75
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KOMBUCHA FILTRATE AS AN INHIBITORY FACTOR FOR CUCUMBER MOSAIC *CUCUMOVIRUS* AND BACTERIAL CONTAMINATION IN BANANA TISSUE CULTURES

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ABSTRACT

Great problems facing banana crop production and tissue culture are virus infection and bacterial contamination. Banana suckers, cultivar Maghrabi (50 cm height) confirmed to be infected with Cucumber mosaic *cucumovirus* (CMV) *via* indirect enzyme linked immunosorbant assay (I-ELISA) and specific polyclonal antiserum were used for such investigation. As a control total bacterial plate count and CMV I-ELISA values were determined for ten untreated banana shoot tips (1 cm in length). Another ten shoot tips of the same banana variety, age, length and growing conditions were determined once more, after disinfection with 4% sodium hypochlorite or with undiluted kombucha (growing with or without adding 0.2% folic acid) filtrate obtained using 0.45 µm Millipore® filter for removing microbes. Data proved that sodium hypochlorite was more effective as a disinfectant, while kombucha filtrate slightly reduced virus I-ELISA values. After disinfection with sodium hypochlorite, banana meristem tips (0.5 cm in length) were cultured on Murashige and Skoog medium (MS) supplemented with 3 mg BA, 3% sucrose and integrated with 1.5 ml kombucha (growing with or without adding 0.2% folic acid) filtrate per 15 ml medium. At time intervals of one and three month banana tissues were studied for bacterial count and CMV I-ELISA values. Best results were obtained with kombucha growing with 0.2% folic acid as an enhancer, as its addition prevented bacterial contamination by 50-60% after one and three months, respectively. On the other hand it decreased CMV I-ELISA values and produced a reasonable number (60%) of virus-free bananas. The contaminating predominant bacteria were identified to be related to *Bacillus cereus* depending on morphology and biochemical tests. Kombucha filtrate protein content was determined spectrophotometry and by gel electrophoresis. CMV was detected and identified in bananas using immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) for the coat protein gene (*cp*) and found to be related to CMV subgroup I

INTRODUCTION

Banana (*Musa sapientum* L.) is one of the most economical crops worldwide, containing many important nutrition factors for human health (**Gommaa *et al* 2000**). Production of banana plants through *in vitro* micropropagation has become routine work in many countries. The efficiency of tissue culture came to light when **Hwang *et al* (1984)** reported the production of a

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total of one million pathogen free plantlets of banana for commercial planting in Taiwan through meristem culture.

Banana production is threatened by different biotic agents such as bacteria, fungi or viruses, such as the Cucumber Mosaic *cucumovirus* (CMV) **(Lockhart and Jones, 2000)**.

Plant tissues growing *in vitro* are considered to be under some degree of stress and may be predisposed to direct infection, even by bacteria not normally pathogenic to them. The medium may contain many different bacterial nutrients, both original constituents of the medium and exudates from the plant cells. Thus pathogens, endophytes, epiphytes and incidental contaminants may all occur and may interfere with growth of the plant tissue **(Habiba *et al* 2002)**.

Gram positive bacterial strains isolated from the contaminated banana culture were *Cellulomonas uda, C. flavigena, Corynebacterium paurometabolum* and *Bacillus megaterium*. The gram negative isolates were *Klebsiella* sp., *Erwinia cypripedii* and *Pseudomonas* sp. All of them were non spore former except *Bacillus megaterium* **(Habiba *et al* 2002)**. CMV, which consists of a spherical particles of 28-30 nm in diameter containing ssRNA, is naturally transmitted to banana in field by aphid vectors **(Lockhart and Jones, 2000)**, so starting with virus-free planting material has a great advantage. **Hu *et al* (1995)** have identified CMV banana isolates to be belonging to the subgroup I. Symptoms depend on the strain of the virus pathogen and growth temperature. Thus, mild or severe chlorosis and necrosis on leaves and pseudostem can be observed, causing significant yield quantity and quality losses **(Helliot *et al* 2004)**.

