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IN VITRO ERADICATION OF BANANA BUNCHY TOP VIRUS FROM NATURAL INFECTED GRANDNAN BANANA BY USING CHEMOTHERAPY

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Abstract

The aim of this study was to evaluate the efficacy of chemotherapy treatments using four different chemical substances (as antiviral) to eradicate the banana bunchy top virus from the infected banana plantlets grown *in vitro*. Therefore, *in vitro* cultured shoot tip explants of naturally infected mother Grandnan banana plants were treated by different concentrations of chemical substances which were added to the culture medium (MS) for four weeks. Ribavirin was used at 0, 20, 40 and 60 mg/l; Jasmonic acid was used at 0, 0.5, 1 and 2 mg/l; Salicylic acid was added at 0, 17.3, 34.5 and 69 mg/l; Benzoic acid was used at 0, 18, 36 ad 72 mg/l. After chemotherapy treatment, the shoots were subcultured on chemicals free MS medium for another 8 weeks (subcultured each four weeks). Samples of leaves were taken for analysis by PCR before chemotherapy treatment and then after 8 weeks of culture on chemicals free MS medium. The results of PCR analysis proved that the use of Ribavirin at 40 mg/l and 1 mg/l of Jasmonic acid produced the highest virus-free rate of banana shoots 66.62% and 44.42% with survival percentage 93.20% and 100%, respectively. While, the use of Salicylic acid and Benzoic acid produced the lowest virus-free rate of banana plantlets 11.11% with 17.3 mg/l Salicylic acid and 33.31% with 18 mg/l Benzoic acid treatments, respectively.

***Keywords**:* virus-free, antiviral, chemical substances, banana, BBTV, PCR.

Introduction

Banana *Musa spp.* that belong to family Musaceae one of the most important crops in the tropical and subtropical regions. It is cultivated in over 130 countries, source of carbohydrate and income for millions of people in these regions (Tripath *et al*., 2016). Banana bunchy top virus (BBTV) is the most dangerous virus that has the devastating effect on banana crop production (Lassois *et al*., 2013). BBTV genus *Babuvirus*, family *Nanoviridae* was discovered in Egypt 1901. The virus now eliminates 30 % of banana crop in Egypt. Therefore, it was necessary to limit the development of the situation. Therefore, the use of virus-free plants is considered the basis of viral diseases control. A lot of kinds of therapeutic *in vitro* were applied like cryotherapy and thermotherapy, meristem tip culture and chemotherapy, which one method was used either individually or several ways combined (Hazaa *et al*., 2006; Kabir Shiragi *et al*., 2008; Lassois *et al*., 2013). Applying chemotherapy as antiviral treatments *in vitro* has been used to produce virus-free plantlets for important crops. So, it was necessary to search for a way to eliminate the virus and produce virus-free 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). It is necessary to test varying concentrations and treatment time for a lot of antiviral compounds to establish an optimal balance between the virus elimination rate and the plant survival rate. Among all antiviral substances, Ribavirin (virazole), Salicylic acid, Jasmonic acid and Benzoic acid were tested. This antiviral compound acts on virus synthesis rather than through a direct inactivation of the existing virus. It is also necessary to apply all of them for extended periods of time to eradicate viruses from infected tissues. The antiviral compound works directly or indirectly on stopping synthesis of new virus particles while the existing virus particles are decreased in the course of their ontogeny (Lassois *et al*., 2013).

The aim of this study was to evaluate the efficacy of chemotherapy treatments using four different chemical substances (as antiviral) to eradicate the banana bunchy top virus from the infected banana plantlets grown *in vitro*. Also, to detect the most effective treatment to eradicate the banana bunchy top virus from the infected banana plantlets. The detection of BBTV in banana plants was performed using the enzyme-linked immune-sorbent assay, "ELISA" (Wu and Su, 1990; Thomas and Dietzgen, 1991). Currently, the polymerase chain reaction "PCR" (Xie and Hu, 1995; Mansoor et al., 2005) is the most sensitive protocol for detecting BBTV compared to ELISA. Therefore, in this study, PCR protocol was used to detect the presence of the BBTV in *in vitro* banana plantlets.

