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GENETIC STABILITY IN TISSUE CULTURE-DERIVED BANANA VIRUS TESTED USING THIOURACIL

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ABSTRACT

Banana bunchy top virus (BBTV) is a destructive viral disease in many countries including Egypt. It causes severe economic losses because infected banana plants produce no fruit. The tissue culture approach was used to permit the recovery of BBTV-free plantlets, genetic stability followed by chemotherapy and early screening to facilitate the efficient production of virus-free plantlets. Results demonstrated that application of 10,20,30,40 mg/L thiouracil *in vitro* gave an 85,72,35,20 survival and 60, 83, 91 and 95.5% BBTV-free plantlets ,respectively. Furthermore, the obtained virus free micropropagated plantlets were subjected to DAS-ELISA detection. Tissue culture-derived genetic stability banana plants and virus tested plants were screened by RAPD- PCR. Only two RAPD primers (among 10 tested) were chosen as producing polymorphic DNA bands differentiating the investigated micropropagated plants. Based on DNA markers, the genetic stability between micropropagated plants were estimated. The morphological variations were recorded in shoots of micropropagated clones more than healthy clone. The developed RAPD profiles of different micropropagated clones were untypical to that of the healthy clones. The phylogenetic tree recorded that the plantlets derived from healthy and sanitary (10 mg/L thiouracil) plants showed close similarity within the first group while sanitary (20 mg/L) second group and sanitary (30 and 40 mg/L) plants third group showed 70 and35% similarity with first group. The results demonstrated that application of 10 to 20 mg/L thiouracil *in vitro* gave survival plantlets higher than30 and 40 mg/L thiouracil.

Keywords: RAPD-PCR, banana clones, genetic variation, chemotherapy, somaclonal variation.

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INTRODUCTION

Tissue culture plays an important role for the mass multiplication of crops of economical interest which are unable to be bulked up to greater quantities to meet the ever rising market demands of quality plants. Thus , for the provision of *Banana bunchy top virus* (BBTV) diseases and multiplication of virus free banana plants on a massive scale to replenish the infected banana plantations, micropropagation is almost important as thousands of disease free and virus tested plants can be produced in a very short time. A common concept of tissue culture is that all the plants produced will be true to type as tissue culture is considered to be a method of clonal propagation therefore theoretically, all the individuals produced have the same genetic constitution of the plant. Present techniques do not permit this in every case and irregularities sometimes occur, resulting in genetic variants. A valid concern arises with respect to the genetic stability of tissue cultured plants. Genetic variability develops spontaneously during tissue culture and somaclonal variation is of interest as a basic genetic process since it contradicts the concepts of clonal uniformity. These cells and tissue are expected to produce true to type plants through the process of de-differentiation divisions and re- differentiation **(Bushra, 2006).**

Tissue culture techniques classical breeding is a well recognized tool for increasing yield, conferring resistance and improving agricultural trials. *In vitro*, plant production involves the application of plant growth regulator such as auxins for initiation process. Nevertheless, these auxins are known to be associated with genetic stability in plants, a phenomenon called somaclonal variation **(Cullis, 1992).**

Somaclonal variation may be used as a source for variation to get superior clones, it could be also a very serious problem in the plant tissue culture industry resulting in the production of undesirable plant-off-types **(Karp, 1993).** In addition, plant viruses, such as BBTV may be assailed with genetic instability in plants **(El-Dougdoug *et al.*, 2007).**

Since somaclonal variation was first defined, it has been widely documented in tissue culture-raised plants at morphological chromosomal, biochemical and

molecular levels in many plant species and extensively reviewed **(Brown, 1991).** Polymorphism DNA level among the somaclonal families which phenotypically normal was reported in banana **(El-Dougdoug *et al.*, 2007)** and in strawberry **(Damiano *et al.*, 1997).**

Traditional methods, based on morphological karyotypic analysis of metaphase chromosomes and isozyme analysis have been used to determine genetic variations in somaclones and to identify parental hybrids and cultivars. Numerous investigators have successfully employed Randomly Amplified Polymorphic DNA[RAPD] to find molecular markers that could be used for genetic analysis of micropropagated and regenerated plants **(El- Dougdoug *et al.*, 2007)** , genotypic screening and breeding programmes **(Chun uongase *et al.*, 1993) .**

Therefore, this study was designed to employ isozyme and RAPD analysis for genetic integrity and tissue culture- derived plants.