The kombucha colony mat represents a symbiotic relationship between bacteria and yeasts. *Acetobacter xylinum* has been shown to be the major bacterium in such colony, while *Saccharomyces* occurred more frequently among kombucha growing yeasts **(Mayser *et al* 1995)**. Kombucha filtrate is a sour beverage reported to have potential human health effects and a safe organic antimicrobial factor, which can be prepared safely at home without pathogenic risk **(Morales and Sanchez, 2003)**.

Reports on Kombucha suggested that it has antimicrobial activity against a broad spectrum of organisms and this activity was mainly attributed to its acidic contents. The microbial species which highly affected by brewer were *Serratia marcescence, Bacillus subtilis, B. cereus, Micrococcus roseus and Alternaria solani* **(Shehata and Abdel Aty, 2005)**.

Therefore, the aim of this investigation is to evaluate kombucha filtrate as a safe organic inhibitory factor integrated in banana tissue culture for preventing or inhibiting bacterial contamination especially during the first stages of tissue culture. To produce CMV-free banana starting plant material, preparing for future production of virus-free banana seedlings. Identification of the predominant bacteria contaminating tissue culture, and CMV isolate depending on molecular technique, morphology and biochemical tests.

MATERIALS AND METHODS

Kombucha and banana suckers source

Kumbucha was obtained from Gunther W. Frank, Genossenschaftsstr, Birkenfeld, Germany, and cultured as described by **Shehata and Abdel Aty (2005)**, modified (by growing culture with addition of 0.2% folic acid) by **Shehata and Ali (2007)**.

Banana suckers showing mosaic symptoms (about 50 in length) of cultivar Maghrabi (*Musa sapientum*) were obtained from a plant nursery in Cairo, Egypt.

With the aid of I-ELISA (according to **Koenig, 1981**) and CMV subgroup I specific polyclonal antibodies (Agdia Inc., USA), only CMV infected plants were selected for the investigation.

Determination of bacterial load and CMV ELISA values before and after shoot tips disinfection

The outer leaf primordia of banana shoot tips were removed and tips with 2 leaf primordial were excised (about 1 cm in length and 1 g in weight). Ten tips were used to detect CMV level using I-ELISA, and bacterial counting was carried out on nutrient agar medium using the standard dilution method **(Black, 1996)**.

Ten shoot tips were excised as mentioned and used for each of the following treatments after been rinsed 5 times with sterile distilled water. Disinfection treatments were performed by soaking plant materials for 15 min in 4% sodium hypochlorite solution, undiluted kombuch (incubated for 10 days at 28 °C with and without folic acid addition) filtrate (pH 2.5) filtered using 0.45 µm Millipore® filter.

Integration of kombucha filtrate within banana tissue culture medium

Kombucha filtrate was added to MS medium **(Murashige and Skoog, 1962)** supplemented with 3 mg benzyl adenine (BA), 3% sucrose and 7 g agar per liter, pH 6 according to **Gommaa *et al* (2000)** after autoclaving and cooling to 50 °C with a rate of 1.5 ml filtrate per 15 ml medium within each medium jar (the addition was tested before and found to lower the medium pH to 5). After solidification banana meristem tips (0.5 cm in length), excised from shoot tips disinfected with 4% sodium hypochlorite were cultured as one explant for each jar. Ten explants were used for each treatment (with or without folic acid), note that 30 tips were used at the beginning and because of tissues failed to grow only 10 growing tissues were selected for the experiment. As a control ten banana explants were cultured on the mentioned medium without adding filtrate. Jars were incubated under conditions of 28±2°C, lighted by 40W fluorescent tubes for 16 h per day, and started to be subcultured on fresh medium with same conditions and additives every two weeks and after one month from starting culture. Tissues were tested for bacterial contamination per 1 g (samples were taken from the basic part of the plantlet) and CMV I-ELISA values at time intervals of one and three months post culturing.

