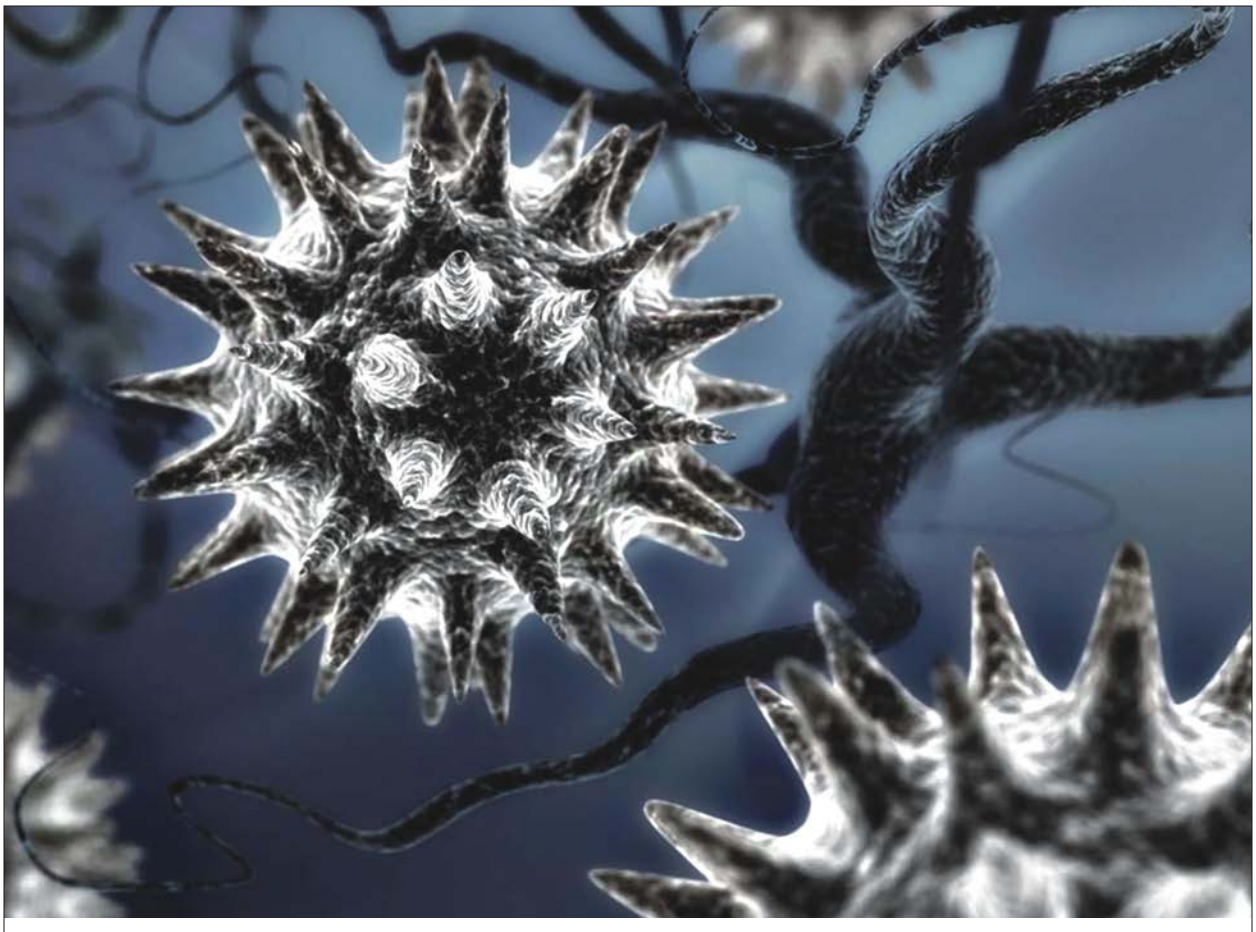

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Antiserum Production and Control Measures of Pepper Mottle Potyvirus Using Different Applications