MATERIALS & METHODS

Plant materials: Tissue culture derived banana plantlets (*Musa paradisca* cv.williams) were initiated from shoot tip explants BBTV infected and healthy ones.(Virology Lab, Fac. Agric. Ain Shams Univ.). Time course needed for the attainment of explant shoots is approximately 5 months old culture (subculture 4^{Th}).

Phytotoxicity: To monitor the assessment of mutation effects of thiouracil as well as virus-free were performed on MS medium supplemented with 10, 20, 30 and 40 mg/L medium of thiouracil. After 24 days treatment regenerated plantlets were transferred to an MS medium without thiouracil (regular MS medium) used as control. The mutant effect was evaluated based on viability and analysis of DNA using RAPD-PCR as well as the percent of plantlets virus tested.

BBTV Screening: Initially the health and BBTV status of regenerated materials were checked on the survived *in vitro* plantlets through DAS-ELISA using polyclonal antibodies of BBTV **(El- Dougdoug *et al.*, 2006).** The eradication rate was calculated in fine for each treatment as follows:

$$\text{Eradication \%} = \frac{\text{negative in vitro plantlets}}{\text{Tested in vitro plantlets}} \times 100$$

Genomic DNA isolation: DNA of 5 different tissue culture derived subculture banana plants and mother plants was extracted using CTAB method **(Doyle and Doyle, 1990).**

RAPD–PCR analysis: Twenty 10 mer oligonucleotide primers (Oderon technology, USA) were randomly chosen for the study. PCR conditions were performed to a total volume of 40 ml reaction mix containing 1ml of 10 X reaction buffer (2mM $MgCl_2$, 2µl dNTD at 0.2 mM ,0.1µl (0.5µ) of Tag DNA polymerase, (promega USA), 30 µg of genomic template DNA and 10 p mol primer in a preheated thermocycler (Biometrica UnO) . PCR was initiated by a denaturation step at 92°C for 3 min and the reaction was subjected to 45 cycles of 92°C for 30 sec , at 35°C for1 min , 72°C for 2 min and a final elongation step of 10 min at 72°C . In order to select the optimal conditions of the RAPD – PCR different optimization experiments were carried out.

The amplification products were resolved by electrophoresis on 1.5% agarose gel with ethidium bromide and visualized under UV. The presence and absence of bands among samples was scored and data were transcribed into binary format (1, 0, respectively).

Based on the matrix of genetic similarity, cluster analysis was performed. The UPGMA method (unweighted pair-group method with arithmetic averages) was used for clustering employing the NISYS-PE program **(Rohlf, 2001).**

RESULTS

Chemotherapy is an alternative *in vitro* technique traditionally used for virus elimination. To avoid the phytotoxicity of thiouracil it was found that using 10 or 20 mg thiouracil gave the high percentage of sanitary survival (85 and 72%) and BBTV elimination (60 and 83 %) respectively **(Table1)** .

On the contrary 30 and 40 mg^{-1} thiouracil due to induced phytotoxicity and decreased sanitary survival banana plants (35 and 20 %) respectively, whereas such treatments increased BBTV elimination (91.5 and 95.5%) respectively. The BBTV elimination from sanitary shoots was determined by DAS-ELISA. It was found that the BBTV concentration was decreased along

with increasing thiouracil concentration **(Table1).** Thiouracil compound inhibits BBTV and appeared to cause phytotoxicity or mutation in the tissue culture-derived treated banana .