Identification of the predominant contaminating bacteria

The most occurring bacterial colonies in contaminated cultures were isolated as a pure culture. Bacteria were observed under microscope after proper staining (Simple, Gram and Spore staining). Essential biochemical tests were carried out according to **Collins and Lyne (1984)**, **Krieg and Holt (1984)** and **Sneath (1986)**. For identification, characterized bacterial strains were compared with the standard strains of Bergey's Manual **(Sneath, 1986)**.

Detection and identification of CMV isolate using IC-RT-PCR

IC-RT-PCR was carried out on banana tissues giving negative I-ELISA values after the first and the third month of culturing explants, and on control tissues (without adding kombucha). Immunocapturing and cDNA synthesis was carried out as described by **Minafera and Hadidi (1994)**. The following primers (from Invitrogen Corp., USA) were used for the isolation and amplification of CMV *cp* gene:
5'ATGGACAAATCTGAATCAAC 3' (Sense) and 5'TCAAACTGG GAGCACCCCAG 3' (Antisense).

PCR was carried out according to **Ghosh *et al* (2002)**. Aliquots of the resulting cDNA, 5 µl each were transferred to tubes each containing 45 µl PCR reaction mixture contained primers (1 µM final concentration each) *Taq* DNA polymerase (1 unit), 200 µM of each dNTPs, 5 µl 10X PCR buffer (500 µM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0)), completed to 45 µl with Sterile deionized water and the reaction was overlaid with 50 µl sterilized mineral oil.

PCR program was 94°C initial melting for 3 min followed by 35 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min and 72°C/10 min final extension (using PerkinElmer thermal cycler, PerkinElmer Inc., USA).

For PCR product analysis 1.5% agarose gels were used. Electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad®, USA) at 80V. The gels were photographed under UV-transilluminator using a gel documentation system.

Spectrophotometry and electrophoresis of kombucha filtrate

Using a spectrophotometer (Shimadzu UV 1201) absorbance of kombucha filtrate (incubated for 10 days at 28 °C with and without folic acid addition) was performed at 280 nm. Concentrations were calculated according to **Layne (1957)** by the following equation: Concentration (mg/ml) = Absorbance at 280 nm/ path length (cm) (the length between cuvette opposite walls, usually 1 cm).

Filtrates protein contents were determined (after been treated for 5 min in a boiling water bath) using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking and 15% resolving gels and buffer system with the aid of vertical slab gel electrophoresis (Bio-Rad®, USA) as described by **Laemmli (1970)**.

RESULTS

Determination of bacterial load and CMV ELISA values before and after shoot tips disinfection

Data presented in **Table (1)** proved that using sodium hypochlorite as a disinfectant was more

efficient compared with the use of kombucha filtrate. Using kombucha slightly decreased ELISA values which assumed that it has an inhibitory effect on CMV when it partially penetrated tissue during disinfection especially with kombucha growing in the presence of folic acid. Also kombucha filtrate with folic acid reduced bacterial count compared with folic acid-free medium.

Integration of kombucha filtrate within banana tissue culture medium

Figure (1) demonstrating some of banana tissue culture stages used during the present investigation. Data in **Tables (2 a&b)** revealed that best results were obtained with medium integrated with kombucha filtrate of 10 days incubation age and with addition of folic acid as a growth enhancer. After one month post culturing low numbers of bacterial counts and slightly decreased CMV I-ELISA values were obtained (compared with the controls growing without kombucha addition), in spite of that there are no bacterial colonies were observed inside jars.

Lower numbers of bacteria appeared after incubation for 3 months, on the other hand, CMV I-ELISA values decreased significantly and 60% & 50% tissues were virus-free with cultures incubated with and without kombucha integration, respectively.