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ABSTRACT

Pepper mottle virus (PepMoV) was purified with a high degree of purity and sufficient quantities for its antiserum production. The absorption spectrum of the purified virus had a min at 245 nm and a max at 260 nm. The A260/280, A280/260 and Amax/min ratios were 1.30, 0.76 and 1.29, respectively. The estimated yield of the purified virus was 3.35 mg/100 g leaf tissues. Electron micrographs of the purified virus preparation revealed the presence of filamentous flexuous virus particles about 730-745 nm long. Antiserum obtained after the second bleeding was 1/1024. The optimum concentrations of IgG and IgG conjugate were 1.0 μg mL^{-1} and 1/1000, respectively. The antigen dilution end point was 1/800. Ribavirin had a higher inhibitory effect (80%) when sprayed at lower concentration (the minimum inhibiting concentration is 0.0001%) before 1 h of inoculation. Salicylic acid and Parahydroxy benzoic acid has the same effect (80% reduction) at concentration of 0.01% and 0.1, respectively in the treatments inoculated 1 h after sprays. Six different chemical compounds applied as foliar sprays with two concentrations for each were used to study their effect on the percentage of virus infection in pepper treated plants: Actellic application gave superior results (46.7%) in decreasing the infection percentage followed by either Sumithion or K.Z. oil (55.0%), whereas Potassium Soap spray gave infection percentage of 60.0% followed by Bio-fly (61.7%). Moreover, Supper Royal treatment gave the lower effect in the percentage of virus infected plants (63.3%). Although, actellic treatment produced a higher yield as well as a higher weight of fruit followed by Sumithion and K.Z. oil (57.7 and 19.48, 54.3 and 19.08 and 53.11 and 18.17, respectively). Beside that Supper Royal treated plants produced more average fruit weigh followed by those treated with Bio-fly and Potassium Soap (16.99, 16.66 and 16.57, respectively) comparing with those of untreated plants.

Key words: Pepper mottle potyvirus, purification, IgG, IgG conjugate, ELISA, ribavirin, parahydroxy benzoic acid, salicylic acid, actellic, potassium soap, bio-fly, supper royal, sumithion, K.Z. oil

INTRODUCTION

Purified virus preparation are a pre-requisite for studying the intrinsic properties of a virus and for raising an antiserum against the virus. However, the purification of plant viruses is more of an

art than as science (Matthews, 1993). When plant pathologists, become involved immunology the goal, generally, is to generate an antibody probe which, will significantly identify a target antigen in the assay (Regenmorted van, 1982).

Important advances in virus chemotherapy have been made during the last few years. A variety of compounds with potent and selective antiviral activity has been found. The majority of these antiviral agents affects viral macromolecular synthesis. However, interference with processes which are associated with the initial phases of viral replication or inhibition of virus specific events that occur during viral maturation as assembly also represent important approaches to virus chemotherapy (Streissle *et al*., 1985).

The aim of the current study was: firstly, purify and produce ELISA reagent which can be used as a rapid serological method for PepMoV, secondly, identify possible components that could be used to develop a sustainable approach for the management of PepMoV and other aphid-borne viruses of peppers.

MATERIALS AND METHODS

Purification and production of polyclonal antibodies against PepMoV

Virus purification: The isolated virus (reported by Badr *et al*., 2008) was purified according to the method described by Purcifull *et al*. (1975), except that polyethyleneglycol was not used for virus precipitation from the supernatant and sucrose gradients were used for density gradient concentration instead of CsCl gradients.

Virus concentration was estimated Spectrophotometrically with a Spectronic 2000 Spectrophotometer using an extinction Coefficient of 2.8 (Purcifull, 1990).

Purified virus preparation was negatively stained with 2% uranyl acetate, pH 7.0 according to Noordam (1973) and examined in Electron Microscope Unit (sea Sumy electron optics), Faculty of Science, Al- Azhar University.

Production of polyclonal antibodies against PepMoV: Antiserum was prepared by injecting two 6-month-old New Zealand White rabbits (about 3 kg weight for each) intramuscularly a total of 6 mg purified virus preparations for each rabbit Freund's complete adjuvant (1:1, v/v) was used for the first (0.5 mg of purified virus and Freund's incomplete adjuvant for six subsequent injections (1 mg of purified virus) at weekly intervals (Hampton *et al*., 1990). The rabbit was bled one week after the last injection a long 3 weeks from the marginal ear veins. Antiserum was processed and stored at -20°C until used for titer dermination (Noordam, 1973) using indirect ELISA (Hampton *et al*., 1992).

