Efficiency of eugenol oil nanoemulsion against Banana bunchy top virus and contamination with fungi in plant tissue culture

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ABSTRACT

Development of micropropagation technique for mass propagation of virus-free banana plantlets and reduction of the frequency of fungal contamination is critical. For this purpose the experimental trials in the current study aimed to evaluate the effectiveness of eugenol oil nanoemulsion (EON) on elimination of Banana bunchy top virus (BBTV) and fungal contaminants of banana under in vitro conditions. To achieve our aims, the virus has been identified on the basis of symptoms from naturally infected banana seedlings with its suckers (Musa spp. cv. Grand Nain), grown in Qalyubia Governorate, and later by serological detection (DAS-ELISA) as BBTV then used as a source for virus-infected explants in a tissue culture line. Three fungal contaminants (Aspergillus flavus, Aspergillus niger and Penicillium expansum) were identified in growth media of banana in vitro cultures on the basis of colony morphology and microscopic appearance. The rate of occurrence of aspergilli (Aspergillus flavus and A. niger) was found to be higher than Penicillium expansum. Different concentrations (125, 250 and 500 mg/L) from EON were studied in vitro. All tested concentrations exhibited high inhibitory activities (94.81, 95.5and 96.66 %) against Penicillium expansum and almost the same effect (88%) on Aspergillus niger but fluctuated their effect on A. flavus causing a decrease in the size and rate of growth by 79.99, 80.73 and 77.77%, respectively. On the other side, the inhibition rate of virus activity caused by treatments was evaluated using DAS-ELISA. Results showed that there was no antiviral effect of EON at concentration 125 mg/L compared with concentrations of 250 and 500 mg/L of EON which caused a very low antiviral activity through primary establishment stage. The antiviral effect of EON seems to be based on the incorporation of EON particles, three successive times, into culture MS media during implementation of the in vitro multiplication. Preliminary results indicate a possibility of therapeutically application of EON at high concentration (500 mg/L) against BBTV with completely decontamination of culture medium in plant tissue culture technique. Moreover, root induction was achieved on MS medium containing 1.0 mg/L indole-3-butyric acid and complete BBTV-free banana plantlets were obtained after five weeks in vitro.

Keywords: *Banana, Banana bunchy top virus, Micropropagation, Eugenol oil nanoemulsion, Antiviral activity, Antifungal activity.*

INTRODUCTION

Banana bunchy top disease (BBTD), caused by *banana bunchy top virus* (BBTV) and gets its name from the bunchy appearance of infected plants, is one of the most serious diseases affecting many banana-cultivated areas of the world. It has a major impact on the local agriculture, which reduces banana yields and market value. Now, the disease is endemic in 11 countries in Africa and BBTD alone can cause yield losses up to 100%. BBTV (genus *Babuvirus*, family *Nanoviridae*) is primarily transmitted through infected planting material, spread by banana aphid (*Pentalonia nigronervosa*), and can lead to stunted growth of the banana plant. The first symptoms are dark green streaks near the base of the petiole (or leaf stalk), and later along the veins of the leaf. The virus can move systemically to infect the entire banana corm and any new shoots derived from the corm, and in advanced stages of the disease, the infected plants produce deformed fruits or not edible (Watanabe *et al*., 2013 and Mukwa *et al*., 2014).

Plant tissue culture is a standard practice in banana propagation, aiming to provide virus-free planting material to banana farmers with superior quality. However, microbial contamination represented a prevailing and serious obstacle in plant tissue culture laboratories that mainly related to different contamination sources. Like the main explants or may be the sterilization technique by laboratory workers is not performed correctly, makes it harder to control contamination levels. In different experimental procedures, contamination problems have been also associated with a wide variety of antimicrobial treatments, including chemical sterilization, antibiotics and fungicides (Leifert and Cassells, 2001). Additionally, the culture medium also serves as a good source of nutrients for microbial growth and the presence of these contaminants usually results in an increase of culture mortality rates (Odutayo *et al*., 2007), loss of high-value crops and time or effort spent for developing cultures and setting up experiments that may need to re-establish new cultures.

Losses due to contamination reached up to 15% at every subculture in the majority of research organizations and commercial plant tissue culture laboratories as well as about 40-60% of banana explants destroyed under *in vitro* conditions in spite of using main aseptic reliable procedures (Msogoya *et al*., 2012 and Helaly *et al*., 2014).

In ancient times, the Egyptians were the first civilization used the aromatherapy and aromatic herbs in cosmetics and for medicinal purposes. In recent times, there has been an increased interest in looking at antimicrobial properties of aromatic oily liquids (essential oils, EOs) obtained from different plant materials using modern extraction technologies (Brenes and Roura 2010). Studies have shown that the antimicrobial activity of EOs is attributable to eugenol (Fig.1), one of the major constituent of EOs from *Syzygium aromaticum*. *S. aromaticum* or common clove (Chaieb *et al*., 2007).