Table 1. Effect of banana shoot tip disinfection on bacterial count (CFU/g) and CMV I-ELISA values

Treatment / Samples	Untreated			4% sodium hypochlorite			Kombucha filtrate (undiluted)					
							10-Folic			10+Folic		
	$C\times10^7$	EV	R	$C\times10^2$	EV	R	$C\times10^3$	EV	R	$C\times10^3$	EV	R
1	6.3	1.055	+	0.0	0.825	+	6.1	0.781	+	2.8	0.596	+
2	7.0	1.105	+	0.0	0.891	+	5.4	0.651	+	4.1	0.691	+
3	5.3	0.911	+	1.2	0.995	+	5.1	0.890	+	2.3	0.616	+
4	4.8	0.817	+	3.0	0.715	+	3.8	0.655	+	3.1	0.589	+
5	6.3	1.100	+	0.6	0.950	+	2.9	0.918	+	2.5	0.750	+
6	7.5	0.995	+	0.0	0.859	+	4.5	0.855	+	4.2	0.711	+
7	5.5	0.899	+	2.4	0.922	+	6.5	0.891	+	5.2	0.695	+
8	7.4	0.751	+	3.2	1.015	+	3.5	0.900	+	2.8	0.900	+
9	5.8	0.946	+	0.0	0.805	+	7.1	0.597	+	2.5	0.671	+
10	6.5	0.858	+	3.2	1.022	+	3.9	0.914	+	3.6	0.862	+
EV controls	I	1.132	+									
	H	0.231	–									

C: Bacterial count (CFU/g), EV: I-ELISA values, R: Result, I: Infected, H: Healthy, 10±Folic: Kombucha culture growing for 10 days with+ or without- folic acid addition.
Each I-ELISA value was the average of three readings.
Counts after sodium hypochlorite disinfection were representing bacilli and yeasts.
Samples were 1.0 cm in length banana shoot tips.

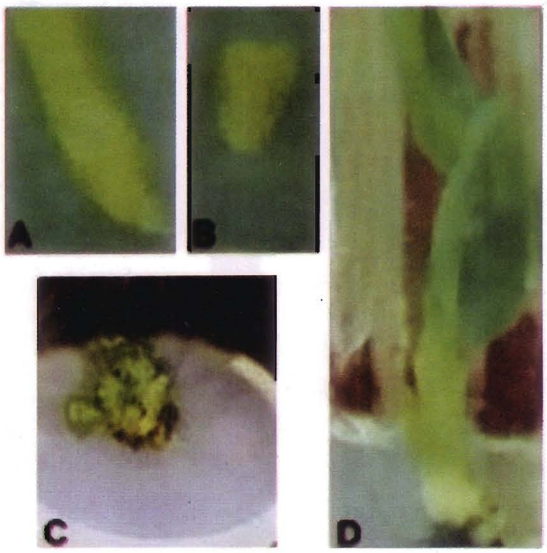

Figure 1. Stages of banana tissue culture: A) Shoot tip (1 cm), B) Meristem tip (0.5 cm), C) Tissue after one month post culturing and D) Single banana plantlet (age of 3 months).

Table 2. Effect of adding kombucha filtrate to banana culture medium on bacterial count (CFU/g) and CMV I-ELISA values

(a) One month

Treatment / Sample	Control*			1.5 ml Kombucha filtrate (undiluted) per 15 ml MS medium					
				10-Folic			10+Folic		
	C×10²	EV	R	C×10²	EV	R	C×10²	EV	R
1	0.0	0.918	+	0.0	0.800	+	0.0	0.615	+
2	3.2	0.855	+	2.1	0.792	+	0.0	0.600	+
3	2.1	0.711	+	0.0	0.799	+	1.2	0.681	+
4	0.0	0.785	+	0.0	0.612	+	0.0	0.510	+
5	4.0	0.815	+	1.6	0.599	+	0.8	0.700	+
6	0.0	0.900	+	1.2	0.692	+	0.0	0.602	+
7	2.5	0.659	+	0.0	0.751	+	0.4	0.651	+
8	1.5	0.799	+	0.2	0.715	+	1.5	0.701	+
9	0.0	0.691	+	1.5	0.698	+	1.3	0.705	+
10	4.1	0.781	+	0.4	0.720	+	0.0	0.581	+
EV controls	I	1.132	+						
	H	0.231	–						