Gamaglobulin were purified using the caprylic acid method described by Steinbuch and Audran (1969). IgG was conjugated with alkaline phosphatase Clark and Adams (1977). The concentrations of IgG and IgG conjugate were determined by a checkerboard test (Converse and Martin, 1990). Dilution end point of PepMoV was determined using indirect ELISA.

Control measures of PepMoV using different applications

Antiviral and induced systemic resistance against PepMoV: Ribavirin, Salicylic acid and Parahydroxy benzoic acid were used as, antiviral induced resistance agents to prevent PepMoV infection. The substances were each diluted with distilled water to final concentrations of 0.1, 0.01, 0.001 and 0.0001. Ten young pepper seedlings (California Wonder) for each treatment in pots (25 cm), kept under greenhouse conditions were sprayed with the compounds under investigation.

The whole plant was sprayed especially lower surface where stomata abundantly exist, each plant received about 20-30 mL solution. Plants for each treatment were inoculated after sprayed the substances three times at 1 h intervals. An equal number of seedlings were sprayed with water and subsequently inoculated later with the virus isolate to serve as controls. Leaves of tested plants dusted with Carborundum, 600-mesh were mechanically inoculated with the virus isolate inoculum. After 2-3 weeks plants were observed for systemic symptoms described by Devi *et al.* (2004) using the following equation:

$$\text{Inhibition \%} = \frac{A - B}{A} \times 100$$

where, A is controlled and B is treatment.

Control of PepMoV disseminated by its aphid vector on pepper plants: An experiment was conducted under field conditions at Emirate's Farm (62 km Cairo- Alexandria Road). Six different chemical compounds to study the effect of reducing the dissemination of PepMoV by natural infection aphid. Insecticides (Actellic, Sumithion and Potassium Soap), mineral oils (Supper Royal and K.Z. oil) and bio-agents (Bio-flay) were applied as foliar sprays with two concentrations (A and B) for each. Marconi pepper cultivar seeds were grown as a Nili crop and then transplanted on August/2008. The experiment was carried out using randomized split plot design with three replicates. Each replicate contained six whole plots. The six foliar sprays were randomized in whole plots, whereas, sprayed plants (with concentrations A and B) as well as unsprayed check plants were randomized in subplots. All replicates received care as regards cultivation, maturing and fertilization as recommended. Insecticides were applied weekly as soon as first appearance of alate aphids before and after transplanting. However, mineral oils and bio-agents were sprayed weekly after transplanting using Compression Sprayer (10 L). Untreated plants were sprayed at the same times with tap water. Fourteen days after transplanting, percentage of virus infection in each plot of the three replicates of sprayed and unsprayed check plants were determined. At the end of the season peppers were harvested as soon as they reached marketable size. Marketable fruits were separated from each treatment in labeled pepper pags until they weighted. Data were recorded as follows:

- Percentage of virus infection in sprayed and unsprayed check plants
- Effect of virus infection on the average total yield per plant in gram
- Effect of virus infection on the average weight of individual fruit in grams

RESULTS

Virus purification: After sucrose density gradient centrifugation, one zone was observed and no pellets were seen at the bottom of the tubes. The absorption spectrum of the purified virus preparation had a min at 245 and a max at 260 nm. The A260/280, A280/260 and Amax/min ratios were 1.30, 0.76 and 1.29, respectively. The estimated yield of the purified virus was 3.35 mg/100 g leaf tissues. Electron micrographs of the purified virus preparation revealed unaggregated filamentous flexuous virus particles (Fig. 1) of about 730-745 nm long.

Fig. 1: Electron micrograph of purified particles of PepMoV, negatively stained with 2% uranyl acetate, pH 7.0. Magnification 75.000x

Polyclonal antibodies

Produced against PepMoV: Antiserum developed against PepMoV after rabbit immunization from bleeding taken 3 times at weekly intervals after the last injection had antibody dilution titers of 1/512, 1/1024 and 1/256 for the first, second and third bleeding respectively (Table 1), using indirect ELISA.

Antiserum obtained after the second bleeding was used in the subsequent experiments. ELISA reactions were considered positive when the A_{405} values were greater than twice of healthy controls. The optimum concentrations of IgG and IgG conjugate were 1.0 μg mL^{-1} and 1/1000, respectively according to the schematic diagram of checkerboard arrangement test (Table 2).

