



جامعة الإسكندرية
كلية الزراعة - سابا باشا

دراسات على بعض الفيروسات التي تصيب القرعيات

مقدمة من

صلاح الدين مليجي الشاعر

رسالة علمية مقدمة استيفاء لمتطلبات منح درجة

دكتور الفلسفة في العلوم الزراعية

(تخصص أمراض النبات)

قسم النبات الزراعي

من

جامعة الإسكندرية



Alexandria University
Faculty of Agric. (Saba-Basha)

**STUDIES ON SOME VIRUSES
INFECTING CUCURBITS**

BY

SALAH ELDIN MELEIGI ELSHAIR

**A Thesis Submitted in Partial Fulfilment of the Requirements
Governing the Award of the Degree of**

DOCTOR OF PHILOSOPHY IN AGRICULTURAL SCIENCES

(PLANT PATHOLOGY)

Department of Agricultural Botany

From

Alexandria University

2009

لجنة الإشراف

الأستاذ الدكتور/ إبراهيم عبد السلام السمرة

أستاذ أمراض النبات المتفرغ

كلية الزراعة - ساها باشا - جامعة الإسكندرية

الأستاذ الدكتور/ جابر إبراهيم فجلة

أستاذ أمراض النبات المتفرغ

كلية الزراعة - جامعة الإسكندرية

الدكتور/ حسنى على عبد الحميد يونس

أستاذ أمراض النبات المساعد

كلية الزراعة - ساها باشا - جامعة الإسكندرية

ADVISOR'S COMMITTEE

Prof. Dr. Ibrahim A. El-Samra

**Professor of Plant Pathology,
Faculty of Agriculture,
Saba-Bacha, Alexandria University.**

Prof. Dr. Gaber I. Fegla

**Professor of Plant Pathology,
Faculty of Agriculture,
Alexandria University.**

Dr. Hosny A. Younes

**Associate Prof. of Plant Pathology,
Faculty of Agriculture,
Saba-Bacha, Alexandria University.**

ACKNOWLEDGMENT

FIRSTLY, THANKS AND MERCIFUL TO GOD

Sincere gratitude is due to **Prof. Dr. Ibrahim A. El-Samra**, Emeritus Professor of Plant Pathology, Faculty of Agriculture, Saba-Bacha, Alexandria University, for supervision, his kind support and generous personal encouragement.

Sincere thanks and deep appreciation are extended to **Prof. Dr. Gaber I. Fegla**, Emeritus Professor of Plant Pathology, Faculty of Agriculture, Alexandria University for suggesting the problem, guidance, supervision, continuous encouragement, valuable advice, continuous help throughout the investigation and preparation of the manuscript and putting the thesis in its final form.

Sincere thanks and deep appreciation are extended to **Dr. Hossny A. Younes** Associate Professor of Plant Pathology, Faculty of Agriculture, Saba-Bacha, Alexandria University for supervision, guidance, his kind support continuous encouragement and valuable helpful to finish this thesis.

Grateful acknowledgement is expressed to **Dr. Mervat Fathalla** associate professor of plant pathology, Plant Pathology Institute, Agriculture Research Center for faithful cooperation, kind support to determine coat protein molecular weight and use RT-PCR and multiplex PCR techniques for virus detection.

Also, sincere thanks are due to **Prof. Dr. Mohamed R. Rasmy** Professor of Plant Pathology, Plant Pathology Institute, Agriculture Research Center for helping in molecular assays.

The author wishes to express his deepest gratitude and sincere appreciation to Dr. **Maha Kawanna** and **Dr. Sandy Hammed** for their encouragement and for helping in statistical analysis.

Thanks are also to **Prof. Dr. Ahmed Zaiton**, Entomology Dept. Faculty of Agriculture, Alexandria University for helping in the identification of aphids.

It is with sincere appreciation that thanks my friends **Mr. Hossny El-Wazan** and **Mr. Yousry Ghattas** for contribution toward the creation of this work.

Acknowledgments are finally due to all the staff members, colleagues, technicians and laborers of the Agriculture Botany Department for their goodwill cooperation and help.