Different methods, the disk diffusion method and agar well test, were used to quantify the antimicrobial activity of EOs. However, these methods are not accurate because the hydrophobic properties of EOs do not allow them to diffuse in aqueous systems. Emulsions by definition are dispersion of two immiscible liquids where one liquid is dispersed as droplets in the continuous phase of other liquid, and when the droplet size reaches sub-micron scale, such emulsions are called as nanoemulsions. Nanoemulsions have already proven successful in inhibiting the

growth of a wide range of microorganisms including viruses, fungi, and bacteria (Hamouda *et al*., 2001; Hemmila *et al*., 2010 and Pedro *et al*., 2013). This, in turn, reduces the undesirable contaminants and; therefore, nanoemulsions can act as an available, cheap and eco-safe way or may be critical to the antiviral and antifungal activities in plant tissue culture line. In the light of the above, the present study aimed to (I) evaluate the antiviral activity of eugenol oil nanoemulsion (EON) on eliminating BBTV from naturally infected banana plants and produce virus-free banana plants, (II) identify fungal contaminants of *in vitro* banana cultures and (III) evaluate the potential of EON on the suppression of the identified microbial contaminants and reduce their occurrence frequency.

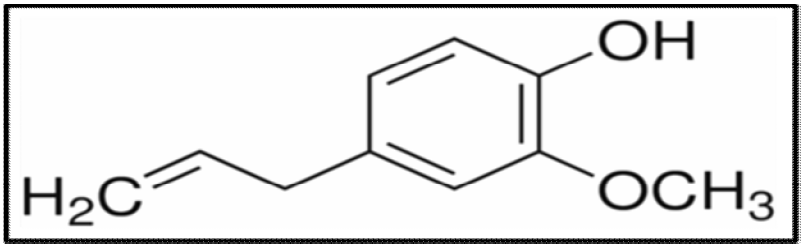

Fig. (1): Chemical structure of eugenol (4-Allyl-2-methoxyphenol).

MATERIALS AND METHODS

Plant materials and serological detection

Suckers of banana plants (*Musa* spp. cv. Grand Nain) showing the characteristic symptoms of viral infection were obtained from a banana plantation located in Qalyubia Governorate, Egypt. Banana plant sap of 63 samples was tested for the presence of the viruses (BBTV and CMV, *Cucumber mosaic virus*) serologically by using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adam, 1977). Absorption values at 405 nm were recorded using a microplate reader (Dynatech MR 7000). Reactions were considered positive when the infected/healthy ratio was 2 or more. DAS-ELISA was the main virus assay method used in this study. Testing was performed according to the manufacturer's instructions using specific viral diagnostic kits (Agdia-Products).

Surface-sterilization procedures

The collected suckers were prepared, surface sterilized for 20 min in a fungicide mixture and taken to the lab for further sterilization. From the selected suckers a cube of tissue about 1-2 cm^3 containing the apical meristem is excised, under laminar airflow cabinet. Tissue blocks were placed in 70% ethanol for 30 sec, followed by 50% commercial Clorox solution mixed with few drops of Tween 20 for 20 min, washed four times with sterile distilled water (SDW) every 10 min then were used to initiate shoot culture.

In vitro cultures micropropagation

The procedure for micropropagation of banana plantlets was done at tissue culture unit, Virus and Phytoplasma Department and comprised of four stages: culture initiation, multiplication, regeneration and rooting (Table 1). The cultures were initially incubated under artificial conditions of 26±2°C in darkness.

After 4 weeks, all subsequent subcultures were maintained at the same conditions under fluorescent illumination (intensity about 2000 Lux) with 16 h photoperiod. The pH of the medium was adjusted to 5.8 prior to autoclaving. During the initial phase, infected banana explants (positive-ELISA) were inoculated individually onto shooting initiation solid MS medium with full strength salts (Murashige and Skoog, 1962) supplemented with 4 mg/L BAP for culture establishment. The explants were weekly transferred to similar fresh medium to reduce browning and complete removal of blackish basal tissues. Another 4 weeks, clusters of many shoots were formed. Afterward, the proliferated microshoots were separated from their clumps and then subcultured twice onto similar fresh medium to reach the desired shoot numbers for forthcoming experiments. The total number of cultures derived from BBTV-infected banana explants and contamination rate was scored at the end of each culturing period.

Isolation and identification of fungal contaminants

Emerging contaminant plants were cut into small pieces (1cm), then surface sterilized by soaking in 2% sodium hypochlorite solution for 3 minutes and then washed thoroughly with SDW for two times. Surface sterilized pieces were put on sterilized filter papers to dry and transferred to Petri-plates (9 cm diameter) containing 25 ml potato dextrose agar (PDA) medium. Plates were incubated at 25±1°C for 5-7 days. The obtained isolates were divided into three categories according to their external features and growing characters. Most frequently occurring fungal species were further validated by culture at Seed Pathology Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt. Individual fungal colonies were selected and further purified by repeated subculturing on fresh PDA medium. Identification procedures for the isolated fungi were carried by using the morphological characteristics and their microscopic features according to Neergaard (1973) and as recommended by International Seed Testing Association, ISTA (1976 a,b); confirmed by Mycology and Plant Disease Survey Department, Plant Pathology Research Institute, ARC, Giza, Egypt.