For determination of antigen dilution end point, it was found that, IgG and IgG conjugate can be ready applied for virus detection in pepper extracts at dilution up to 1: 800 (Table 3).

Control measures of PePMoV using different application

Antiviral and induced systemic resistant agents against PepMoV: Three different compounds were tested for their ability to inhibit PepMoV infection in pepper plants. Recorded results in Table 4 showed that the three substances inhibit PepMoV infection when applied as a spray. Ribavirin had a higher inhibitory effect (80%) when sprayed at lower concentration (the minimum inhibiting concentration is 0.0001%) before 1 h of inoculation. Salicylic acid and parahydroxy benzoic acid have the same effect (80% reduction) at concentration of 0.01% and 0.1, respectively in the treatments inoculated 1 h after spray. Other concentrations of the compounds have almost the same inhibitory effect (60-70%) on PepMoV infection when applied one, two and three hrs before inoculation.

Table 1: Determination of PepMoV antiserum titer in relation to time of blood collection

	ELESA reading of PepMoV Antisera collected at weekly intervals (A405 nm)					
	1st		2nd		3rd	
Antiserum dilution	I	H	I	H	I	H
1/1	1.999	0.654	1.985	0.615	1.898	0.598
1/2	1.887	0.585	1.892	0.550	1.798	0.512
1/4	0.981	0.411	0.971	0.391	0.811	0.315
1/8	0.951	0.401	0.901	0.381	0.800	0.307
1/16	0.901	0.390	0.892	0.305	0.799	0.252
1/32	0.853	0.351	0.850	0.299	0.751	0.242
1/64	0.821	0.301	0.800	0.289	0.701	0.242
1/128	0.751	0.285	0.701	0.277	0.689	0.231
1/256	0.602	0.235	0.691	0.231	0.566	0.200
1/512	0.565	0.225	0.590	0.221	0.372	0.199
1/1024	0.390	0.225	0.499	0.211	0.301	0.189
1/48	0.351	0.211	0.311	0.200	0.300	0.185

Reading after 30 min incubation with the substrate. I: Infected sap, H: Healthy sap

Table 2: Schematic diagram of checkerboard arrangement determination of approximate working dilutions of IgG and IgG conjugate to PepMoV for ELISA test

	Concentration of IgG (μg mL^{-1})							
	4.0		2.0		1.0		0.5	
Dilution of IgG conjugate	I	H	I	H	I	H	I	H
1/250	0.982	0.399	0.819	0.30	0.610	0.290	0.401	0.252
1/500	0.869	0.334	0.792	0.301	0.529	0.250	0.395	0.230
1/1000	0.642	0.240	0.532	0.224	0.409	0.202	0.290	0.202
1/2000	0.398	0.289	0.275	0.230	0.125	0.106	0.092	0.090

I: Infected plants, H: Healthy plants

Table 3: Determination of antigen end point

	Absorbance at (405 nm) ELISA reading	
Antigen dilution	I	H
1/50	0.999	0.400
1/100	0.989	0.348
1/200	0.985	0.329
1/400	0.890	0.312
1/800	0.698	0.299
1/1600	0.421	0.258

Reading after 1 h, incubation with the substrate; I: Infected plants, H: Healthy plants

Control of PepMoV disseminated by its aphid vector on pepper plant

Percentage of virus infection in sprayed and unsprayed check plants in grams: The results presented in Table (5) show that the concentration B in all sprayed treatments was the most effective on decreasing the percentage of virus infected plants than did concentration A. Moreover,

Table 4: Effect of ribavirin, salicylic acid and Parahydroxy benzoic acid on PepMoV inhibition

	Percentage of inhibition after treatment with								
	Ribavirin infected after			Salicylic acid infected after			Parahydroxybenzoic acid infected after		
Chemical concentration%	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
0.1	60	60	60	60	60	70	80	60	60
0.01	70	70	70	80	70	70	70	70	70
0.001	70	70	70	70	70	70	70	60	70
0.0001	80	70	70	60	70	70	70	60	60
0	0	0	0	0	0	0	0	0	0

