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CHARACTERIZATION OF *CELERY MOSAIC VIRUS* ISOLATED FROM SOME APIACEAE PLANTS

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ABSTRACT

Celery mosaic Potyviruses (CeMV) was detected and isolated from naturally infected celery plants showing mosaic symptoms suspected to be caused by viral infection, also, it was isolated from other hosts. The isolated virus was biologically purified from single local lesions formed on *Chenopodium amaranticolor*. CeMV was identified by its host range, symptom expression, modes of transmission and particle morphology. CeMV able to infect only 20 plant species and varieties from 22 tested by mechanical inoculation. The virus was transmitted in a non-persistent manner using *Myzus persicae* Sulz. Infected plants were reacted positively only with the specific antiserum for the CeMV using double antibodies sandwich–enzyme linked immunosorbent assay (DAS-ELISA). It was successfully purified from infected *N. tabacum* L. var. White Burley leaves and virus particles had filamentous flexuous shape. Only one band of purified virus preparation was observed 1.5 cm bellow the meniscus of the density – gradient column. Infectivity test of the viral zone was found positive. The absorption spectrum of the purified virus isolate had a maximum at 260 nm and a minimum at 243 nm. The max/min and $A_{260/280}$ ratios of the virus isolate were 1.12 and 0.96, respectively. The yield of purified -virus was 6.7 mg/100g of tobacco leaves. Electron micrographs of the purified virus isolate revealed the presence of filamentous particles with average length of 780 nm and 15 widths. The polyclonal antibody raised against the virus under study had a virus – specific titer of 1:7500 in bled one and 1: 7000 in bled two. Incidence of the disease reached 75% in some apiaceae crops and many of the infected plants were unfit for marketing. It is suggested that control of the disease may be achieved by introducing a celery-free period to break the cycle of virus transmission between successive crops. This work records of the first time the prescience of this disease in Egypt.

Keywords: *Celery mosaic virus, Potyvirus,* host range, transmission, purification, antisera, DAS-ELISA, incidence, apiaceae, control.

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INTRODUCTION

Celery and parsley plants that become infected in the early to middle part of their growing cycle are unmarketable while plants that become infected late may be marketable but with a reduced shelf life.

Celery mosaic virus (CeMV) causes a serious disease of celery (*Apium graveolens* L.) It also infects other members of the plant family it belongs to (Apiaciae). *CeMV* was first identified in South Australia in the 1980's but has now spread throughout all worldwide **(Alberts *et al.*, 1989)**. CeMV is transmitted by aphids in a non persistent manner **(Severin and Freitag, 1938) and Simons and Sylvester (1953).** It is a member of the genus *Potyvirus* and is restricted in host range to the Apiaceae. CeMV is the most common viral disease of celery. CeMV infected plants usually are stunted and have a flattened appearance. Leaflets often exhibit a prominent mosaic, although sometimes only a faint mottle will appear. Leaflets often are considerably more narrow than normal. Sometimes the leaflet margins curl upward and raised areas occur on upper leaf surfaces. Foliage on some plants is yellow or bronze. Petiole mottling frequently appears as light patches against a darker background. CeMV affects only celery, carrot, and closely related weeds. CeMV is spread by aphids and by mechanical means **(Latham and Jones ,2003)**

The virus is not reported to be seed borne; it is vectored by aphids. Celery is the primary host of this disease. *Celery mosaic virus* (CeMV) is filamentous with a length about 780 nm to 870 nm; width of 15 nm **(Walkey *et al.*, 1970)**.

Hence, the aim of this study is to isolate and identify CeMV from celery and parsley using host range and symtomatology, mode of transmission, enzyme-linked immunosorbent assay examine and elucidate the effects of some plant species for antiviral activity in form of oils of some medicinal plants on plants before inoculation with CeMV on *Nicotiana* and *Chenopdium* plant.

MATERIALS & METHODS

Virus source and symptoms:

Samples of celery and parsley plants showing typical systemic mosaic, vein clearing, chloroatic spots, and yellowing symptoms of

CeMV were collected from different location in Mina governorate. The symptoms of CeMV in celery plants are severe vein clearing of leaves, leaf up-curling, leaf chlorosis and plant stunting.