Antifungal activity assay

Three concentrations (125, 250 and 500 mg/L) of EON used in this study were obtained by Dr. Kamel. Institute of Phytopathology, Agricultural Research Centre, Giza, Egypt. Researcher and co-author have identified the characterization of EN by stability analysis, particle size analyzer and transmission electron microscope (Abd-Elsalam and Khokhlov, 2015). One hundred microliters of each concentration was added aseptically on Petri-plates (9 cm-diam.) containing PDA media before solidification. Disks (5 mm-diam.) of each isolated fungi taken from seven day-old cultures were placed on the centre of sterilized Petri-plates. Each concentration test was replicated three times. Another set of untreated PDA medium was used as a positive control. Plates were incubated at 25±1°C until the tested fungi reached full growth in the check treatment and the growth was measured in millimeter. At the end of each treatment, the efficacy of EON in terms of percentage of inhibition of mycelial growth was calculated according to Fokemma (1973) using the following formula: Inhibition (%) = [(dc – dt) / dc] x 100, where dc = average increase in diameter of mycelial growth in control, dt = average increase in diameter of mycelia growth in treatment.

Antiviral activity assay

To evaluate the effect of treatment with EON on BBTV-elimination from banana plants and survival rates, four concentrations of nanoemulsion were tested: 0, 125, 250 and 500 mg/L. 100 µl of each level were applied individually through sterilized filter 0.22 µm pore size onto semi-solid MS medium after autoclaving (~25 ml per culture jar). Ten infected explants with BBTV were used for each treatment and the experiment was conducted twice. All cultures were incubated under the same artificial conditions for 4 weeks. Treatment efficiency (TE) was determined according to the rate of success in removing virus using DAS-ELISA and percentage of survival using following formula: TE (%) = [Number of survived virus-free explants / Total number of explants in each treatment] ×100

Efficacy of EON against BBTV and fungal contaminants

Studying the effect of the nanoemulsion against BBTV and fungal contaminants was done at the same time through multiplication stage in three subcultures under the same artificial conditions for 4 weeks each. The concentrations investigated were added at each subculture as above and starting medium was replaced with shooting one. Ten proliferated shoots were individually separated from contaminated cultures and used in the beginning of each treatment. In the second and third subcultures, the multiple shoots were separated and trimmed into 2 pieces or in some cases into 3, and then inoculated into the same culture media. The rates of contamination were evaluated at the end of each subculture as well as the percentage of surviving plantlets while, the rate of success in removing virus was determined based on DAS-ELISA assay at the end of multiplication stage before taking either steps, regenerating and rooting.

Plantlets regeneration and rooting stage

After about three months in shoot multiplication medium, shoots clusters were transferred to the fresh media free of EON for 4 weeks, then separated into individual shoot and kept three weeks for shoot elongation. The regenerated shoots of about 3 cm in height were transferred in half strength rooting medium containing hormonal supplements for root induction under the same incubation conditions for 4-5 weeks. Samples were randomly taken from several parts of plant body in order to confirm diagnosis and their freeness of viral infection using DAS-ELISA.

Statistical analysis

Data obtained from antifungal activity study were analyzed by the Analysis of Variance method, and the means were compared using the Least Significant Difference (LSD) test at the 0.05 level, as recommended by Snedecor and Cochran (1982).

Table (1): Chemical composition of culture media for different growth stages of banana micropropagation.

Constituents (g/L)	MS Medium of growth stages		
	Starting	Multiplication	Rooting
Sucrose	30.00	30.00	30.00
Agar	7.000	5.000	7.000
MS salt	4.200	4.200	2.100
Myo-inositol	0.100	0.100	0.100
BAP	0.004	0.005	0.000
IBA	0.000	0.000	0.001
Activated charcoal	0.000	0.000	0.500

MS = Murashige and Skoog (1962). BAP = 6-benzylaminopurine. IBA = Indole-3-butyric acid.
The pH of the medium was adjusted before autoclaving to 5.8
All media were sterilized by autoclaving at 121ºC/25 min.

Fig. (2): Naturally symptoms of Banana bunchy top virus showed stunting and narrow leaves with yellow (chlorotic) margins.

RESULTS AND DISCUSSION

Symptomatology and virus detection

Naturally infected banana plants showed symptoms of viral infection such as stunting and narrow leaves with yellow (chlorotic) margins - bunched together at the top of the stem (Fig. 2). Results of serological detection found to be free of CMV while, BBTV infection was detected in 29 of 63 tested samples by using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). It is one of the most popular serological technique which has been developed by Clark and Adams (1977) to detect a considerable number of economically important plant viruses, and successfully used to confirm infection with BBTV in suckers and tissue cultures of banana (Prakash *et al*., 2010).

Cultures establishment and microfungi identification

Under *in vitro* conditions, initiation stage was successfully performed, followed by transferring of explants to similar culture medium and two subsequent subcultures that established from the infected small banana suckers in order to improve the numbers of explants necessary for the present study. It was

possible to observe a small green leafy shoot or multiple shoots on solid medium after one month and/ or 3 months (Fig. 3A). With increasing the number of subcultures, the browning percentage decreased and the contamination percentage was increased around the base of explants with visually apparent growth of microorganisms (Fig. 3B). This led to decrease the number of explants throughout the cultures establishment stage as listed in Table 2.

Table (2): Effect of subcultures on primary establishment of BBTV-infected banana explants and contamination rates.