Table 2 (b). Three months

Treatment / Sample	Control*			1.5 ml Kombucha filtrate (undiluted) per 15 ml MS medium					
				10-Folic			10+Folic		
	C×10²	EV	R	C×10²	EV	R	C×10²	EV	R
1	0.0	0.861	+	0.0	0.401	-	0.0	0.301	-
2	2.2	0.911	+	2.2	0.555	+	0.0	0.205	-
3	2.0	0.688	+	1.2	0.351	-	0.5	0.591	+
4	0.0	0.707	+	0.0	0.345	-	1.0	0.400	-
5	3.1	0.895	+	2.1	0.615	+	0.0	0.315	-
6	0.0	0.845	+	0.0	0.595	+	1.2	0.550	+
7	0.0	0.751	+	0.8	0.313	-	0.0	0.222	-
8	1.6	0.691	+	0.0	0.411	-	0.4	0.321	-
9	4.0	0.793	+	1.5	0.600	+	0.0	0.611	+
10	1.8	0.810	+	0.0	0.710	+	0.0	0.592	+
EV controls	I	1.132	+						
	H	0.231	–						

C: Bacterial count (CFU/g), EV: I-ELISA values, R: Result, I: Infected, H: Healthy, 10±Folic: Kombucha culture growing for 10 days with+ or without- folic acid addition.
Each I-ELISA value was the average of three readings.
* Meristems (0.5 cm) cultured on MS medium without adding kombucha filtrate.
Counts were mainly representing bacilli.
Samples were 0.5 cm in length banana meristem tips.

Identification of the predominant contaminating bacteria

Identification was performed as described by **Sneath (1986)** and proved that the studied isolate was related to *Bacillus cereus* depending on the following: It was found to be Gram positive, long rod shaped, endospore forming and motile. Gave positive result with Voges-Proskauers (VP), producing acid from glucose, hydrolyze casein, gelatin, starch and urea, utilize citrate, galactose, lactose, sucrose, fructose and glucose. Optimum growing temperature and pH were 30-40°C and 5.7-6.8, respectively.

Detection and identification of CMV isolate using IC-RT-PCR

By analyzing the PCR product on agarose gel, bands representing CMV *cp* gene were observed with a size of about 657 bp **(Figure 2)**. Results indicated that band produced from tissues giving negative I-ELISA values after one month has a very weak intensity compared with control tissues. On the other hand tissues with negative I-ELISA results after 3 months of treatment, gave negative results with PCR confirming CMV absence.

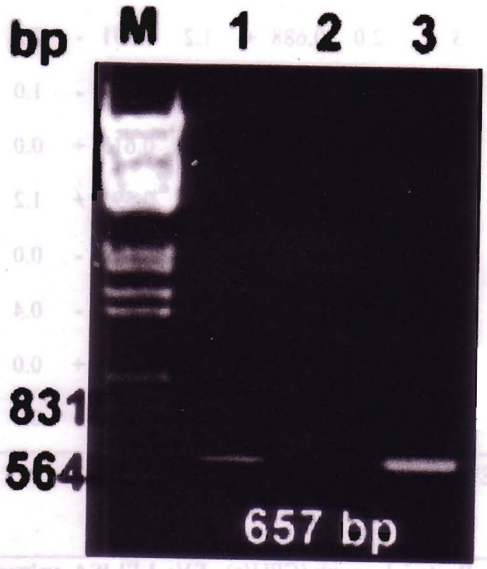

Figure 2. IC-RT-PCR for amplification of CMV *cp* from banana tissues after one month (lane 1) or three months (Lane 2) from growing banana tissues in the presence of kombucha filtrate. Control tissues growing without kombucha (Lane 3). M: Lambda DNA/*Eco*RI+*Hind*III marker (Promega, USA).

Spectrophotometry and electrophoresis of kombucha filtrate

Results of UV absorption at 280 nm indicated the presence of protein with a concentration of 0.87 and 0.66 µg/µl for kombucha filtrate growing with and without folic acid, respectively. Electrophoresis lanes **(Figure 3)** showed degrees from blue colour compared with control (tea filtrate), this may confirm the presence of a low moleculer weight protein, which was slightly higher with kombucha growing with folic acid addition.