Virus isolation and propagation:

Diseased leaves of celery (*Apium graveolens* L.) and parsley plants (*Petroselinum crispum* L.) showing a mosaic symptoms suspected to be virus infections were collected from El-Mina governorate. Detection of CeMV in the collected leaves samples was carried out using polyclonal antibody as demonstrated by **Clark and Adams (1977) and Converse and Martin (1990).** Antiserum for CeMV was kindly provided by Diederichsen, Alex at Institute of Plant Genetics and Crop Plant Research, Gatersleben / International Plant Research, Genetic resources Institute, Rome., Italy.

The virus isolate was biologically purified through a single local lesion technique repeated two times on *Chenopodium amaranticolor Coste&Reyn* plants **(Kahn and Monroe, 1963) and Kuhn (1964).** The virus was transmitted mechanically to *Nicotiana tabacum L. White Burly* for virus propagation and used as a source of virus in the following experiments.

Mechanical transmission and host range:

Different plant species belonging to 6 different families (*Amaranthaceae, Apiaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Solanaceae*) were mechanically inoculated with infectious sap in preliminary transmission tests; the transfer of CeMV from infected celery to herbaceous hosts was found to be satisfactory (1g w/ml 0.01 M phosphate buffer pH 9.4, containing 0.001 M Na-DIECA).

Five seedlings of each host were inoculated and observed daily from symptoms development. A number of healthy seedlings of the same species and age were left without inoculation to serve as a control. Two weeks after inoculation, plants were visually examined for symptoms appearance and assays using serological techniques (DAS-ELISA test).

Insect transmission:

Aphid transmissibility of the present viruses was evaluated under controlled conditions on four weeks old (*N. tabacum* cv. White Burly)

seedlings. Colonies of aphid species used in tests were collected in March and April, at the ARC (Horticulture Institute) *Myzus persicae* aphids were collected from peach orchard from trees and associated herbaceous ground cover in and around the orchard. And *Aphis gossypii* were collected from a bean field at the ARC. Also, in addition virus- free aphids were originally obtained from the stock culture of Aphid Section, Plant Protection Research Institute, ARC; Giza.

Virus purification:

The isolate virus was partially purified by a modification of the method described by **Shepard and Grogan (1967 and 1971) and Gamal El-Din *et al.* (1997) and.** Two hundred grams of frozen systemically infected tobacco leaves were homogenized for 5min. in Braun blender with freshly 0.5 M potassium phosphate buffer, pH 7.0 (1:2W/V) containing 0.5% 2-mercaptoehanol, 0.5 M urea, 1% Na_2So_3 and 0.05M sodium EDTA. The homogenate was squeezed through two layers of cheesecloth and clarified with 7% n-butanol and 50% chloroform clarification was undertaken by stirring the homogenate with chloroform, in the blender for 5 min, then with n-butanol for 30 min at 4C. The emulsion was centrifuged at 8000 rpm for 15 min. The virus was precipitated from the supernatant by adding 4% PEG and 3% NaCl slowly (W/V) with stirring for 30 min. at 4C, then incubating the mixture overnight.

The virus was collected by centrifugation at 10000 rpm for 30 min and the pellets were resuspend in 0.1 M potassium phosphate buffer, pH 7.0, containing 1.0 M urea, 0.1% 2-mercaptoehanol and Triton X-100 (V/V) and with stirring overnight at 4 C. After a low speed centrifugation to remove insoluble material the virus was concentrated by one cycle of high speed 40,000 for 1.30 h, followed by 30% sucrose cushion 35,000 for 2 h.

Purified virus was layered onto 10-50% sucrose gradient columns, centrifuged (50,000/1.30 hr) using SW 60 rotor. Virus bands were collected from the sucrose gradient using a syringe, diluted in 0.01 M potassium phosphate buffer, pH 7, centrifuged (45,000 rpm/1.30 hr/4 °C), then resuspended in 0.01 M potassium phosphate buffer. The virus was estimated spectrophotometrically

using extinction coefficient $E^{0.1}$ 1 cm 260 of 2.8 **(Purcifull, 1990).**

Electron microscopy

Virus preparation was examined with an electron microscope Model SEO TEM at the Electron Microscopy Unit, Military Veterinary Hospital, Cairo, Egypt after staining with 2% phosphotungstic acid (PTA).

Rabbit immunization and bleeding:

A total 10 mg purified CeMV was used for subcutaneous injection. Purified virus was emulsified with an equal volume of Freund's incomplete adjuvant at weekly intervals. Rabbit was bleed 10, 15 days after the last injection.