Parameters	No. of starting explants	Total number of non-contaminant explants (%)	Contamination rates (%)
Initiation stage*	29	23 (79.3)	20.7
1st Subculture	23	21 (91.3)	8.7
2nd Subculture	51	41 (80.4)	19.6
3rd Subculture	123	84 (68.3)	31.7

Incubation period ~4 weeks under artificial conditions 26±2°C (darkness*) and 16h photoperiod.

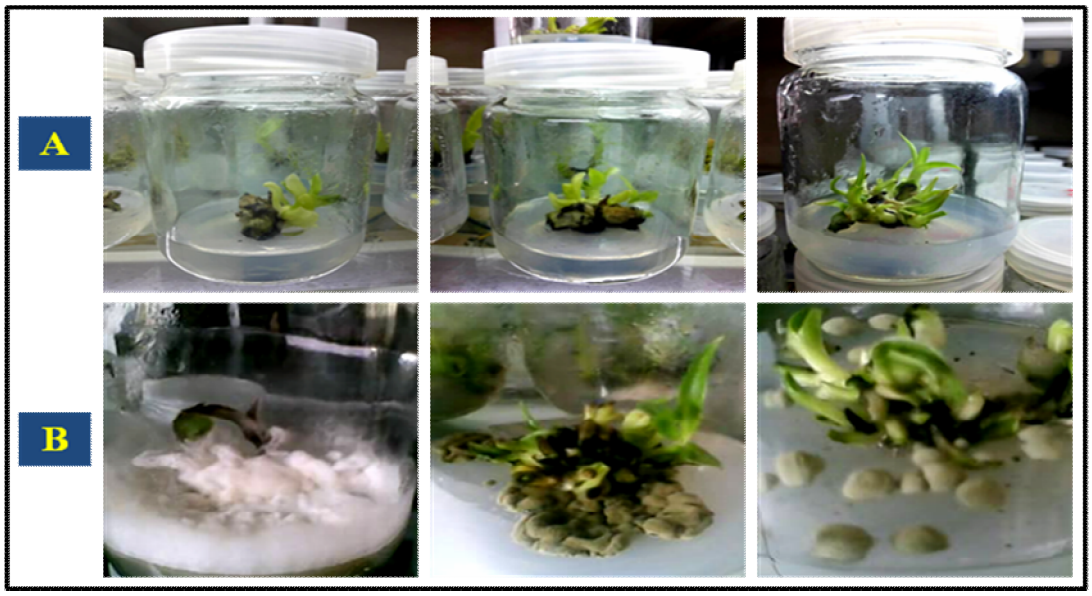

Fig. (3): In vitro culture initiation stage. (A): Shoot initiation of banana explants (Musa spp. cv. Grand Nain) and establishment of cluster of shoots on medium fortified with 6-Benzylaminopurine. (B): Spread of fungal contaminants around the base of the banana explants in tissue culture.

Altogether, three fungal genera were identified as *Aspergillus flavus*, *A. niger* and *Penicillium expansum* (Fig. 4 and 5). The rate of occurrence of *Aspergillus* species was higher than that of *Penicillium expansum*. However, among all the fungal strains, *Aspergillus flavus* was found to be the most prevalent on all the sampled plant tissue material. The isolated fungal contaminants were identified in the level of species based on mycelia (color, size and shape) and morphological characteristics (conidia size and conidiophores). They are of the traditional practices for observing sporulation characteristics of most fungi (Al-Hindi *et al*., 2011). Several studies have reported the incidence of exogenous fungal contaminants (*Aspergillus flavus*, *A. niger* and *Penicillium* spp.) during one or more stage of the developmental stage of the explants micropropagation due to insufficient surface sterilization. This is due to the concealed microorganisms within explants and then, introduce in cultures after a certain period of growth as well as inefficient sterilization of tissue culture-tools like forceps or scalpels which could cause cross contamination in culture medium (Odutayo *et al*., 2007; Msogoya *et al*., 2012 and Helaly *et al*., 2014). These results suggest that, the stress of the contamination or its effect was very strong and directly influenced on the multiplication rate and possibly due to interference of plant pathogens or incidental contaminants with growth of the plant tissue culture. This competes for nutrients in MS media and also the production of phytotoxins (aflatoxin and oxalate by *Aspergillus flavus* and *A. niger*, respectively) led to death of plant tissues, reduce shoot proliferation (Obuekwe and Osagie 1989) and therefore slowed the multiplication rate one of the important factors affecting the efficiency of plant tissue culture technique as the ratio of shoot number at the end of subculture to the initial number of shoots (Mendes *et al*., 1999).