Materials and Methods

Plant materials

In vitro cultured shoot tip explants which used in this experiment were taken from suckers of naturally infected mother Grandnan banana plants with Banana Bunchy Top Virus (BBTV). All suckers used were about 50-70 cm in length, and collected from a field in Bader center and transferred to the Plant Biotechnology Research Laboratory (Plant Physiology Division, department of Agricultural Botany) Faculty of Agriculture, Cairo University. The explants were soaked into a 100% Clorox (5% sodium hypochlorite) for 20 min with a few drops of Tween 20. Then, the explants were rinsed 3 times with sterile distilled water. Afterwards, the explants were soaked in mercuric chloride (0.1 %) for 5 min, then were rinsed 3 times with sterile distilled water. Finally, the explants were soaked into a 50% Clorox (5% sodium hypochlorite) for 40 min with a few drops of Tween 20, and then the shoot tip explants were rinsed 3 times with sterile distilled water.

Nutrient Medium

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3 mg/l Benzyl adenine

(BA) + 7 g/l agar + 30 g/l sucrose deposited in 200 ml jars (25 ml/ jar) and autoclaved at 121 ºC for 20 min and incubated at 24 ºC ± 2 ºC for 3 days at least before using it.

Culture of infected shoot tip explants

The sterilized BBTV infected shoot tip explants with 4 cm in length were cultured on MS medium supplemented with 5 mg/l BA + 7g/l agar + 30g/l sucrose, and shoot tip explants were incubated for 3 subcultures (4 weeks for each subculture) in the growth chamber at 24 ºC ± 2 ºC in darkness. After this step, the culture jars were transferred to the light by fluorescent tubes given intensity of 1500 Lux for 16 hours per day for 2 subculture (21 days for each) in the growth chamber at 24 ºC ± 2 ºC.

Chemotherapy Treatments

Four different compounds (as antiviral) were used for production of *in vitro* BBTV-free banana plantlets by using four different concentrations of each compound; Ribavirin (0, 20, 40 and 60 mg/l), Jasmonic acid (0, 0.5, 1 and 2 mg/l), Salicylic acid 0 µM, 125 µM (17.3 mg/l), 250 µM (34.5 mg/l) and 500 µM (69 mg/l), Benzoic acid 0 µM, 125 µM (18 mg/l), 250 µM (36 mg/l) and 500 µM (72 mg/l). The stock solutions of chemical compounds were filtered through sterile 0.2 µm filters and different concentrations were added to the sterilized solution of MS medium then mixed with the sterilized agar 7g/l and poured in 200 ml jars in laminar airflow cabinet. Each concentration was added in MS medium which supplemented by 3 mg/l BA + 7 g/l agar + 30 g/l sucrose. Twenty seven BBTV infected shoot tip explants were used for each treatment and divided into 3 replicates (3 jars for each replicate) and each jar contains 3 BBTV infected shoot tip explants which were incubated at 24 ºC ± 2 ºC, and the light provided by fluorescent tubes given intensity of 1500 Lux for 16 hours per day. After 4 weeks, the BBTV infected shoot tip explants were transferred and cultured on chemicals free MS medium (control) supplemented with 3 mg/l BA + 7g/l agar + 30g/l sucrose for 8 weeks (subcultured each four weeks).

DNA virus detection

BBTV genome contain six circular single-stranded (ss) DNA, these are designated as DNA-R (rolling circle replication initiation protein), DNA-S (coat protein peptide of 19.6 kDa, DNA-M (movement protein), DNA-C (cell cycle link protein), DNA-N (nuclear shuttle protein), and DNA-U3 (unknown protein function) Thomas and Dietzgen (1991), Beetham *et al*., (1999).

DNA extraction from banana leaf tissues

The positive control ssDNA of BBTV was isolated from collected leaves of infected banana mother plants grown in the field. Samples were collected from leaves of *in vitro* banana plantlets before subculture on growth media of different treatments. Afterwards, the samples were collected from leaves of *in vitro* banana plantlets grown for 8 weeks on chemicals free MS medium. Genomic DNA was extracted from leaves of *in vitro* banana plantlets for PCR detection analysis of BBTV. DNA was isolated from leaf tissues of banana plantlets using the modified CTAB method (Shankar, *et al*., 2011). The purity of DNA was determined spectrophotometrically using the ratio ($A_{260}/_{280\ nm}$) and the yield of each sample at $A_{260\ nm}$. Then, 2 µg of each sample was loaded on a 0.8% agarose gel to estimate the quality of the isolated genomic DNA.