Titer of antiserum:

The titer of the induced antiserum was determined using indirect ELISA. Healthy and infected leaves of *Nicotiana tabacum L.White Burly* with CeMV were extracted (1:10 W/V) in coating buffer (0.05 M carbonate, 0.02% sodium azide, pH 9.6) at 1:10 (wt/vol) and clarified by centrifugation at 10,000 rpm for 1 min. The antigen was further diluted in coating buffer to 1:10 (vol/vol) for CeMV detection at 200 µl/well and incubated at 4°C overnight. After washing the plate, 200 µl IgG were added per well and plates were incubated at 4°C overnight. Plates were washed and alkaline phosphataseconjugated goat anti-rabbit IgG (Sigma, A.8025) was added at a 1/7,000 dilution and incubated 2 h at 37°C. *p*-Nitrophenyl phosphate (200 µl/well) was added at 1 mg/ml and incubated at room temperature for 30 to 60 min for color development. Readings of reactions were carried out after 1 h at 405 nm in an ELISA Reader (ELx800 universal Microplate). Samples were considered positive when absorbance readings (*A*405) of sample wells were greater than twice times the mean value of three healthy controls.

Purification of immunoglobulin (IgG) from CeMV specific antiserum:

Anti-CeMV immunoglobulin was purified from the antiserum of CeMV according to the method described by **Brattey and Burns (1998).** A mixture of 1 ml of crude antiserum and 9 ml of distilled water was added to an equal volume of saturated ammonium sulfate, pH 7.0, while stirring. Contents were incubated for 60 min

at room temperature. The precipitate was collected by centrifugation for 15 min at 9000 rpm, and dissolved in 2 ml of half-strength PBS. The dissolved precipitate was dialyzed 3 times against 500 ml phosphate buffer, pH 7.2 for 3 h/each to remove ammonium sulfate. The optical density (O.D) was measured at a wavelength of 280 nm and the gamma globulin concentration was then adjusted in half strength PBS to read 1.4 (1mg/ml). The resultant γ globulin was stored at –20°C.

Incidence of *Celery Mosaic Virus* in *Apiaceae* crops:

Polyclonal antibodies specific to CeMV produced earlier wasused in detected the virus in survey and evaluation the affective of mineral oils were used to inhibit the virus infection and also in Indirect ELISA assay (the secondary antibody, goat antirabbit conjugate with alkaline phosphatase sigma A4503) was used to detect the virus in the host plants. Leaf samples were collected from different farms in El-Minia and Kalubia Governorates.

Virus inhibition:

Preparation of the inhibitor:

The oils produced from of plant material (Jasmine oil and Camphor oil) was mixing and shaking well in distilled water (0.5:100v/v) as antiviral inhibitor. These oils product were used as foliar spraying 1, 2 and 24 hours before virus inoculation to *Ch. amaranticolar* and *N. tabacum* White Burly and were keeping under observation to record the results.

Serological test to detect inhibition:

CeMV detected in apiaceae and other different host range, samples were tested using conventional double antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA) **(Clark and Adams, 1977)**, with coating IgG and IgG conjugates produced in this investigation. Optical density (O.D.) was measured at $\lambda = 405$ nm in an ELISA micro well reader (Dynatech Immunoassay MR 7000). Lyophilized samples obtained were used as positive and negative controls in each ELISA plate. Positive threshold values were set at twice average value for the negative controls **(Clark and Adams. 1977)**. Incidence levels were considered as all positive samples within total number of samples for each province.

CeMV was detected in the inoculated and control plants by ELISA test after two weeks of virus inoculation.

Local lesion assay: The local lesion assay was performed on three plants of *Chenopodium amaranticolr* for each treatment in greenhouse controlled at 25 ± 2°C. The third to sixth leaves from the top of the plant were used. Carborandum of 600 meshes was sprinkled on the *Chenopodium* leaves and CeMV inoculum was rubbed on the leaf surface with a small cotton ball. Local lesions on the surface were counted after seven to ten days after inoculation (numbers of local lesions per leaf then calculated the mean numbers of local lesion) according to an inoculation scheme **(Noordam, 1973) and Jayashree and Sabitha (1999).**

The inhibition percentage of virus infectivity was calculated by the formula:

Inhibition percentage = (C-T/C) x 100.

Where >C = average number of local lesion on control leaves *Ch.amaranticolr*.