In vitro micro propagation of banana-BBTV tested from shoot tip, through the proliferation of meristem on MS medium treated with 10, 20, 30 and 40 mg/L thiouracil followed by shoot recovery give rise to three types of the shoots (fig.1). The types can be clearly detected after prolonged periods of sub culturing. Normal shoots comparable to healthy control were the majority of the 3 month old culture and treated with 10 and 20 mg/L as a result of prolonged in vitro culture the plants were with the same phenotypic.

On the contrary the culture treated with 30 and 40 mg/l thiouracil showed variable visible phenotypic. Phenotypic variations including plants stunting, rosy cluster, faint green leaves, and twisted leaves can be detected in 3 month old culture **(Figure 1).**

Based on the phenotypic polymorphism which was clearly observed in five tissue culture-derived banana plants cv. williams, a simple molecular marker for the identification of the genetic variations among tissue culture-derived plants is required.

Preliminary RAPD analysis technique was performed to standardize a reproducible protocol for banana genome analysis. It was found that PCR conditions, especially thermal profiles and source of tag polymerase resulted in disappearance or appearance of DNA bands.

On the basis of the number, intensity and reproducibility of RAPD bands two primers (OPA2 and OPC5) were selected out of 5 primers, which were previously tested. Bands with the same mobility were treated as identical fragments. Weak bands with negligible intensity and smear bands were both excluded from final analysis. **Figure 1** demonstrates the RAPD profiles obtained with two primers (OPA2 and OPC5). The number of scored bands were 8 and 7 bands per OPA2 and OPC5, respectively with different molecular weight ranged from 1450 to 210 bp. The DNA amplified fragments of BBTV infected plantlets, treated healthy

and infected with 10, 20, 30 and 40 mg^{-1} MS medium (sanitary) as well as ones were varied in number, intensity and molecular weight. The variability among 6 banana plantlets appeared some DNA amplified fragments absent or / and present **(Table 2).**

The polymorphism among 5 banana plantlets; 7 monomorphic amplified bands are common in all banana plantlets with 46.6%; 6 polymorphic amplified bands (specific bands) with 40% and 2 unique bands (genetic markers) with 13.3%. The genetic markers were 975 and 315 bp found in sanitary plantlets with 40mg/l thiouracil and BBTV infected while were absent in other sanitary banana plantlets 10, 20, 30 mg^{-1} thiouracil as well as healthy ones.

In order to confirm the genetic variation (mutations at molecular level) of the banana plantlets treated with thiouracil in vitro (tissue culture derived), the induced mutation between 5 banana plantlets by thiouracil was screened with the ten random RAPD primers. The DNA was isolated from the level of 4 banana plantlets thiouracil treated and healthy untreated ones. After RAPD amplification, it was obvious that the infected banana plantlet treated with 40 mg^{-1} l thiouracil showed non- identical RAPD profiles which observed polymorphism. As an example **(Figure 2 and Table 2)** presented the 5 unique banding patterns of the RAPD profiles which were amplified using 10 mer OPA2 and OPC5 random primers.

Table 1. Percentage of survival and BBTV elimination in banana plants cv.williams by chemotherapy.

Tissue samples	Thiouracil Mg/l	% of survival	% of BBTV elimination	DAS - ELISA absorbance value (405nm)
Healthy shoot tip	0	100	—	0.075
Infected shoot tip	0	90	0	0.425
Sanitary shoots	10	85	60	0.275
Sanitary shoots	20	72	83	0.172
Sanitary shoots	30	35	91.5	0.185
Sanitary shoots	40	20	95.5	0.098