PCR analysis to detect the presence of banana bunchy top virus (BBTV) in banana plantlets

PCR reactions were performed using 100 ng genomic DNA of banana leaf samples in a 25 ul PCR mixture containing 5 µl of One*Taq* PCR buffer (5X), 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer and 1 unit of One*Taq* Hot Start DNA Polymerase (NEB; M0481). The presence of the highly conserved region of the coat protein gene (DNA-S) among banana bunchy top virus isolates; BBTV (GenBank Accession Number: KT180301.1) in banana plantlets was determined by amplification of a 459 bp fragment of the gene using the specific primer pair (Fwd.) 5'-TCCGAAGAAATCCATCAAGAA-3' and (Rev.) 5'-CCAGAACTACAATAGAATGCCAAA-3'. The PCR amplification was performed by initial denaturation step at 94°C for 5 min. followed by 35 cycles; at 94°C (35 s), annealing (35 s) at 60°C and amplification (40 s) at 72°C and finally holding 10 min at 72°C for extension employing the Applied Biosystems® ProFlex™ 3 x 32-well PCR System. The products of PCR reactions of the samples (three replicates) were electrophoresed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide then photographed using (GelDoc 2000; Bio-Rad).

Statistical analysis

Data were statistically analyzed on the basis of complete randomized design according to (Snedecor and Cochran, 1969), the treatments were replicated thrice and each replicate with 3 jars (3 explants/jar) and mean values were compared using Duncan's new multiple range test (Duncan, 1955).

Results and Discussion

Effect of different concentrations of four antiviral substances on the average of shoots number/explant

The results of the average shoots number/explant as shown in Table (1) and (Fig. 1) indicated that, the Ribavirin and Jasmonic acid were the best among the other antiviral substances, where the rate of shoots number/explant recorded the highest mean value 1.64 and 1.43, respectively. In addition, the mean value of the first concentration of each antiviral substance (Ribavirin, Jasmonic acid and Benzoic acid) was also the best, which recorded 1.66, except for Salicylic acid which recorded the highest value with the second concentration. Moreover, the Ribavirin at the first concentration 20 mg/l recorded the highest rate of shoots number/explants (3.13) compared to the other treatments.

Effect of different concentrations of four antiviral substances on the survival rate percentage

The results in Table (2) showed that, the use of Jasmonic acid and Ribavirin recorded the highest mean value of the survival rate percentage (97.88% and 94.51%) respectively, compared to the other antiviral substances. In addition, the medium supplemented with the first concentration of each antiviral substance (the chemicals free MS medium; control) and the medium supplemented with the second concentration of each antiviral substance showed the highest survival rate percentage (91.55% and 100%), respectively, except for Salicylic acid (91.55% and 73.00%) respectively.

Table 1: Effect of different concentrations of four antiviral substances on the average of shoots number/explant

	Antiviral substances				
Conc.*	**Ribavirin**	**Jasmonic acid**	**Salicylic acid**	**Benzoic acid**	**Mean**
Control	0.79 d g	1.66 b	0.86 c g	1.13 b f	1.11 b
1	3.13 a	1.53 b d	0.73 e g	1.26 b f	1.66 a
2	1.59 b c	1.13b f	1.06 b g	0.52 f g	1.08 b
3	1.06 b g	1.39 b e	0.66 e g	0.33 g	0.86 b
Mean	1.64 a	1.43 a	0.83 b	0.81 b	

* different concentration were added among each antiviral **substances;** Ribavirin (control, 20, 40 and 60 mg/l), Jasmonic acid (control, 0.5, 1 and 2 mg/l), Salicylic acid 0 µM (control), 125 µM (17.3 mg/l), 250 µM (34.5 mg/l) and 500 µM (69 mg/l), Benzoic acid 0 µM (control), 125 µM (18 mg/l), 250 µM (36 mg/l) and 500 µM (72 mg/l).

Table 2: Effect of different concentrations of four antiviral substances on the survival rate percentage

	Antiviral substances				
Conc.*	**Ribavirin**	**Jasmonic acid**	**Salicylic acid**	**Benzoic acid**	**Mean**
Control	91.55 ac	91.55 a	91.55 ac	91.55 a	91.55 a
1	100 a	100 a	73.00 bd	100 a	93.25 a
2	93.20 ab	100 a	100 a	52.80 b	86.50 a
3	93.29 ab	100 a	66.40 cd	33.06 e	73.17 b
Mean	94.51 ab	97.88 a	82.73 bc	69.35 c	

*different concentration were added among each antiviral **substances;** Ribavirin (control, 20, 40 and 60 mg/l), Jasmonic acid (control, 0.5, 1 and 2 mg/l), Salicylic acid 0 µM (control), 125 µM (17.3 mg/l), 250 µM (34.5 mg/l) and 500 µM (69 mg/l), Benzoic acid 0 µM (control), 125 µM (18 mg/l), 250 µM (36 mg/l) and 500 µM (72 mg/l).