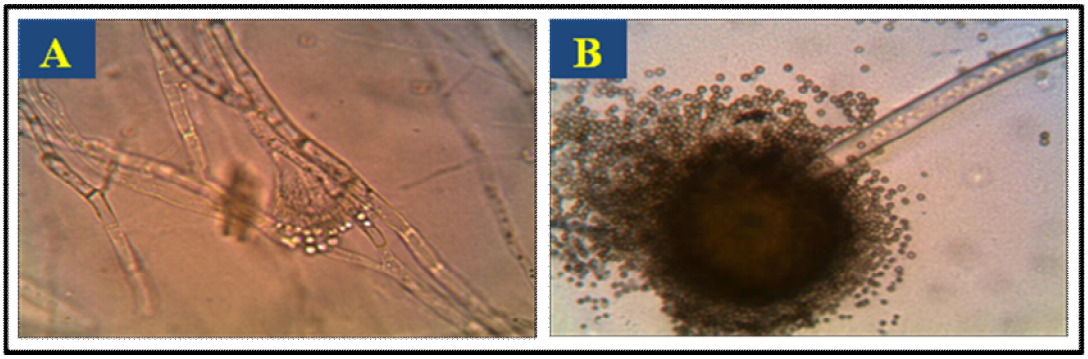

Fig. (4): Microscopic appearance of Aspergillus species. (A): Aspergillus flavus; yellow colonies, radiate conidial heads with phialides arise circumferentially from the globose vesicle, erect hyaline conidiophores, aggregated in compact columns and conspicuously echinulate. (B): Aspergillus niger; dark brown to black colonies, spherical radiated conidial heads extend circumferentially and obscure the vesicle.

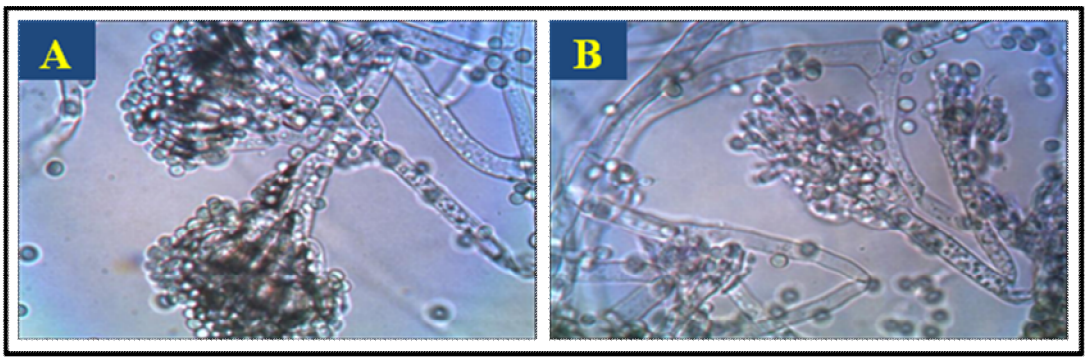

Fig. (5): Microscopic appearance of Penicillium expansum, two conidiophore branching patterns observed in Penicillium. (A) Biverticillate. (B) Terverticillate.

Assessing EON treatment *in vitro*

In the present study, the evaluation specifically focuses on the effectiveness of the eugenol oil nanoemulsion (EON) to eliminate *Banana bunchy top virus* (BBTV) that readily transmitted through tissue culture in different cultivars of banana plants (Thomas *et al*., 1995). Three major fungal contaminants (*Aspergillus flavus*, *A. niger* and *Penicillium expansum*) were reported in banana tissue cultures at the same time (Helaly *et al*., 2014).

Effect of EON on the growth of isolated fungi

All nanoemulsion - concentrations investigated individually were found to inhibit the fungal growth compared to control (Table 3). Data indicated that *Penicillium expansum* being to be the most sensitive to EON than all tested species, causing a decrease in the size and rate of growth by (94.8, 95.5 and 96.6%) at 125, 250 and 500 mg/L, respectively. On the other hand, nanoeugenol emulsion showed the same activity toward *Aspergillus niger* (88.51, 88.88 and 88.88%) and a moderate inhibitory effect for *Aspergillus flavus* growth (79.9, 80.7 and 77.7%) at 125, 250 and 500 mg/L, respectively.

Table (3): Effect of various concentrations of EON on growth inhibition of the isolated fungi in vitro.

EON conc. (mg/L)	*Aspergillus flavus* (%)	*Aspergillus niger* (%)	*Penicillium expansum* (%)	LSD 0.5
125	79.99 ± 1.69^{b}	88.51 ± 0.97^{a}	94.81 ± 2.90^{a}	6.97
250	80.73 ± 1.77^{c}	88.88 ± 2.56^{b}	95.55 ± 0.64^{a}	6.35
500	77.77 ± 2.71^{c}	88.88 ± 1.95^{b}	96.66 ± 1.28^{a}	7.14
(Control)	0.00	0.00	0.00	

The values are means ± SE. The mean values with different small letters within a raw indicate significant differences ($p \leq 0.05$).

From the data dictated in Table 3, the EON exhibited varying antifungal activity against the tested isolates. Based on latest conducted research, it was reported that the chemical structure of eugenol determines their antifungal properties and the phenolic component of eugenol possess a high level of toxicity against plant pathogenic fungi (Morcia *et al*., 2012 and Manganyi *et al*., 2015). Regarding studies on the mechanism of action of eugenol; Campaniello *et al*. (2010) suggested that the antifungal activity could be attributed, in part, to the presence of a phenolic group resulting in damage to the envelope of fungal cells. It is based on the assumption that a disruption of the membrane will cause a release of intracellular components from the fungal cell. In parallel, Sikkema *et al*. (1995) and Gill and Holley (2006a) found that the antibacterial mechanism of action of eugenol is the disruption of the cytoplasmic membrane, which could be due to the fact that the phenolic hydroxyl group might increase the solubility of this molecule in aqueous suspensions improving the ability to pass through the hydrophilic portion of the cell envelope. In addition, Gill and Holley (2006b) reported that eugenol was able to inhibit the activity of the following enzymes: ATPase, histidine decarboxylase, amylase, and protease. Inhibition of the ATPase may be important for cell killing at high eugenol concentrations because energy generation needed for cell recovery is impaired.