Negative control 0.075 O. D. at 405 nm
Positive control 0.475 O. D. at 405 nm

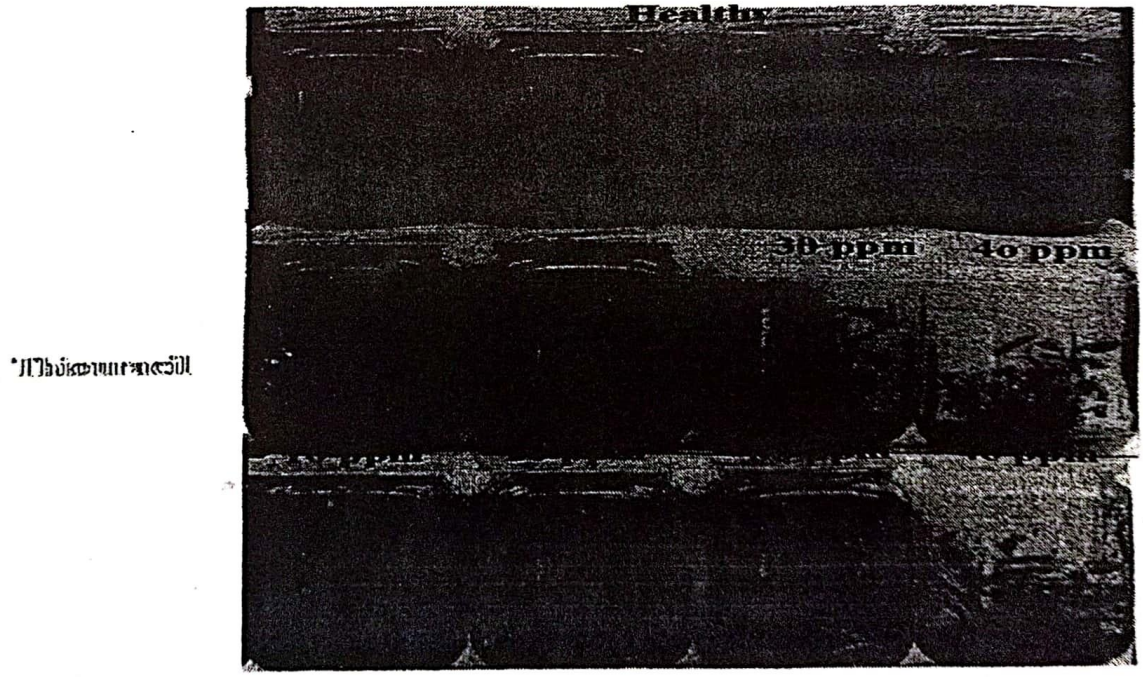

Figure 1. Effect of thiouracil on micropropagation of banana *in vitro*

Table 2. DNA polymorphism and genetic markers of tissue culture derived banana plants by RAPD–PCR analysis.

Genetic markers							MW	polymorphism				primer
	S40	S30	S20	S10		H	(bp)	Unique	MAF	PAF	TAF	
Polymorphic	+	++	—	—		—	1450	2	3	3	8	
Polymorphic	+++	+++	—	—		—	1125					OPA 2
Unique	+++	—	—	—		—	975					
Monomorphic	++++	++++	++++	+++		+++	750					
Polymorphic	++	++	+++	+		+++	625					
Monomorphic	++	++	++	++		++	450					
Unique	—	—	—	—		—	315					
Monomorphic	++	++	++	++		++	210					
Polymorphic	++	++	—	—		—	1450	—	4	3	7	
Polymorphic	++	++	—	—		—	1125					OPC 5
Polymorphic	+++	+++	—	—		—	975					
Monomorphic	++++	++++	+++	+++		++	652					
Monomorphic	++	+	+++	++		++	450					
Monomorphic	++	++	++	++		++	315					
Monomorphic	+	++	+	+		+	210					
	14	13	8	8		8		2	7	6	15	Total

TAF: total amplification fragment
PAF: polymorphic or specific amplification fragment
MAF: monomorphic or common amplification fragment
Unique: genetic marker
(–) Absent band , (+) Weak band, (++) Moderate band, (+++)Strong band, (++++)Very strong band
Lane H:healthy shoots, Lane V: BBTV infected shoots, Lane S10: Sanitary shoots 10 mg thiouracil
Lane S20: Sanitary shoots 20 mg thiouracil, Lane S30: Sanitary shoots 30 mg thiouracil
Lane S40: Sanitary shoots 40 mg thiouracil.

