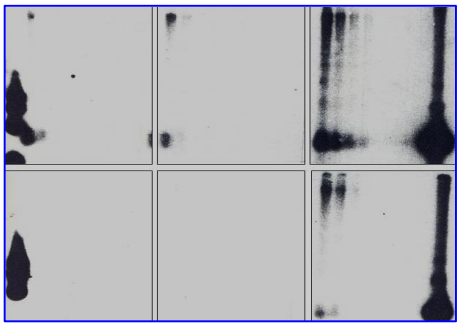

Figure-4: Southern blot hybridization using different dilutions (1/8, 1/16, 1/32, 1/64, 1/128 and 1/256) of nucleic acid extracts from root (1), corm (2), midrib (3), pseudostem (4), leaf (5) and meristem (6). The insert of pBT338 and all nucleic acid extracts were analyzed on a 1% agarose, blotted onto Hybon-N probe membrane and hybridized with ^{32}P-labelled insert from pBT338.

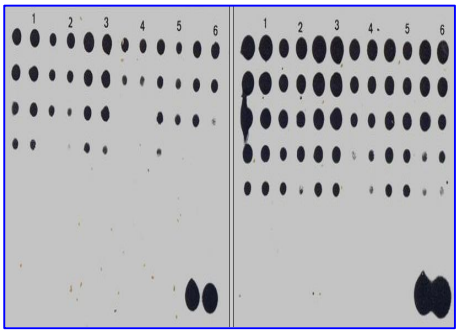

Figure-5: Blot analysis of nucleic acid extracts of different dilutions (1/16, 1/32, 1/64, 1/128 and 1/256) from BBTV-infected root, corm, midrib, pseudostem, leaf and meristem (2 replicates for each). Nucleic acid from healthy plant and the insert from pBT338 were used as negative and positive controls. All nucleic acid extracts were blotted onto Hybond-N probe membrane and hybridized with ^{32}P-labelled insert of pBT338. The autoradiograph was developed after overnight (b) and 36 hours.

This conclusion agrees with that found by Eweida *et al.* (1989). According to Thomas and Dietzgen (1991) extracts of midrib tissue consistently resulted in higher A_{405} values in the double antibody sandwich (DAS-ELISA (1.3 to 4.6 fold) than did extracts from the leaf lamina. Wu (1992) studied the distribution of BBTV in different parts of diseased plant in Taiwan using the ELISA test. They found that, BBTV was detected in leaf, midrib and petiole but not in the root and rhizome. He also reported that the midrib and petiole of plant No.2 contained higher concentrations of BBTV than other parts of the leaf. BBTV also was detected in the sheath of oldest healthy looking leaf.

Detection of BBTV in plant materials obtained from tissue culture: Results of

Southern blot hybridization (Figure- 6) and dot blot hybridization (Figure- 7) showed that, the insert of pBT338 labelled with ^{32}P hybridized strongly with all nucleic acid extracts (10µl/plant) from ten symptomless banana plants, prepared from infected banana meristem plants using tissue culture. On the other hand, no hybridization was found (10µl/plant) from those ten plants prepared from health banana meristem plants.

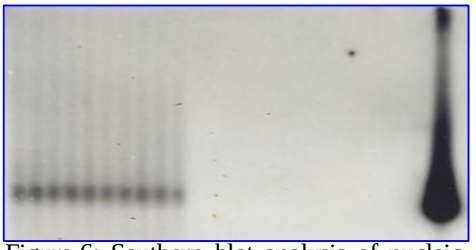

Figure-6: Southern blot analysis of nucleic acid extracts from healthy plant (a) and BBTV-infected plant (b) obtained from the tissue culture. Nucleic acid extracts were analyzed on a 1% agarose gel, blotted onto Hybond-N probe membrane and hybridized with ^{32}P-labelled insert of pBT338. Unlabelled insert was also used as a positive control (c).

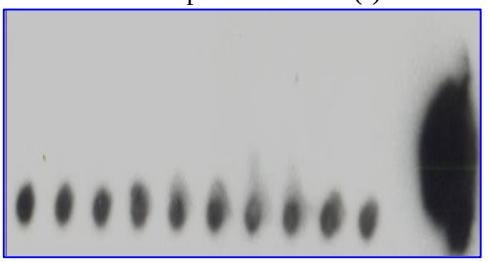

Figure-7: Blot analysis of nucleic acid extracts from health (a) and BBTV-infected plants (b) provided by the tissue culture. The extracts were blotted onto Hybond-N probe membrane and hybridized with ^{32}P-labelled insert of pBT338. Unlabelled insert was also used as a positive control (c).

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