Effect of EON on BBTV elimination

Data in Table (4) showed the effect of different concentrations of EON in production of virus-free banana plantlets. Nearly similar results were obtained during the first and second experiment on the same period. Results indicated that all propagated explants on a EON-free medium were positive for BBTV and resulted in 100% of regenerated explants with pale yellow symptoms (Fig. 6A). Incorporation of 125, 250 or 500 mg/L of EON into MS medium resulted in comparatively larger shoots and faster growing without any effect on the rate of survival whereas, the efficiency in elimination of virus was very low (20% or less) across all treatments (Figs. 6B, C and D).

Table (4): Evaluation of various concentrations of EON on the rate of survival and the percentage of virus-free plantlets.

EON conc. (mg/L)		Survival (%)	Virus-free (%)	TE (%)
1st Experiment	125	100	0	0
	250	100	0	0
	500	100	20	20
2nd Experiment	125	100	0	0
	250	100	10	10
	500	100	20	20
Control	0	100	0	0

Ten explants/treatment - Data are based on DAS-ELISA detection. TE: Therapy Efficiency.

Table (5): Effect of various concentrations of EON on the percentages of survival and virus-free plantlets throughout the shoot multiplication phase.

EON conc. (mg/L)	Multiplication rate (Survival %)			No. of Virus-free plantlets	Virus-free (%)
	1st subculture	2nd subculture	3rd subculture		
125	6 (60%)	12 (100%)	29 (100%)	12	41.37
250	6 (60%)	13 (100%)	35 (100%)	19	54.28
500	8 (80%)	19 (100%)	53 (100%)	33	62.26
0 (Control)	4 (40%)	0	0	0	0

Ten explants in the beginning of each treatment - Data are based on DAS-ELISA assay

Effect of EON on the elimination of BBTV and fungal contaminants

In third tissue culture experiment during multiplication stage, the green shoots of clusters that appeared in Figure (3B) were transferred onto shooting semi-solid medium. It took 2.5 ~ 3 months through three successive subcultures using infected materials with BBTV and fungal contaminants. Results summarized in Table (5) clearly showed that, most of untreated explants in control groups could not survive under the artificial conditions *in vitro*. However, the rate of survival was about 40% or less and in some cases the initial shoots stopped to develop and failed to proliferate. Their color gradually became reddish brown and died during incubation period of the second subculture (Fig. 6E). Incorporation of 125, 250 and 500 mg/L of EON into MS medium resulted in 60, 60 and 80%, respectively, of survived explants after first subculture (Fig. 6F) and (100%) after the second subculture across all treatments (Fig. 6G) as well as (41.37, 54.28 and 62.26%) of virus-free plantlets, respectively, at the end of multiplication stage without any effect on the rate of survival (Fig. 6H).

It was also observed that the effect of treatments on the decontamination of explants using the high concentration of emulsion (500 mg/L) showed a massive reduction in the contamination area on the surface of media through the first and second subculture (Figs. 6F and 6G). This then was completely disappeared in the third subculture as compared with other concentrations (Fig. 6H, data not shown for other treatments). Contamination became not visible when explants were transferred to similar fresh medium free of EON without any phytotoxicity (Fig. 6I) but, with positive and speedy effect, needed only about 12-14 days, on regeneration rate of single shoot (Fig. 6J). Furthermore, at the end of micropropagation procedures, complete healthy banana plantlets derived from infected and contaminated explants were achieved after 35 days on MS medium containing 1.0 mg/l IBA+0.5 g/l activated charcoal (Fig. 6K).