CONTENTS

	<u>Page No.</u>
INTRODUCTION	1
REVIEW OF LITERATURE	2
MATERIALS AND METHODS	29
RESULTS	41
1. Survey studies of some viruses affecting cucurbits	41
1.1. Cucurbits of open fields	41
1.1.1. Visual survey.....	41
1.1.2. Serological diagnosis by indirect ELISA	41
1.2. Cucurbits of plastic houses	69
2. Characterization of the most prevalent viruses	70
2.1. Host range and symptomology	70
2.1.1. WMV	70
2.1.2. ZYMV	70
2.2. Virus stability in sap	70
2.3. Mode of transmission	84
2.3.1. Mechanical transmission	84
2.3.2. Seed transmission	84
2.3.3. Aphid transmission	84
2.4. U.V. absorption spectra of the purified viruses	84
2.4.1. WMV	84
2.4.1.1. Purification	84
2.4.1.2. U.V. absorption spectrum	84
2.4.2. ZYMV	84
2.4.2.1. Purification	84
2.4.2.2. U.V. absorption spectrum	85
2.5. Determination of molecular weight of coat protein subunit of WMV and ZYMV	85
2.6. Detection of the viruses by molecular assay	85
2.6.1. Detection of WMV and ZYMV by RT-PCR	85
2.6.2. Detection of WMV and ZYMV by multiplex RT-PCR	85
2.7. Electron microscopy	85
2.7.1. WMV	85
2.7.2. ZYMV	85
3. Preparation of antisera against WMV and ZYMV	92
4. Some control measures	92
4.1. Effect of distance among hills and corn barrier	92
4.1.1. Effect on infection percentage of squash	92
4.1.2. Effect on number and weight of squash fruits	92
4.2. Effect of other treatments	100
4.2.1. Effect on the Incidence of virus diseases of squash	100
4.2.2. Effect on the number of aphids	100
4.2.3. Effect on number and weight of squash fruits	102

DISCUSSION	105
SUMMARY	114
REFERENCES	117
APPENDIX 1	133
APPENDIX 2	134
ARABIC SUMMARY	

LIST OF TABLES

<u>Table No.</u>	<u>Title</u>	<u>Page No.</u>
1	Reaction of different hosts to WMV	7
2	WMV stability in sap according to different authors	11
3	Reaction of different hosts to ZYMV	17
4	ZYMV stability in sap according to different authors	22
5	Oligonucleotide used for reserve transcription polymerase chain reaction (RT-PCR) detection of two squash plants	37
6	Incidence of virus-like diseases (%) in some cucurbit producing areas of Alexandria governorate during the growing seasons 2004, 2005 and 2006	42
7	Incidence of virus-like disease (%) in some cucurbit producing areas of El-Beheira governorate during the growing seasons 2004, 2005 and 2006	43
8	Incidence of virus-like diseases (%) in some cucurbit producing areas of Kafr El-Sheikh governorate during the growing seasons 2004 and 2006	43
9	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in Alexandria governorate during the growing season 2004	44
10	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of Alexandria governorate during the growing season 2004	45
11	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in Alexandria governorate during the growing season 2005	49
12	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of Alexandria governorate during the growing season 2005	50
13	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in Alexandria governorate during the growing season 2006	51
14	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of Alexandria governorate during the growing season 2006	54
15	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in El-Beheira governorate during the growing season 2004	57
16	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of El-Beheira governorate during the growing season 2004	58

17	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in El-Beheira governorate during the growing season 2005	61
18	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of El-Beheira governorate during the growing season 2005	62
19	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in El-Beheira governorate during the growing season 2006	64
20	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of El-Beheira governorate during the growing season 2006	64
21	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in Kafr El-Sheikh governorate during the growing season 2004	66
22	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of Kafr El-sheikh governorate during the growing season 2004	66
23	Occurrence and relative prevalence of some viruses infecting cucurbits in some cucurbit producing areas in Kafr El-Sheikh governorate during the growing season 2006	67
24	Viruses causing single and mixed infections in samples collected from naturally infected cucurbit plants in fields distributed in some locations of Kafr El-sheikh governorate during the growing season 2006	67
25	Virus like disease incidence, occurrence and relative prevalence of Some viruses infecting cucumber cultivated in some plastic houses in Alexandria, El-Beheira and Kafr El-Sheikh governorates	71
26	Viruses causing single and mixed infection in samples collected from naturally infected cucurbit plants in plastic houses distributed Alexandria, EL-Beheria and Kafr El-Sheikh governorates during growing seasons 2004, 2005 and 2006	72
27	Reactions of different plant species to inoculation with WMV and ZYMV	78
28	Stability in sap of WMV and ZYMV as indicated by number of infected squash plants	83
29	Transmission of WMV by six aphid species	86
30	Transmission of ZYMV by six aphid species	86
31	Indirect ELISA absorbance values (E 405 nm) of extract of WMV- infected squash plants against various dilutions of WMV antiserum	93