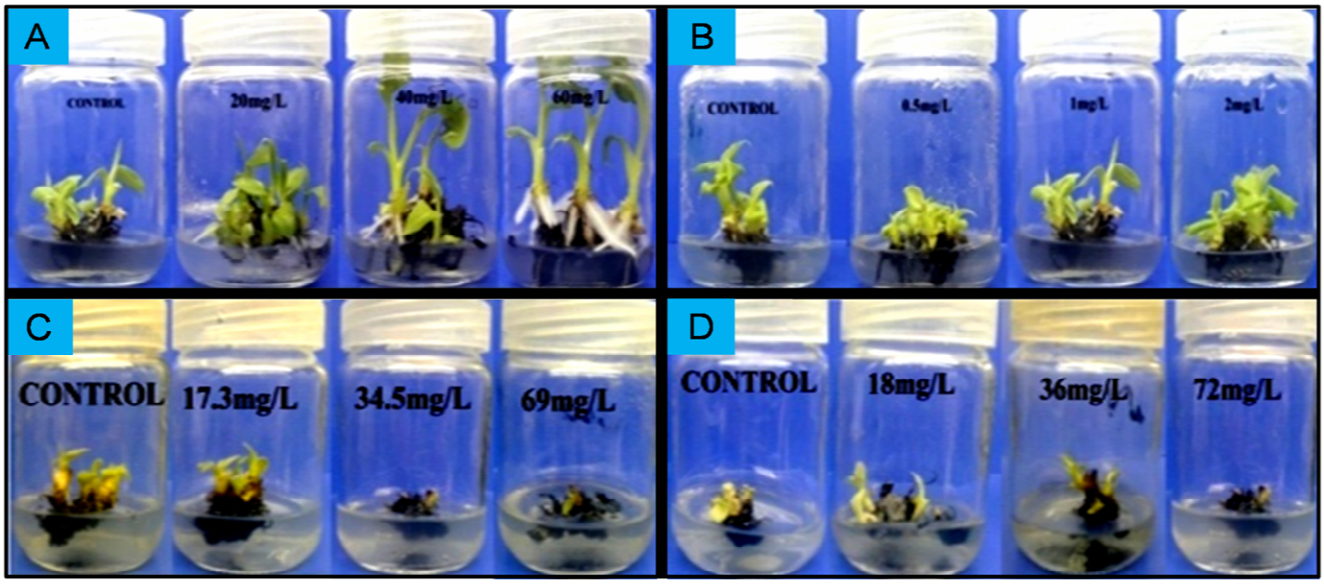

Fig. 1: The BBTV infected shoot tip explants after the treatment with four different concentrations of four antiviral substances. (A) Ribavirin (control, 20, 40 and 60 mg/l). (B) Jasmonic acid (control, 0.5, 1 and 2 mg/l). (C) Salicylic acid 0 µM (control), 125 µM (17.3 mg/l), 250 µM (34.5 mg/l) and 500 µM (69 mg/l), Benzoic acid 0 µM (control), 125 µM (18 mg/l), 250 µM (36 mg/l) and 500 µM (72 mg/l).

Effect of different concentrations of four antiviral substances on the percentage of virus-free plantlets

The results in Table (3) showed that the use of antiviral **substances** with different concentrations have significantly affected the percentage of virus-free banana plantlets. Particularly, the results of Ribavirin treatment at 40 mg/l achieved the highest percentage of virus-free banana plantlets, which reached 66.62% followed by the treatment 1 mg/l of Jasmonic acid (44.42%) and the treatment 125 µM (17.3 mg/l) of Benzonic acid (33.31%) in comparison with the control treatment. On the other hand, the results of the treatment 125 µM (17.3 mg/l) of Salicylic acid were ineffective in comparison with the control treatment. Moreover, the high concentrations 250 µM (36 mg/l) and 500 µM (72 mg/l) of Salicylic acid were significantly reduced the percentage of virus-free banana plantlets.