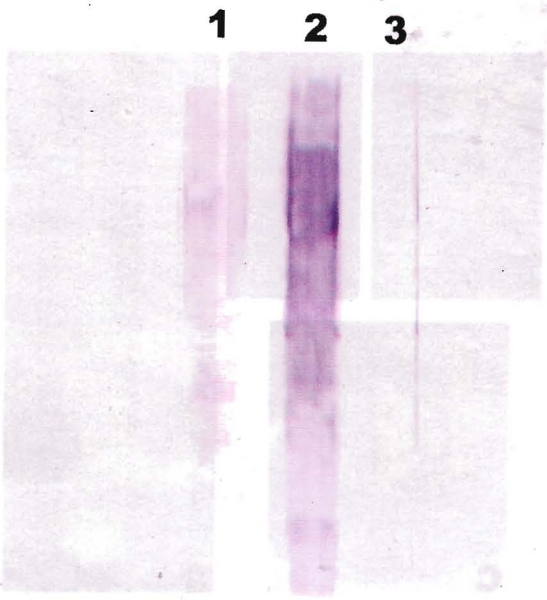

Figure 3. Polyarylamide gel electrophoresis for kombucha filtrate, Lanes 1 and 2 growing without and with folic acid, respectively. Control: Lane 3 tea filtrate without kombuca.

DISCUSSION

Recently *in vitro* micropropagation considered to be the most important and extensively used technique in banana plant production. This way of production is threatened by bacterial contamination and viral infections. Although initially surface sterilization works, latter microbial contamination at the base of the explant can be observed within 7 to 15 days post culturing, and viruses will not be greatly affected by disinfection. Huge number of explants were destroyed in the culture due to endogenous bacteria especially soil-borne spore forming bacteria (*Bacillus megaterium, B. cereus and B. subtilis)* which are difficultly to be killed by disinfectants **(Van den Houwe and Swennen, 2000 and Habiba *et al* 2002)**.

Due to vegetative propagation and insect transmission of banana viruses, it will be so important to start planting bananas with virus-free plant material. This will avoid over-infection by viruses after planting in open field, which will cause great loss in crop yield **(Helliot *et al* 2004)**.

To achieve such goal a first trial was performed to integrate kombucha filtrate in banana tissue culture medium as a bio antimicrobial substance with low risk compared with chemical substances used for bacterial inhibition and virus

elimination. Compared with control, promising successful results were obtained, as contaminant bacteria were killed or inhibited till bananas reached strong growing stage and can be lately transferred to the acclimatization stage.

CMV I-ELISA values were reduced during tissues growth, giving 60% & 50% virus- free plantlets after 3 months of culture growth with kombucha addition produced with and without folic acid addition, respectively.

During kombucha culture fermentation, many acids with an antimicrobial activity were produced, i.e., acetic, glutamic and usnic acids. The acids found in kombucha filtrate and its total acidity (around 33 g/l), which is relatively high, can limit the ability of many microorganisms to grow **(Greenwalt, 1997 and Shehata and Abdel Aty, 2005)**.

Antiviral chemicals were used to eliminate viruses in combination with meristem tip culture and some acids were found to have an antiviral effect, i.e. salysalic and usnic acids. Ribavirin was used extensively as it is the most effective antiviral chemotherapy tool for the production of virus-free plants, but with no effect on bacteria **(Klein and Levington, 1982; Cassels, 1987 and Yi *et al* 2003)**.

Antibiotics are not preferable to be mixed with plant tissue culture media, as it will inhibit tissue growth or evenly can kill tissues when added with its effective concentration. So, the way of application can be the immersion of surface sterilized explants in screened antibiotics for the predominant contaminating bacteria (ampicillin, gentamicin and tetracycline) for different concentrations and durations of time **(Habiba *et al* 2002)**.