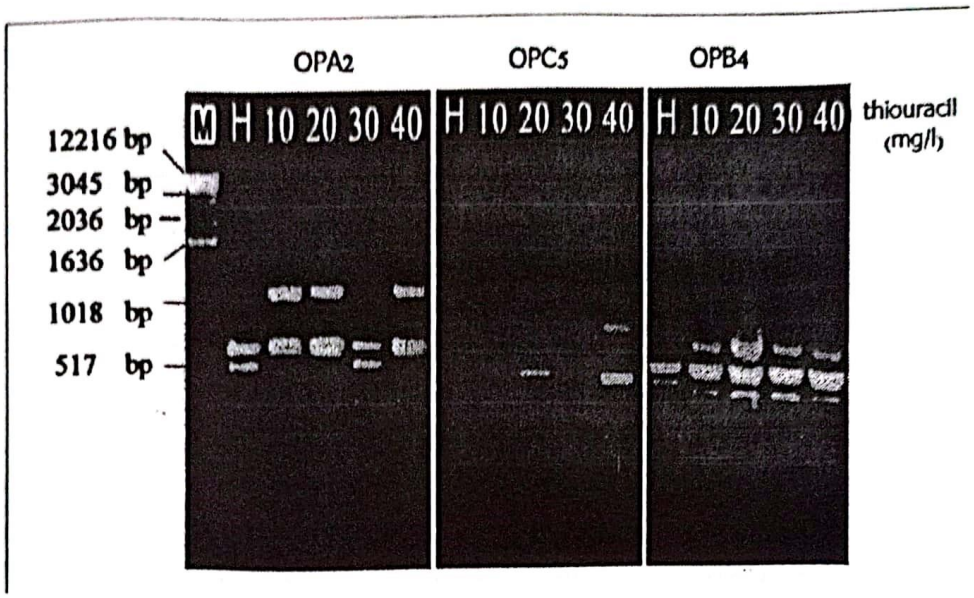

Figure 2. Agarose gel (1.5%) showing DNA amplification profiles of tissue culture-derived healthy, BBTV infected and sanitary banana plantlets by RAPD-PCR. M: 1K bp marker.

DISCUSSION

Tissue culture techniques and classical banana breeding are well recognized tool for propagation of certified banana plants and virus tested **(El-Dougdoug, 2007).** Although there are many references dealing with banana tissue culture, e.g. **(Banerjee and Delanghe, 1985; Bushra, 2006** and **El-Dougdoug *et al.*, 2007**). the analysis of tissue culture derived plants for somaclonal variation or mutation has yet to be established. ELISA test was done to detect BBTV in shoot tip of mother plant and micropropagated shoots which are used to produce banana certified and BBTV tested **(El-Dougdoug *et al.*, 2006)** where the virus infected tissues were serologically reacted specifically with specific BBTV antibodies. The specific virus antibodies was used to detect the presence of BBTV in plant tissues and enabled to detect many samples all at once.

Chemotherapy using thiouracil is an alternative in vitro technique traditionally used for virus

elimination **(Walkey, 1991).** According to phytotoxicity of thiouracil we decided by low concentration 10 or 20 mg^{-1} thiouracil, while 30 or 40 mg^{-1} decreased survival rate and increased the percentage of BBTV elimination. Antiviral compounds is too closely linked with normal metabolic processes in plants further some of substances that inhibit viruses appear to be phytotoxic or cause mutation in the treated plants .