32	Indirect ELISA absorbance values (E 405 nm) of extract of ZYMV-infected squash plants against various dilutions of ZYMV antiserum	93
33	Effect of distances among hills and corn barrier on infection percentage of squash in the growing seasons 2003 and 2004	94
34	Effect of distances among hills and corn barrier on number of squash fruits in the growing season 2003	96
35	Effect of distances among hills and corn barrier on weight of squash fruits in the growing season 2003	97
36	Effect of distances among hills and corn barrier on number of squash fruits in the growing season 2004	98
37	Effect of distances among hills and corn barrier on weight of squash fruits in the growing season 2004	99
38	Effect of different treatments on the incidence of virus diseases of squash in the growing seasons 2005 and 2006	101
39	Effect of different treatments on the number of aphids on the squash plants in the growing season 2005	101
40	Effect of different treatments on the number of aphids on the squash plants in the growing season 2006	103
41	Effect of different treatments on weight and number of squash fruits per plot in the growing season 2005	103
42	Effect of different treatments on weight and number of squash fruits per plot in the growing season 2006	104

LIST OF FIGURES

<u>Fig. No.</u>	<u>Title</u>	<u>Page No.</u>
1	Diagrammatic outline of procedure used for ZYMV purification	34
2	Diagrammatic outline of procedure used for WMV purification...	35
3	Squash leaf naturally infected with WMV	47
4	Natural infection of squash leaf with TRSV	47
5	Squash naturally infected with CMV + WMV + ZYMV	52
6	Squash naturally infected with WMV + ZYMV	52
7	Squash naturally infected with TRSV + WMV + ZYMV	53
8	Squash naturally infected with PRSV + SqMV + WMV	55
9	Natural infection of squash leaf with ZYMV	59
10	Natural infection of squash leaf with WMV	59
11	Squash leaf infected naturally with PRSV + WMV + ZYMV	60
12	Natural infection of squash with CMV	65
13	Natural infection of cantaloupe with WMV + ZYMV	65
14	Natural infection with ZYMV on squash fruit (A) and squash leaf (B).....	68
15	Cucumber fruits infected with WMV in plastic-house	73
16	Symptoms of WMV+ZYMV on cucumber grown in plastic-house	73
17	Necrotic local lesion with red border, caused by <i>Chenopodium amaranticolor</i>	74
18	Mosaic and veinbanding (A), mosaic and blisters (B) caused by WMV on <i>Cucumis melo</i> var. <i>flexuosus</i>	74
19	Mosaic (A), mosaic with few blisters (B) and severe symptoms (C) caused by WMV on <i>Cucumis sativus</i>	75
20	Different patterns of symptoms, mosaic (A) and veinbanding (B & C) on leaves of <i>Cucurbita maxima</i> infected by WMV	75
21	Various symptoms, mosaic (A), mosaic with blisters (B) and severe blisters and malformation (C & D) caused by WMV on <i>Cucurbita pepo</i>	76
22	Mosaic and yellowing on <i>Luffa aegyptiaca</i> infected with WMV .	76
23	Mild mosaic on <i>Vicia faba</i> caused by WMV	77
24	Chlorotic spots (A&B) produced by WMV on <i>Hibiscus esculentus</i>	77
25	Mosaic (A) followed by severe mosaic and blisters (B) on <i>Citrulus lanatus</i> infected by ZYMV	80
26	Mild mosaic (A), severe mosaic (B) and mosaic accompanied with blisters (C) on leaves of <i>Cucumis melo</i> var. <i>flexuosus</i> infected with ZYMV	80
27	Mild mosaic (A), severe mosaic and blisters (B&C) on leaves of <i>Cucumis sativus</i> infected by ZYMV	81
28	Mosaic and veinbanding (A) and mosaic with blisters (B) induced by ZYMV on leaves of <i>Cucurbita maxima</i>	81
29	Different patterns of symptoms mosaic (A&B) and malformation (C, D&E) on <i>Cucurbita pepo</i> infected with ZYMV	82