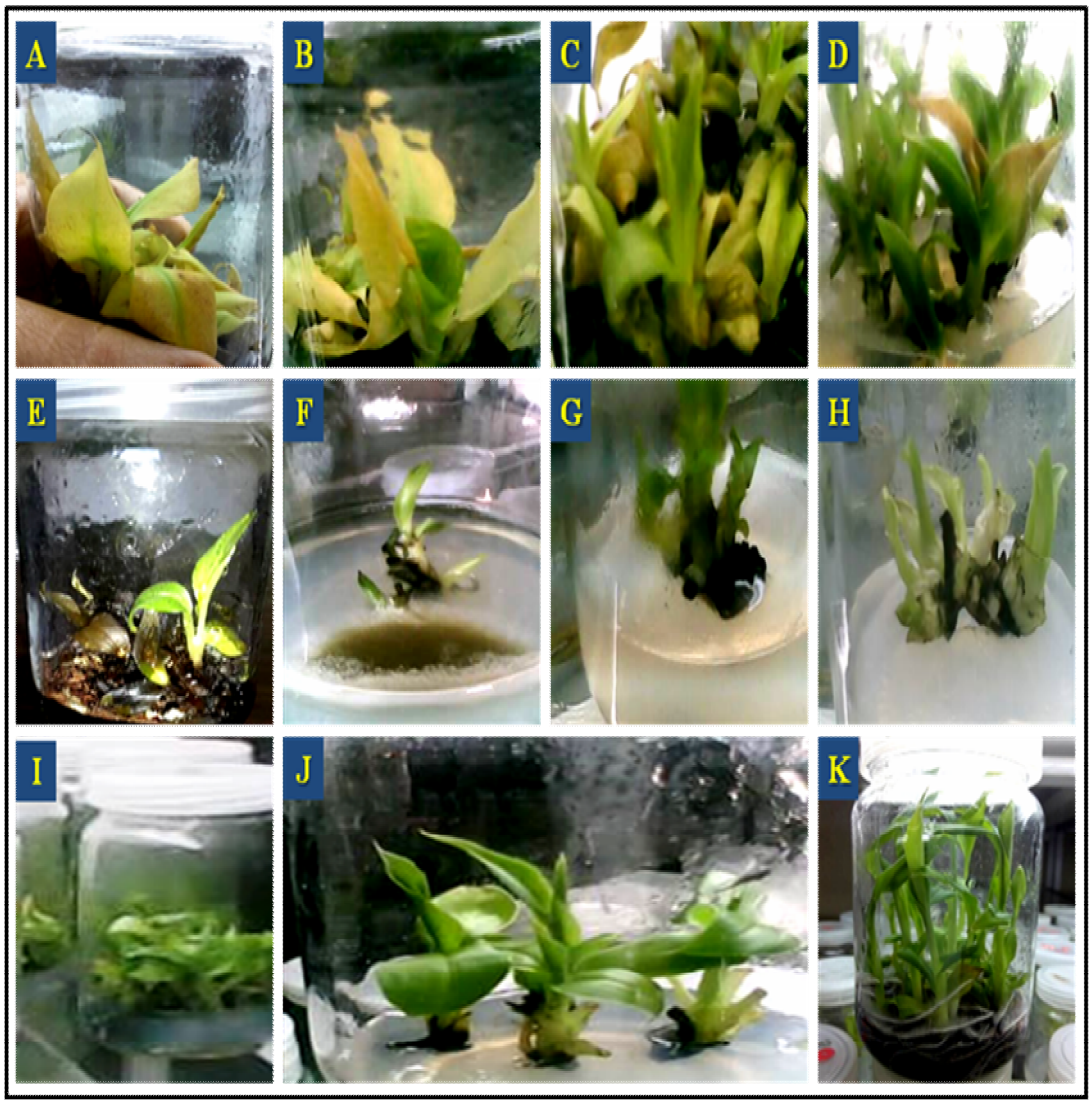

Fig. (6): In vitro cultures treatments. Culture proliferation of banana explant and clusters-shoots development with BBTV symptoms on medium containing 0 mg/L (control) (A), 125 mg/L (B), 250 mg/L (C) or 500 mg/L (D) of EON after 4 weeks. Contamination-induced stress in absence of treatment, control treatment (E). Successful shoot induction and gradual reduction in contamination area after first and second subcultures with high concentration 500 mg/L of EON (F and G). Complete reduction of contamination after third subculture with highest concentration (H) and in subsequent multiplication medium free of EON (I). Elongation of individually regenerated shoots within two weeks (J). Root formation of BBTV-free banana plantlets (K).

Considering the results of serological detection, the results of the first and second assay were disappointing (20% or less) as compared with influence of three successive treatment times during implementation of the *in vitro* multiplication. Results showed that EON-treatments with the highest concentrations (250 and 500 mg/L) had the highest potential for inhibiting BBTV as evidenced by the high percentages (54.28 and 62.26%) of eliminating BBTV, respectively. One possible explanation is that eugenol may be accumulated inside plant cells, resulting in rising the eugenol concentrations and acted directly on the BBTV by tying up some constituents within cell of host plant required for virus multiplication. Specially, one of the reasons for eugenol oil' effectiveness is their lipophilic character and therefore easily absorbed into plant tissues and produce the greatest results dependent on increasing the concentration of oil. This hypothesis was confirmed by the results of DAS-ELISA assay to monitor the effect of antiviral activity of eugenol in the current study and therefore supports its therapeutically utilized as evident by changing in the percentages of healthy banana plants after three successive therapeutically treatments.

Later pharmacological studies indicated that this compound has powerful inhibitory effects against the replication of either RNA or DNA virus (Mancini *et al*., 2009). There have been reports that eugenol has direct virucidal effect (Benencia and Courreges, 2000 and Tragoolpua and Jatisatienr, 2007). When high concentrations of oil are topically applied to the infected area with Herpes simplex virus (HSV) and repeating this protocol 3 or 4 times cause to a complete remission of HSV lesions and prevent the virus from transmitting from cell to cell without toxicity for human body. Antiviral properties of nanoemulsion are believed to result from the small size of oil particles that have a high surface tension which can fuse with and subsequently disrupt the membrane of viruses (Pedro *et al*., 2013). Therefore, the presence of eugenol oil in the nano form helped to increase the activity of the inhibitory effect where, the use of nanoparticles provides a sustained and slow release of the active constituents (Bilia *et al*., 2014). In parallel, variable results were observed in the rate of survival across all treatments and possibly due to changes in culture medium in the presence of EON led to inhibit or eliminate different fungal or viral pathogens with varying degree of sensitivity.