O: Control

Table 5: Percentage of virus infection in sprayed and unsprayed check plants

	Infection percentage of plants			
	Sprayed with conc.			
Foliar sprays	A	B	Unsprayed check	Mean
Actellic	58.3	46.7	71.7	58.9
Sumithion	61.7	55.0	73.3	63.3
Supper royal	66.7	63.3	75.0	68.3
K.Z oil	68.5	55.0	73.3	65.6
Bio- flay	63.3	61.7	73.3	66.1
Potassium soap	68.3	60.0	75.0	67.8
Mean	64.4	56.9	73.6	65.0

LSD at 5% level for, Foliar spray treatments = 3.62, Sprayed treatments = 2.95

Table 6: Effect of virus infection on the average total yield per plant in grams of sprayed and unsprayed check plants

	Average total yield per plant in grams			
	Sprayed with conc.			
Foliar sprays	A	B	Unsprayed check	Mean
Actellic	50.316	57.174	41.000	49.496
Sumithion	46.660	54.391	39.222	42.757
Supper royal	43.289	49.028	34.778	42.365
K.Z. oil	44.297	53.106	40.278	45.893
Bio-flay	46.515	49.150	39.889	45.184
Potassium soap	44.200	49.482	39.389	44.357
Mean	45.879	52.055	39.093	45.675

L.S.D. at 5% level for, Foliar spray treatments = 7.43, Sprayed treatments= 5.71

Actellic application gave superior results (46.7%) in decreasing the infection percentage followed by either Sumithion or K.Z oil (55.0%).

This reduction was less when the plants were sprayed with Supper Royal (63.3%) which may had the lower effect in the percentage of virus infected plants. However, Potassium Soap gave infection percentage of 60.0% followed by Bio-flay (61.7%). No interaction was found between the main plot and subplot (foliar spray and sprayed) treatments in the three replicates.

Table 7: Effect of virus infection on the average weight of individual fruit in grams of sprayed and unsprayed check plants

	Average weight of fruit in grams			
	Sprayed with conc			
Foliar sprays	A	B	Unsprayed check	Mean
Actellic	17.071	19.481	14.024	16.859
Sumithion	16.573	19.078	14.016	16.556
Supper royal	15.229	16.993	14.183	15.468
K.Z. oil	15.640	18.169	14.389	16.066
Bio-flay	14.600	16.663	14.250	15.171
Potassium soap	14.200	16.573	14.071	14.948
Mean	15.552	17.826	14.155	15.844

L.S.D. at 5% level for, Foliar spray treatments = 0.982, Sprayed treatments = 1.006

Effect of virus infection on the average total yield per plant in grams: It is clear from Table 6 that concentration B was selected as optimal concentration because it provided the best aphid control. It was also shown that, the average total yield was significantly higher in plants treated with Actellic (57.17) followed by Sumithion (54.39) and K.Z oil (53.11). Whereas, plants treated with Potassium Soap, Bio-flay and Supper Royal had almost the same effect (49.48, 49.15 and 49.03, respectively) in producing more yield comparing with those of untreated plants (Table 6).

Effect of virus infection on the average weight of individual fruit in grams: Statistical evaluation of the data demonstrated in Table 7 show that the average weight of fruit was significantly greater in plants sprayed with Actellic (19.48) followed by Sumithion (19.08) and K.Z oil (18.17). Similar results were obtained concerning the plants treated with Supper Royal (16.99) and Potassium Soap (16.57) in producing adequate average weight of fruit comparing with the unsprayed check plants.

DISCUSSION

In the present work PepMoV was purified with a high degree of purity and sufficient quantities for its antiserum production. After sucrose density gradient centrifugation, one zone was detected, 7.9 mm below the meniscus of the gradient column. This zone gave a typical UV- absorption spectrum with a min at 245 nm and a max at 260 nm. The A260/280, A280/260 and Amax/min ratios were 1.30, 0.76 and 1.29, respectively. These results almost agree with the results reported by other investigators (Brunt *et al*., 1996; Khattab, 2002).

The estimated yield of the purified virus was 3.35 mg g^{-1} of pepper leaf tissues. This yield was lower than that obtained by El-Kady (1983). Electron micrograph of the purified PepMoV preparation showed unaggregated filamentous flexuous virus particles of about 730-745 nm long. This result was similar to those reported before for *Potyvirus* (Brunt *et al*., 1996; Khattab, 2002). In the present study, the titers of the antiserum prepared were 1/512 to 1/1024 and then dropped to 1/256 from the first, second and third bleeding , respectively. Moreover, Khattab (2002) worked with the same virus reported that the titers of the antiserum were 1/1024, 1/512 and 1/128 for the first, second and the third bleeding, respectively as determined by indirect ELISA.