30	Mosaic with blisters and yellowing on <i>Luffa aegyptiaca</i> caused by ZYMV	82
31	Ultraviolet absorption spectrum of purified W/MW	87
32	Ultraviolet absorption spectrum of the purified ZYMV	87
33	Electrophoresis of WMV and ZYMV coat protein in 12% SDS-PAGE	88
34	Agarose gel electrophoresis showing the PCR amplification products of WMV and ZYMV coat protein gene	89
35	Detection of WMV and ZYMV by RT-PCR and multiplex-RT-PCR	90
36	Area of cytoplasm from thin section of squash leaf infected with WMV showing pinwheel (PW) and laminate inclusion components (La).....	91
37	Transverse section of cylindrical inclusion appeared as pinwheel (PW) and scroll (S) in the cytoplasm of squash leaf infected with ZYMV	91

INTRODUCTION

The cucurbits (family *Cucurbitaceae*) form a diverse group consists of about 90 genera and 750 species, grown around the world under many different conditions for many different purposes. The cultivated species are important crops in the tropics, subtropics and milder portion of the temperate zones.

In Egypt annual cultivated areas (Feddan) with watermelon, squash, cucumber, cantaloupe, sweet melon, snake cucumber, melon (shahd), melon(quoz) and pumpkin were 164526, 91054, 84261, 71362, 22560,10460, 2148, 867 and 242, respectively. Such areas produced 2025185, 698606, 802644, 745953, 211257, 104637, 23917, 7803 and 1672 ton in case of watermelon, squash, cucumber, cantaloupe, sweet melon, snake cucumber, melon (shahd), melon (quoz) and pumpkin, respectively. (A. R. E. Ministry of Agriculture and Land Reclamation. Economic Affairs Sector. Vol. 2. 2006).

There are over 200 known cucurbit diseases of diverse etiologies. Virus diseases are a major problem in cucurbit planting areas all over the world. They are difficult to control and result in tremendous crop losses. At least 45 viruses have been reported to infect cucurbit plants all over the world (Lovisolo, 1980; El-Saleh, 1994; Brunt *et al.*, 1996 and Fegla *et al.*, 2008). Out of these viruses 24 were recorded in Arab regions (Zouba *et al.*, 1997; Hag-Kasem *et al.*, 2005; El-Chaabi *et al.*, 2006 and Fegla *et al.*, 2008). Among of these viruses the most common are *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber mosaic virus* (CMV), *Cucurbit aphid borne yellow virus* (CABYV), *Papaya ring spot virus* (PRSV), *Squash mosaic virus* (SqMV), *Tobacco ring spot virus* (TRSV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV).

Little work has been done on virus diseases affecting cucurbits in northern Egypt; therefore the aim of the present investigation is directed mainly to study the following:

- 1- Distribution and prevalence of cucurbit viruses in different locations of northern Egypt.
- 2- Characterization of the most prevalent viruses (WMV and ZYMV) using host range and symptomology, stability in sap, mode of transmission, U.V. absorption spectra of purified viruses, molecular weight of coat protein subunit, reverse transcription-polymerase chain reaction (RT- PCR), multiplex- RT- PCR and electron microscopy.
- 3- Preparation of antisera against WMV and ZYMV
- 4- Some control measures such as distance among hills, barrier crops, soil mulches, insecticide and oil sprays and their effect on productivity.

MATERIALS AND METHODS

1. Survey studies of some viruses affecting cucurbits

A survey studies were conducted in different locations of three governorates namely Alexandria, El-Beheira and Kafr El-Sheikh from February through October during the growing seasons 2004, 2005 and 2006.

Surveys were performed to study the prevalence and distribution of viruses affecting cucurbits growing in open fields as well as in the plastic houses.

Periodical inspection was done every one month. Plants were randomly evaluated based on symptoms suspected to be caused by virus infection such as chlorosis, vein clearing, mottle, mosaic, yellowing, blister, stunting and leaf malformation and fruit distortion.

Samples of plants exhibiting virus like symptoms were collected and the symptoms of plants were recorded. Each plant sample was kept separately in a plastic bag.

Estimation of diseases incidence was based on external symptoms and serological diagnosis.