The results of PCR analysis revealed that the extracted genomic DNA from *in vitro* banana plantlets was at high quality and a sharp single fragment in a size 459 bp of the BBTV coat protein gene was detected in all banana positive samples without any mismatches in the negative control (healthy) plantlets as shown in Fig. (2). Moreover, the results of different experiments proved that this protocol is an efficient method for diagnosing the BBTV infection of *in vitro* banana plantlets and these results are in accordance with several reports (Mansoor *et al*., 2005; Chen and Hu, 2013; Mahadev *et al*., 2013).

Table 3: Effect of different concentrations of four antiviral substances on the percentage of virus-free plantlets.

Antiviral substances					
Conc.*	**Ribavirin**	**Jasmonic acid**	**Salicylic acid**	**Benzoic acid**	**Mean**
Control	11.11 f	12.73 f	11.10 f	11.12 f	11.52 d
1	45.02 c	22.23 e	11.11f	33.31 d	27.29 b
2	66.62 a	44.42 c	0.00 h	22.22 e	33.32 a
3	55.53b	31.51 d	0.00 h	6.66 g	23.43 c
Mean	44.57 a	27.72 b	5.55 d	18.33 c	

*Different concentration were added among each antiviral substances; Ribavirin (control, 20, 40 and 60 mg/l), Jasmonic acid (control, 0.5, 1 and 2 mg/l), Salicylic acid 0 µM (control), 125 µM (17.3 mg/l), 250 µM (34.5 mg/l) and 500 µM (69 mg/l), Benzoic acid 0 µM (control), 125 µM (18 mg/l), 250 µM (36 mg/l) and 500 µM (72 mg/l).