The concentration of IgG and IgG conjugate were 1.0 g/mL and 1/1000, respectively using direct ELISA.

Results also showed that IgG and IgG conjugate can be readily applied for virus detection in infected pepper extracts at dilutions up to 1/800 for PepMoV. Results of IgG conjugate were agreed with the results reported by Salama (1998). Whereas, Kheder *et al.* (2002) reported that the concentrations of IgG and IgG conjugate were 0.5 mg mL^{-1} and 1:2000, respectively for BYMV using indirect ELISA.

Ribavirin, Salicylic acid and Parahydroxy benzoic acid were used as antiviral and induced resistant agents to prevent PepMoV infection. Ribavirin, Salicylic acid and Parahydroxy benzoic acid has a higher inhibitory effect (80%) when sprayed at concentrations of 0.001, 0.01 and 0.1%, respectively, in the treatments inoculated 1 h after sprays. Hansen (1984) mentioned that visual observation of infected and treated kwanzan trees indicated that foliar treatment with Ribavirin completely prevent development of green ring mottle causal agent and necrotic ring spot virus symptoms. Ribavirin, a guanosine analogue in which the purine ring is open, inhibits the replication of a number of DNA and RNA viruses. Zein (2002) indicated that Ribavirin had the lower effect (70%) on Barley stripe mosaic virus multiplication in barley plants. Salicylic acid is an important signal molecule in plants that is required for the induction of systemic acquired resistance (SAR) against a wide variety of pathogens, including fungi, bacteria and viruses (Dempsey *et al.*, 1999). Regarding benzoic acid, Gupta *et al.* (1980) reported that benzoic acid when injected in tobacco cv. Xanthi leaves induced resistance to TMV. Moreover, Ali (2001) stated that benzoic acid has beneficial effect on the production of virus- free plantlets of Tobacco ring spot virus (TRSV).

This study was designed to evaluate the effect of some foliar sprays on control of PepMoV spread by aphids, i.e. insecticides (Actellic, Sumithion and Potassium Soap), mineral oils (Supper Royal and K.Z. oil) and bio-agents (Bio- flay) with two concentrations for each (A and B). However, concentration B was selected as the optimal concentration, because it provided the best aphid control. Moreover, both insecticides, Actellic and Sumithion as well as K.Z. oil were significantly reduced virus infection (46.7, 55.0 and 55.0; respectively). It was also found that Bio-flay and Potassium soap gave a moderate effect on virus infection (61.7 and 60%, respectively). On the other hand, Supper Royal application had the lowest effect (63.3%).

Ibrahim *et al.* (1998) found that, during two seasons actellic spray reduced the population and percentage of virus infection in the two tested cultivars as compared to control.

Mansour (1997) found that, control of aphid borne viruses in squash using stylet oil and reflected mulch together was greater than using stylet oil and an insecticide.

The use of biocontrol agents is a promising approach to provide good control of aphids, including green peach aphids. Bio-flay and insecticidal soap sprays were reduced aphid population size (Matsumoto *et al.*, 2004).

Results of the field experiment indicated that, the average total yields per plant as well as the average weight of the produced fruit in grams were lower in unsprayed check plants than those of sprayed ones. A delay in disease onset was associated with the increase in either the average total yield per plant or in the average weight of individual fruit. It was obviously that Actellic treated plants produced higher yield as well as higher weight of fruit in grams followed by Sumithion and K.Z. oil (57.17 and 19.48, 54.3 and 19.08) and 53.11 and 18.17, respectively). However, plants treated with Potassium Soap, Bio- flay and Supper Royal had almost the same effect on the average total yield per plant in grams (49.49, 49.15 and 49.03, respectively). There was a little difference in the arrangement of the last three compounds in increasing the average fruit weight.

Supper Royal comes first then followed by Bio-flay and finally by Potassium Soap (16.99, 16.66 and 16.57, respectively). These results were approximately in the same trend with those obtained by other investigators (Bachatly, 1992; Nasser, 1999; Jetiyanon *et al*., 2003).

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