T = average number of lesions leaves inoculated by virus inoculums with inhibitors treated **(Baranwal and Verma, 1992 and 1997).**

RESULTS & DISCUSSION

Virus isolation and propagation:

Virus isolate was obtained from naturally infested celery and parsley plants showing mosaic and malformed leaves (Fig.1 A, B and D) collected from three commercial farms in Minia Governorate. After successive single lesion transferred in *C .quinoa, the* resulting virus isolate was propagated in *N. tabacum* var. White Burley. The symptoms were very similar to those illustrated by **Hollings (1964), Alberts *et al.*, (1989) and Bos *et al.*,(1989).** Subsequent work clearly proved that the virus under study is *Celery mosaic virus*. These results were based mainly on symptomatology, host range, modes of transmission and serology.

Mechanical transmission and host range:

Celery is most common host of this virus. As the name implies this virus causes a mosaic or mottling in the leaves of celery. There can also be malformation of leaflets. In order leaves chlorotic or necrotic spots may occur and the plants can be stunted. In addition to celery, this virus has been identified in other important crops

in *Apiaceae* including carrots (*Daucus carota*), coriander (*Coriandrum sativus*), parsely (*Petroselinm crisum*), dill (*Anethum graveolens*) and Anise (*Pimpinella* anisum).

Results of host range trials showed that CeMV had a wide host range (Table 1 and Fig 1). Seventeen plants of twenty four plant species and cultivars belonging to six families were susceptible with CeMV and many of them were infected systemically showing different symptoms. No symptomless infection was detected by DAS-ELISA in leaves (Table 1). CeMV locally infected *C. quinoa* and *C. amaranticolor* (Fig 1:G and H) In *C. quinoa* irregular chlorotic spots on the inoculated leaves were observed 7 days to 10 days after the inoculation with CeMV.

These results showed that distinct virus is associated with mosaic, chlorotic rings and curling in different hosts. The virus was identified as *Celery mosaic virus* (CeMV).

Insect transmission:

Experiments demonstrated that *Aphis gossypii* and *Muzus persicae* were able to transmit CeMV in non persistent manner from infected celery plants to the same healthy celery or parsley, carrot, coriander and *N. tabacum*. *M. persicae* was more effective than *A. gossypii* in transmitting CeMV. Data in **Table (3)** revealed that *Aphis gossypii* and *Muzus persicae* were able to transmit CeMV from infected celery plants to healthy celery, parsley, carrot, coriander and *N. tabacum*. These results were agree with **Purcifull and Shepard (1967), Zitter (1970) and Latham and Jones (2003).**

Virus purification:

Using the purification method one band was observed, 1.5 cm below the meniscus in the sucrose density gradient columns. The ultraviolet absorption spectrum of CeMV was characteristic of a nucleoprotein, had a maximum absorbance at 260 nm, a minimum absorbance at 243 nm and A260/280 ratio of 1.12 and Amax/min ratio was 0.96. These results are similar to that estimated by **Brunt *et al.*(1996)**. These results suggested that the purification method was quite successful in purifying the virus under study. Virus yield was 6.7 mg/100g of infected tissue. **Mathews (1991)** reported yields

for *Potyvirus* from different laboratories may vary quite widely due to such factors as host species, growing conditions and isolation procedure.

Electron microscope:

Electron microscopic micrograph of the purified preparation revealed the presence of filamentous particles with average length of 700-780 nm and (Fig.1 -L). These results are similar with **Walkey *et al.* (1970) and Brunt *et al.* (1996).**

Titer of CeMV antiserum:

Titers of the induced antiserum measured with indirect ELISA were 17500 from bleeding obtained after the first and 1/7000 in the second one which is an indication of the high immunogenecity of this antiserum. Antiserum obtained after the first bleeding (1:7500) was used in the subsequent experiments. **Mathews (1991)** obtained that the antiserum to be used for virus estimation are best produced in rabbits, from which a large volume of serum. It is worth to try to produce an antiserum of high titer. This allows high dilution of the serum, thus avoiding nonspecific inhibitory effects of serum at low dilution and allowing more tests to be made from a given volume of serum.

Purification and conjugation of IgG with alkaline phosphatase:

Purification of IgG was done according to the procedure described by **Brattey and Burns (1998).** The concentration of IgG was adjusted to 1 mg /ml (A_{280} nm =1.4).

Incidence of celery mosaic virus in *Apiaceae* crops:

From 2008 to 2010, surveys were carried out to determined the incidence of CeMV in carrot, celery, coriander and parsley crops in El-Minia and Kalubia Governorats. CeMV was found on 75% in celery, 72% in carrot, 40% in parsley and 35% in coriander samples. In this investigation **Latham and Jones (2003)** etermined the incidence of CeMV in celery crops in South-west Australia, CeMV was found in allfarms samled in this region. Incidences of infection in individual crops were 43-96%.