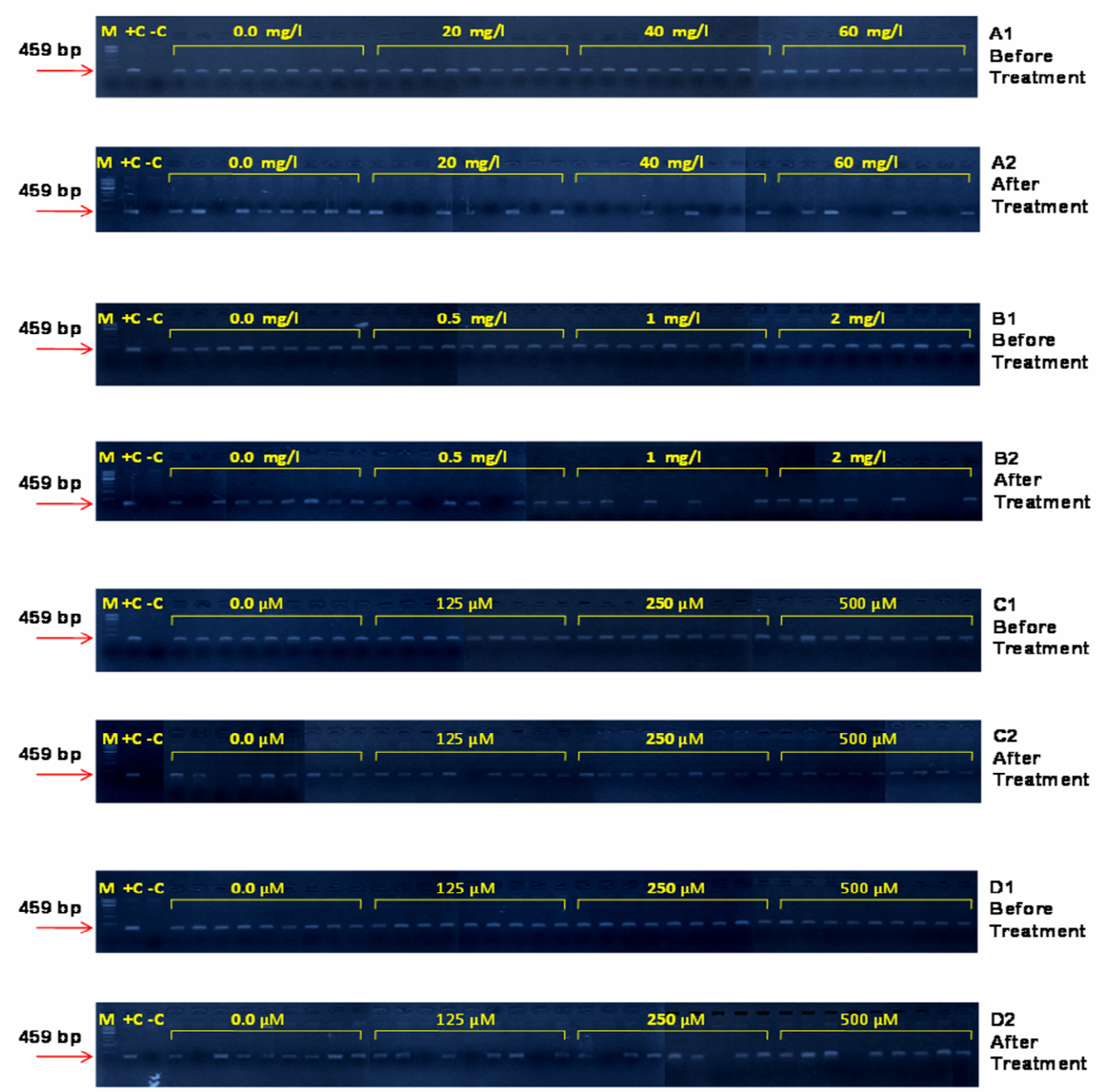

Fig. 2 : PCR analysis using specific primers amplify a 459 bp fragment of the coat protein gene (DNA-S) to detect the presence of banana bunchy top virus (BBTV) in leaves of *in vitro* banana plantlets before chemotherapy treatments and after 8 weeks of treatments (A1&A2) Ribavirin treatments, (B1& B2) Jasmonic acid treatments, (C1&C2) Slicylic acid treatments, and (D1&D2) Benzoic acid treatments. M; 1Kb DNA ladder, +C; positive control ssDNA of BBTV isolated from field grown infected banana plants, -C; negative control of DNA isolated from *in vitro* grown healthy banana plantlets.

Finally, infected BBTV shoot tip explants were cultured on MS medium contains four different antiviral substances with four concentrations to eradicate the virus. The use of Ribavirin at the concentrations 40 and 60 mg/l resulted in the highest percentage of virus-free banana plantlets (66.62 and 55.53%), respectively, compared to other antiviral chemicals investigated in this study. In the same line, the percentage of survival rate was the highest (93.20 and 93.29%, respectively). These results are in agreement with (Hazaa *et al*., 2006) when they tried to eliminate the BBTV from infected banana plant cv. Williams using 30 mg/l of Ribavirin and Salicylic acid which recorded 90% and 93% virus-free, respectively, which is probably higher than the rate we achieved in this study, and also they used the Salicylic acid and were able to reach 93%, on the other hand, this result in contradiction with our results reported here for Salicylic acid. To eliminate plum virus using Ribavirin, Paunvic *et al*. (2013) explained that, the ELISA test was not accurate enough with low concentrations of the virus inside plant cells, as opposed to the PCR, which can allow detection of low concentrations of the virus. Ribavirin is the most widely used among other antiviral substances and has been used to eliminate many viruses in many laboratories, with a low influence on the plantlets survival rate (Gulseker *et al*., 2015). In addition, the results of the present work proved that Jasmonic acid also was also an effective treatment to eliminate the BBTV from *in vitro* banana plantlets.

Conclusion

In this study, four different chemical substances (as antiviral) were used to evaluate the efficacy of chemotherapy treatments to eradicate the banana bunchy top virus from the infected banana plantlets grown *in vitro*. Therefore, *in vitro* cultured shoot tip explants of naturally infected mother Grandnan banana plants were treated by different concentrations of chemical substances which were added to the culture medium (MS) for four weeks. Ribavirin was used at 0, 20, 40 and 60 mg/l; Jasmonic acid was used at 0, 0.5, 1 and 2 mg/l; Salicylic acid was added at 0, 17.3, 34.5 and 69 mg/l; Benzoic acid was used at 0, 18, 36 ad 72 mg/l. After chemotherapy treatment, the shoots were subcultured on chemicals free MS medium for another 8 weeks (subcultured each four weeks). Samples of leaves were taken for analysis by PCR before chemotherapy treatment and then after 8 weeks of culture on chemicals free MS medium. The results of PCR analysis proved that the use of Ribavirin at 40 mg/l and 1 mg/l of Jasmonic acid produced the highest virus-free rate of banana shoots 66.62% and 44.42% with survival percentage 93.20% and 100%, respectively. While, the use of Salicylic acid and Benzoic acid produced the lowest virus-free rate of banana plantlets 11.11% with 17.3 mg/l Salicylic acid and 33.31% with 18 mg/l Benzoic acid treatments, respectively.