Assuming the presence of microbial proteins with antimicrobial activity (Ribosome-Inactivating Proteins), spectrophotometry and electrophoresis were performed for confirmation. Low concentration of unknown proteins was calculated according to the UV absorption at 280 nm, and a degree of blue color was observed along acrylamide gel lanes confirming protein presence of microbial origin. The protein was relatively higher in concentration with kombucha growing with folic acid addition.

Ribosome-Inactivating Proteins (RIPs) are enzymes that trigger the catalytic inactivation of ribosomes and other substrates and so having a translational inhibitory activity for viruses and bacteria. They are present in a large number of plants and have been found also in fungi, algae and bacteria as a competitive tool **(Girbes *et al* 2004)**. RIPs are currently classified as type 1, those formed by a single polypeptide chain with the enzymatic activity, and type 2, those formed by 2 types of chains, i.e. a chains equivalent to a type 1 RIPs and B chains with lectin activity **(Girbes *et al* 2004)**.

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حوليات العلوم الزراعية
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راشح الكمبوشا كعامل مثبط لفيروس موزيك الخيار و التلوث البكتيري في مزارع أنسجة الموز

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من أكبر المشاكل التي تواجه مزارع انسجة الموز هى الاصابة الفيروسية و التلوث البكتيري. استخدم في هذه الدراسة خلفات موز صنف مغربي (بطول ٥٠ سم) تم التاكد من اصابتها بفيروس موزيك الخيار وذلك باستخدام اختبار الأليزا الغير مباشرة و الاجسام المضادة عديدة النسل المتخصصة لهذا الفيروس. كمقارنة تم عد البكتريا بطريقة الاطباق وتقدير قيم الأليزا لعشرة من اطراف ساق الموز الغير معامل (بطول ١ سم). تمت التقديرات مرة اخرى على عشرة من اطراف ساق الموز بنفس الصنف والعمر و الطول و شروط النمو و لكن بعد التطهير السطحي باستخدام ٤٪ هيبوكلوريت الصوديوم وكذلك راشح الكمبوشا الغير مخفف (المنمى في وجود أو عدم وجود ٠,٢٪ حمض الفوليك) والذي تم الحصول عليه باستخدام فلتر Millipore® باقطار ثقوب 0.45µm للتخلص من الميكروبات. اثبتت النتائج ان هيبوكلوريت الصوديوم اقوى كمطهر سطحي كما ادى استخدام الكمبوشا الى خفض قيم الأليزا بشكل نسبي. بعد التطهير باستخدام هيبوكلوريت الصوديوم تم زراعة القمم النامية للموز (بطول ٠,٥ سم) على بيئة MS المضاف لها ٣ مج بنزيل ادينين و٣٪ سكروز والمخلوطة ب ١,٥ مل من راشح الكمبوشا الغير مخفف (المنمى في وجود او عدم وجود ٠,٢٪ حمض الفوليك) لكل ١٥ مل بيئة. بعد شهر و كذلك ثلاثة شهور من عمر المزرعة تم تقدير عدد البكتريا الكلي وكذلك قيم الأليزا للفيروس هدف الدراسة. افضل النتائج تم الحصول عليها باستخدام راشح الكمبوشا المنماة في وجود ٠,٢٪ حامض الفوليك كمنشط نمو حيث تم منع بنسبة من ٥٠-٦٠٪ بعد شهر الى ثلاث اشهر من التحضين مع الكمبوشا على التوالي، كذلك تم تقليل قيم الأليزا وانتاج عدد مقبول (٦٠٪) من نباتات الموز الخالية من الفيروس هدف الدراسة. حدد قرب انتماء البكتريا السائدة والمحدثة للتلوث الى *Bacillus cereus* وذلك على اساس الشكل المورفولوجي وبعض الاختبارات البيوكيمياوية. نسب فيروس موزيك الخيار الى Subgroup I اعتمادا على الكشف عن جين الغطاء البروتيني باجراء تفاعل البلمرة المتسلسل والنسخ العكسي IC-RT-PCR بعد ربط الجزيئات الفيروسية بالاجسام المضادة المتخصصة.

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