Virus inhibition:

CeMV is easily transmitted by mechanically inoculation. Also, in nature, it is transmitted by aphids in a non-persistent manner. Once the virus is detected in the field, the major control method is to immediately eradicate plant materials which developed symptoms (**Zltter ,1970**).

When study the effect of some oils medicinal plant species (Jasmine and Camphor), for antiviral activity in the form of foliar spraying. Data in Table (3) revealed that the O.D. values determined by ELISA reader (indicate CeMV concentration in *N. tabacum* White Burly) was 0.421 with jasmine oil treated, 0.668 with camphor oil treated and 1.114 for untreated- virus infection leaf samples CeMV infectivity was determined by counting the average number of local lesions (6 leaves were used in each trail) subsequently formed after 6 and 7 days of back inoculation on *Chenopodium amaranticolr* and then switched to percentage the inhabitation and reduction virus infection were: Jasmine oil 80% and 76.5% ; *camphor oil* 74% and 78.2%,55 respectively.

These results agree with studies of Milbrath and Ryan (1938). **Peters and Lebbink 1973). Sugimura *et al.*,(1995), D'Antonio *et al.*,(2001) and Shoresh *et al.*, (2005).**Also, they motin that yet, there were no efficient chemical treatments that protect plants from virus infection.

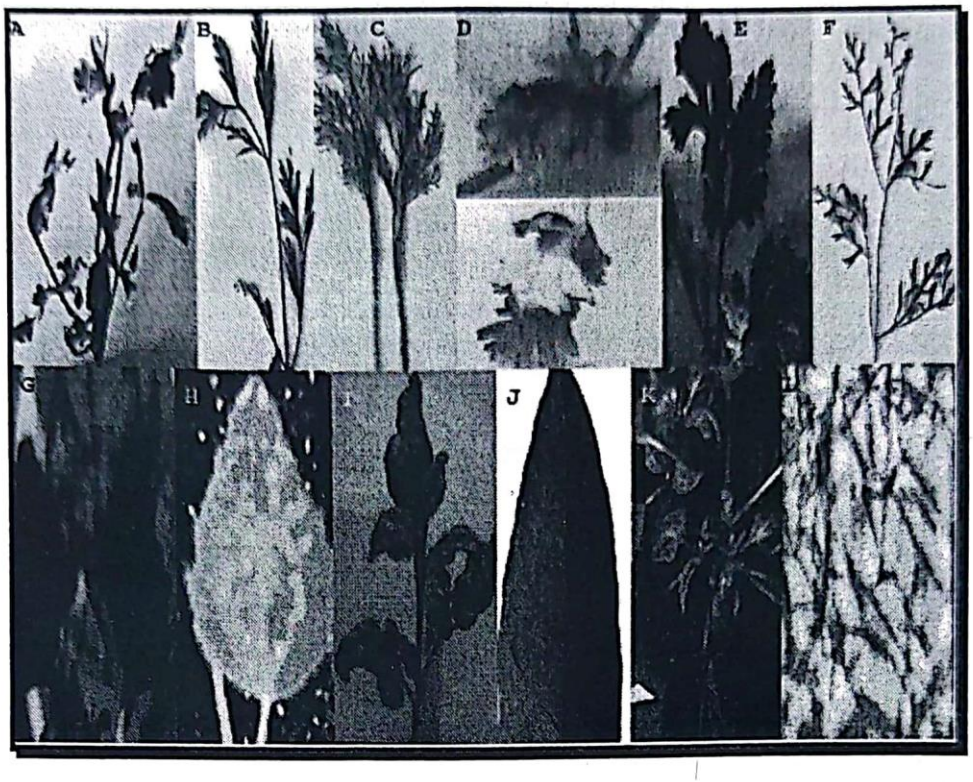

Figure 1. Symptoms of CeMV on naturally and artificially infected host plants: A: Mosaic, chlorotic spots and deformation appeared on naturally infected celery. **B**: CeMV symptoms (mosaic and browning) appeared on artificially infected celery leaves. **C**: Symptoms on dill plants artificially infected showing yellow leaflets. **D**: Mosaic and chlorotic on artificially infected parsley plants. **E**: Mosaic and yellow symptoms on coriander artificially infected leaves. **F**: Mosaic and deformation in carrot leaves infected with CeMV. **G**: Chlorotic spots in *C. quinoa* CeMV infected leaves. **H**: Chlorotic local lesions appeared on *Ch. amaranticolor*.**I**: Symptoms on infected *N. tabacum* var. White Burley showing sever mosaic and deformation. **J**: *N. rustica* reacted the severe mosaic after infected with CeMV. **K**: pepper artificially infected showing mosaic and epinasty. **L**: Electron micrographs of purified CeMV showing filamentous; flexuous shaped virus particles stained with 2% uranyle acetate (40,000).