References

Beetham, P.R.; Harding, R.M. and Dale, J.L. (1999) Banana bunchy top virus DNA-2 to 6 are monocistronic. *Arch Viro*., l144: 89-105.

Chen, Y. and Hu, X. (2013) High-throughput detection of banana bunchy top virus in banana plants and aphids using real-time TaqMan® PCR. J. Virological Methods, 193: 177– 183.

Duncan, D.B. (1955). Multiple range and multiple "F-test". Biometrics, 11: 1-42.

Falcioni, T.; Ferrio, J.P.; Del Cueto, A.I.; Gine, J.; Achon, A.M. and Medina, V. (2013). Effect of Salicylic acid treatment on tomato plant physiology and tolerance to potato virus X infection. Europe Journal Plant Pathology, 138:331-345.

Gulseker, M.; Suzerer, V.; Elibuyuk, I.O.; Ozden and Ciftci, Y. (2015). *In vitro* elimination of PPV from infected apricot shoot tips via chemotherapy and cryotherapy. International Journal of Agriculture, 17: 1066-1070.

Hazaa, M.M.; El-Dougdoug, Kh.A. and Abo El-Maaty, S. (2006). Eradication of banana viruses from naturally infected banana plants 2.Production of certified banana plants and virus tested. Journal of Applied Sciences Research, 2: 714-722.

Kabir Shiragi, M.H.; Baque, M.A. and Nasiruddin, K.M. (2018). Eradication of banana bunchy top virus (BBTV) and banana mosaic virus (BMV) from infected plant of banana cv. Amritasagar through meristem culture. South Pacific Studies, 29:17-41.

Lassios, L.; Lepoivre, P.; Swennen, R.; VandenHouwe, I. and Panis, B. (2013) Thermotherapy, chemotherapy and meristem culture in banana. Humana Press, 994: 419-433.

Mahadev, S.R.; Thamilarasan, S.K. and Kathithachalam, A. (2013). PCR Detection of Banana Bunchy Top Virus (BBTV) at Tissue Culture Level for the Production of Virus-free Planting Materials. Intern. Res. J. Biol. Sciences, 2: 22-26.

Mansoor, S.; Qazi, J.; Amin, I.; Khatri, A.; Khan, I.A.; Raza, S.; Zafar, Y. and Briddon, R.W. (2005). A PCR-based method, with internal control, for the detection of Banana bunchy top virus in banana. Mol. Biotechnol., 30: 167–170.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassaya with tobacco tissue culture. Physiologia Plantarum, 15: 473-497.

Paprstein, F.; Sedlak, J.; Svobodova, L.; Polak, J. and Gadiou, S. (2013) Result of *in vitro* chemotherapy of apple cv.Fragrance-Short communication. Hort. Sci., 40: 186-190.

Paunovic, S.; Ruzic, D.; Vujovic, T.; Milenokovic, S. and Jevremovic, D. (2013). *In vitro* production of plum pox virus-free plums by chemotherapy with ripavirin. *Biotechnology & Biotechnological Equipment*, 21: 417-421.

Shankar, K.; Chavan, L.; Shinde, S. and Patil, B. (2011). An improved DNA extraction protocol from four *in vitro* banana cultivars. Asian J. Biotechnology, 3: 84-90.

Snedecor, G.W. and Cochran, W.G. (1969). Statistical Methods (6th Ed.)Iowa State University Press, Ames, USA.

Thomas, J.E. and Dietzgen, R.G. (1991). Purification, characterization and serological detection of virus-like particles associated with banana bunchy top disease in Australia. J. Gen. Virol., 72: 217–224.

Tripathi, S.; Prabhu, B.; Patil, B. and Verma, R. (2016). Viral diseases of banana and their management. Springer Science, 2: 289-308.

Wu, R.Y. and Su, H.J. (1990). Production of monoclonal antibodies against banana bunchy top virus and their use in enzyme-linked immunosorbent assay. J. Phytopathol., 128: 203–208.

Xie, W.S. and Hu, J.S. (1995). Molecular cloning, sequence analysis and detection of banana bunchy top virus in Hawaii. Phytopathology, 85: 339–347.