1.1. Visual survey

Incidence of viral disease symptoms was estimated visually by counting the number of plants showing symptoms out of 100 consecutive plants in each patch selected, at random, from each inspected field and plastic house.

1.2. Serological diagnosis

Serological diagnosis was carried out using indirect ELISA.

1.2.1 Source of antisera

Antisera of *Cucumber mosaic virus* (CMV), *Cucumber green mottle mosaic virus* (CGMMV), *Squash mosaic virus* (SqMV), *Papaya ring spot virus* (PRSV), *Watermelon mosaic virus* (WMV), *Zucchini yellow mosaic virus* (ZYMV) and *Tobacco ring spot virus* (TRSV) were used.

The antiserum to CMV was locally prepared in laboratory of Plant Virology, Faculty of Agriculture, Alexandria University (Younes, 1995), ZYMV antiserum was supplied by Agricultural Genetic Engineering Research Institute (AGERI), ARC, Egypt, CGMMV antiserum was kindly supplied by Dr. M. EL-Mazaty, Faculty of Agriculture, El-Mansoura University, TRSV antiserum was supplied by antiserum-Bank, Institute of Seed Pathology for Developing Countries, Denmark while SqMV, PRSV and WMV antisera were provided by Agdia, the catalog numbers were CAB 26400, CAB 53500 and CAB 5400, respectively.

1.2.2. Indirect ELISA

The indirect ELISA was carried out as described by Younes (1995) and Fegla *et al.* (1997). Disposable polystyrene flat bottom micro ELISA plates were used. The plant

samples were ground in coating buffer (0.5 M carbonate, pH 9.6) (Appendix 1) 1:10 (w/v) using a mortar and pestle, then strained through double layered cheesecloth. Wells were coated with antigens by adding 100 µl of each sample to the bottom of the well and incubated for 3 hours at 37°C or overnight at 4°C. The plates were rinsed three times by flooding wells with phosphate buffer saline + Tween 20 (PBST) (Appendix 1), 5 minutes each. To reduce non specific reactions, antisera requiring cross adsorption were diluted 1:500 with filtered extract from healthy tissues 1:20 (w/v) in serum buffer [PBS-Tween 20 containing 2% soluble ployvinylpyrrolidone ((PVP) and 0.2% bovine serum albumin (BSA)] (Appendix 1), and incubated for 45 min. at 37°C.

The precipitate, which had formed, was removed by centrifugation for 10 min. at 500 rpm, 100 µl aliquots from the diluted antisera were added to each well, after which the plates were incubated at 37°C for 2 hours or at 4°C overnight , then washed as before.

Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (whole molecule, enzymatic activity 475 units /ml) obtained from Sigma Chem. Co. St Louis, Mo (Production # A 8025) was diluted 1:1000 in serum buffer and 100 µl were added to each well , followed by one hour incubation at 37°C , then washed as before.

One hundred µl of the enzyme substrate, 0.5 mg/ml para-nitrophenylphosphate (Sigma#104) in 10% diethanolamine buffer, pH 9.8 (Appendix 1) were added to each well and incubated at room temperature (25°C) for about 30 minutes. The enzyme activity was stopped by adding 50µl of 3M NaOH.

The ELISA values, measured by multiskan MS ELISA reader, were expressed as absorbance at 405 nm. Absorbance values of at least double that of healthy control, were considered positive.

In each set of test, wells lacking antigen (coating buffer only) were included as a blanks.

2. Characterization of the most prevalent viruses

The most prevalent viruses were *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV). WMV and ZYMV which used in this study were isolated from squash plants. The isolated viruses were separately maintained on squash plants cv. Eskandrani that served as a source for subsequent studies. Except otherwise stated, virus inocula were prepared by grinding infected leaf tissues 1:10 (w/v) with a mortar and pestle in 0.1M phosphate buffer, pH 7.0. Leaves of plants to be inoculated were first dusted with carborandum (600 mesh) and then inoculated with freshly prepared inoculum using forefinger. Inoculated plants were rinsed with tap water shortly after inoculation.

Characterization of the isolated viruses was based mainly on host range and symptomology, stability in sap, mode of transmission, U.V. absorption spectra of purified viruses, molecular weight of coat protein subunit, reverse transcription-polymerase chain reaction (RT-PCR) and electron microscopy.