This was positively reflected in the rising of the survivability percentages or by another meaning, increase towards full or almost full recovery of plant tissues. Even though available information on toxicity or positive biological effects of eugenol in the form of nanoemulsion on banana cultures *in vitro* was too scarce. However, few studies proved that eugenol in the normal or nano form is a safe and non-toxic compound (DeLoney and Schiller, 2000 and Tragoolpua and Jatisatienr, 2007). The compound is most suitable compound for inhibiting aflatoxin production (Bilgrami *et al*., 1992) and has a great ability to suppress growth of the isolated fungi (*Penicillium* and *Aspergillus* spp.) with limiting the capacity for the generation of resistance (Dzamic *et al*., 2009; Campaniello *et al*., 2010 and Mihai and Popa, 2015). In addition, only one report by Helaly *et al*. (2014) studied the ability of nano znic and nanoznic oxide particles on biological contamination of banana cultures, *in vitro*. They found that the different concentrations of nanoparticles (50, 100 and 200 mg/L) did not show any negative effect on banana regeneration and significantly prevented the growth of four fungal contaminants

(*Penicillium* spp., *Aspergillus* spp., *Fusarium* spp. and *Candida* spp.) as well as nine strains of bacterial contaminants.

Based on the findings of this study it may be concluded that incorporation of EON into culture medium was very effective and inexpensive to produce BBTV-free banana plantlets, or could be particularly beneficial to reduce the frequency of fungal contamination with *Aspergillus* and *Penicillium*. Also, using EON as basic constituent of culture media in plant tissue techniques may be have a great commercial importance and will result in a more efficient method in order to maintain the high-value plants and expensive contaminated cultures.

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الملخص العربي

فعالية مستحلب زيت الاوجينول النانوى ضد فيروس تورد قمة الموز والملوثات الفطرية فى زراعة الانسجة النباتية

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تطوير تقنية الإكثار الدقيق لإنتاج أكبر عدد من شتلات الموز الخالية من الفيروس والحد من التلوث الفطرى فى بيئة زراعة الانسجة أمر بالغ الأهمية. ولهذا الغرض كان الهدف من التجارب التى أجريت فى الدراسة الحالية هو تقييم فعالية مستحلب زيت الاوجينول النانوى فى التخلص من فيروس تورد القمة فى الموز والملوثات الفطرية الناتجة عند إكثار الموز معملياً. ولتحقيق هدفنا تم التعرف على الفيروس على أساس الأعراض فى خلف الموز المصابة طبيعياً (صنف جراند نان) من محافظة القليوبية وايضا من خلال الفحص السيرولوجى باستخدام اختبار الاليزا المباشرة DAS-ELISA ثم استخدامها كمصدر للفيروس فى خط زراعة الانسجة. تم عزل وتعريف ثلاثة أنواع من الملوثات الفطرية النامية على بيئة زراعة انسجة الموز وهى *Aspergillus flavus*, *Aspergillus niger* and *Penicillium expansum* بناءا على الخصائص المورفولوجية والفحص المجهرى وقد أظهرت الدراسة أن معدل انتشار كلا من الفطر *Aspergillus niger* or *Aspergillus flavus* أكثر من الفطر *Penicillium expansum* .تمت دراسة تأثير تركيزات مختلفة ١٢٥، ٢٥٠ و ٥٠٠ ملجم/لتر من مستحلب زيت الاوجينول النانوى معملياً. أظهرت جميع التركيزات التى تم اختبارها فعالية تثبيطية عالية (٩٤.٨، ٩٥.٥ و ٩٦.٦% على التوالى) على نمو فطر *Penicillium expansum* وأظهرت تقريبا نفس التأثير (٨٨%) على تثبيط نمو فطر *Aspergillus niger* واختلف تأثيرها على فطر *Aspergillus flavus* وأدى الى تناقص حجم ومعدل نمو الفطر بنسبة ٧٩.٩٩، ٨٠.٧٣ و ٧٧.٧٧% على التوالى.
على الجانب الاخر، تم تقييم معدل تثبيط نشاط الفيروس باستخدام طريقة DAS-ELISA وأظهرت النتائج عدم وجود تأثير مضاد للفيروس من مستحلب زيت الاوجينول النانوى عند تركيز ١٢٥ ملجم/لتر بالمقارنة مع تثبيط منخفض جدا لنشاط الفيروس عند تركيز ٢٥٠ ملجم/لتر و تركيز ٥٠٠ ملجم/لتر خلال مرحلة التأسيس الاولية. كما أكدت الدراسة أن تاثير مستحلب زيت الاوجينول النانوى المضاد للفيروس قد يكون ناتجا عن ادماج جزيئاته ثلاث مرات متتالية فى ببيئة الزراعة MS-medium خلال تنفيذ مرحلة التضاعف. وتشير النتائج الاولية الى امكانية استخدام مستحلب زيت الاوجينول النانوى بتركيز عالى (٥٠٠ ملجم/لتر) كتطبيق علاجى ضد فيروس تورد القمة فى الموز مع ازالة التلوث تماماً من بيئة الزراعة فى تقنية زراعة الانسجة وعلاوة على ذلك تم تحفيز تكوين الجذر على وسط غذائى يحتوى على ١ ملجم/لتر من اندول بيوتريك اسيد والحصول معملياً على شتلات موز كاملة خالية من فيروس تورد القمة فى الموز بعد خمسة اسابيع.