Table 1. Symptomatological responses of herbaceous host ranges to CeMV isolated from Celery.

Host plant	Inoculation(days)	Symptoms	ELISA assay
Family: Amaranthaceae			
Gompherena globosa	30	-	-
Family:Chenopodiaceae			
Ch .amaranticolor Cost & Reyn	6	LL	+
Ch. quinoa Willd.	7	SCS	+
Family: Cucurbitaceae			
Cucumis sativus L.	30	-	-
Family: Fabaceae			
Pisum sativum cv. Little marvel	30	-	-
Vicia faba L. cv. Giza	30	-	-
Family: Solanaceae			
Capsicum annuum L.var.Chili	15	LD&B	+
Datura stramonium	30	-	-
Physalis floridana	30	-	-
Lycopersicon esculentum Mill. cv. Ice	30	Cu	+
Nicotiana. tabacum var. White Burley	30	MM&B	+
N. glutinosa	14	VB	+
N. rustica	15	M	+
Solanum nigrum.	30	-	-
Nicandra physalodes	30	Cu	+
Family: Apiaceae			
Anethum graveolens	15	M	+
Apium graveolens L.	21	M.&Y	+
Carum carvi	15	MN	+
Coriandrum sativum L.	12	Y&LD	+
Cuminum cyminum	15	MM	+
Daucus carrota	15	CS&M	+
Foeniculum vulgare	14	Y	+
Petroselinum crisum	12	VC&D	+
Pimpinella anisum(Anise)	21	St &W	+

B= Blisters, CS= Chlorotic Spots, Cu= Curling, Co= Chlorotic, VB= Vein Banding, VC=Vein Clearing, LD= Leaf Deformation, M.= mosaic , MN= Marginal leaflet Necrosis, St= Stunting, D= Death of plant, LL. = Local Lesion, MM = Mild Mosaic, Y=Yellow, W= Wilting. (+) Positive reaction, (-) Negative reaction.

Table 3. Results of transmission test CMV and PPV by aphids from apricot to apricot, peach .

Host plants	CeMV Symptoms	*Myzus persicae* Infection %	*Aphis gossypii* Infection %
Apium graveolens L.	Mosaic	60	50
Petroselinum crisu	Vein clearing	45	30
Coriandrum sativum L.	Leaf deformation	40	30
Daucus carrot	Mosaic	20	20
Nicotiana. tabacum var. White Burley	Mosaic & blisters	70	30

+ = Virus was transmitted,
- = Virus was not transmitted.

Table 2. Determination of CeMV antiserum titer in relation to time of blood collection.

Antiserum dilution	ELISA reading of CeMV Antisera collected at weekly intervals (A405nm)			
	Bled.1		Bled 2	
	Inf.	H.	Inf.	H.
500	1.301	0.220	1.215	0.672
1000	1.238	0.257	1.118	0.613
1500	1.121	0.212	1.206	0.500
2000	1.209	0.184	1.236	0.584
2500	1.213	0.196	1.204	0.3.29
3000	1.014	0.160	1.228	0.753
3500	0.624	0.141	0.859	0.218
4000	0.826	0.150	0.702	0.188
4500	0.454	0.130	0.676	0.166
5000	0.390	0.176	0.549	0.441
5500	0.521	0.136	0.471	0.179
6000	0.371	0.127	0.311	0.110
6500	0.286	0.120	0.211	0.107
7000	0.219	0.119	0.200	0.10
7500	0.214	0.105	0.11	0.0.9

Table 3. Effect of foliar spraying with *Jasmine oil* (0.5 %) and *Camphor oil* (0.5 %) of certain medicinal oils on *celery mosaic virus* infected *N. tabacum* cv. White Burly (as a systemic host) and *Ch. amanticolor* (as Local lesion host).

Treatments	ELISA test on *N. tabacum*	No. of L.L. on *Ch.. amanticolor*
Jasmine oil	0.421(+)	24 28
Camphor oil	0.668(+)	31 26
Untreated -Virus infection	1.114(+)	84

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