Chemical analogous to purine and pyrimidine bases of nucleic acid that has been extensively tested against both DNA and RNA viruses. Many investigators demonstrated that their incorporation in culture media resulted in an increased percentage of virus-free progeny. These chemicals proved to be effective in animal cell cultures by reducing the availability of ATP for DNA and RNA protein synthesis and GTP derivatives such as GDP-glucose inactivation of intact virus particles presence in a cell by breaking of their RNA or DNA **(Kanovalova, 1990).**

Screening of the thiouracil concentrations for virus elimination using tissue culture revealed that banding profiles obtained by OPA2, OPC5 and OPD4 primers were enough to distinguish all the concentrations. The results indicated that the RAPD technique is effective to develop genotype-specific banding patterns valuable for the mutant identification. The obtained results confirm the usefulness and stability of RAPD markers for genetic variation detection. Our results are in agreement with **EL-Dougdoug *et al.*, (2007)** who used RAPD markers to detect and assess the level of somaclonal variations in tissues culture-derived banana plants, they have confirmed the value of RAPD markers for banana plants-derived tissue culture and selection of subculture suitable for transplanting of genetic stability.

Since RAPD technique does not require previous DNA sequence information and uses very small quantity of DNA, it is considered as one of the most widely used techniques for genetic diversity studies. However, there is a problem with RAPD regarding its reproducibility. The reproducibility of amplification profiles of RAPD is influenced by any variation in the method used for DNA isolation

(Korbin *et al.*, 2000), concentration of template DNA and primer. Tag DNA polymerase concentration, temperature of annealing, number of thermal cycles and $MgCl_2$ concentration **(Kernodle *et al.*, 1993).** Several researchers have reported that the majority of RAPD band are reproducible if one take care in developing a standardized protocol which is strictly followed in each reaction **(Gibbs *et al.*, 1994).** In order to ensure high RAPD reproducibility, it is essential to optimize the PCR reaction.

RAPD has been used for genetic stability and somaclonal variation in banana tissue culture **(El-Dougdoug *et al.*, 2007, 2008).** In our study, only three RAPD primers (20%) were able to generate polymorphisms among banana sub culture on MS media. This result is in accord with what have found by **EL-Dougdoug *et al.* (2007, 2008)** and where only three primers were sufficient to identify all the studied material.

In this study, 10 random primers were used in RAPD analysis to prove the clonal fidelity (i.e. genetic stability) of the tissue culture-derived banana plants. Identical banding patterns were observed with all primers tested. These results confirmed the genetic stability of the tissue culture derived banana plants. Molecular markers are believed to be reliable in monitoring variability at the DNA level in plants. RAPD technique was used by several research groups to examine genetic variability and it has been found to be very efficient and reliable **(Jayanthi and Mandal, 2001).** As found in the present study, various investigators have observed the absence of variations in date palm **(Saker *et al.*, 2005)**, *Picea mariana* **(Isabel *et al.*, 1993)** and *Festuca pratensis* **(Valles, 1993)** using RAPD technique. On the contrary , somaclonal variations were found in banana **(EL-Dougdung *et al.*, 2007)**, *Populus deltoids* **(Rani *et al.*, 1995)** and peach **(Hashmi *et al.*, 1997).**

In contrast to the RAPD results, minor morphological variations were observed in case of the leaves of tissue culture - derived banana plants. The variability observed at the morphological level may be caused by the clonal growth habit of the banana **(Dekroons and Hutching, 1995).**

These results mean that molecular tools are more reliable than the phenotypic observations for evaluating variations and monitoring genetic stability. It also highlights the need for alternative methods of definitive identification based on molecular techniques such as RAPD or amplified fragment length polymorphism (AFIP).

We demonstrated that RAPD analysis can detect sufficient polymorphism to differentiate among micropropagation of banana plants tissue culture derived banana plants and that it is suitable for studying their genetic relationships. This study is considered as a useful report on the assessment of genetic variability of cultivated banana genotypes by RAPD molecular markers. Our results showed a much higher level of genetic variability among subcultures of tissue culture-derived banana plants. While no variation was detected among the free subculture of tissue culture - derived banana plants, which indicated higher genetic stability within each cultivar **(EL-Dougdoug *et al.*, 2007)**.Therefore, the results of molecular characterization of banana cultivar and their genetic relationships provide important parameters for breeding and can be used in the further development of new banana cultivars.

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