UNIVERSITY OF JORDAN

FACULTY OF GRADUATE STUDIES

PURIFICATION AND PARTIAL CHARACTERIZATION OF ZUCCHINI YELLOW MOSAIC VIRUS IN JORDAN

BY

NASER MOHAMMED AHMED ABDULLAH

عميد كلية الدراسات العليا

SUPERVISED BY

Prof. Dr. ABDULLAH M. AL-MUSA

Submitted in partial fulfillment of the requirements for the degree of master of science in Plant Protection / Plant Pathology, Faculty of Graduate Studies, University of Jordan.

December, 1994.

This thesis was defended successfully on Dec. 20,1994 .

Commitee Members	Signature
Dr. Abdullah M. Al-Musa, Professor	A. Musa
Dr. Walid I. Abu-Gharbieh, Professor	W. I. Abu-Gharbieh
Dr. Husien M. El-Mosa, Professor	Husein Elmosa
Dr. Akel N. Mansour, Assistant Professor	A. Mansour

Dedication

TO THOSE WHO MADE IT POSSIBLE,

MY BELOVED MOTHER,

MY DEAREST FATHER,

AND MY WIFE.

NASER

DEC. 20, 94

Acknowledgments

I wish to express my deep appreciation to Dr. Abdullah M. Al-Musa, for his patience, guidance, encouragement and supervision throughout the course of this study. I wish also to thank Dr. Walid Abu-Gharbieh, Dr. Husein El-Mosa and Dr. Akel Mansour for their cooperation, valuable comments, and reviewing this manuscript.

Special thanks are extended to AMC group, especially to Mr. Mustafa Jarrar for his patience, continuous encouragement, and support.

Thanks are also extended to my colleague Iyad Takrouri for his help and encouragement, Mr. A. Abu-Halawa for his help and all my colleagues in the virology laboratory throughout the course of study for their support and patience.

I am indebted to my parents, sisters and brothers for their help, support, encouragement and understanding.

LIST OF CONTENTS PAGE

List of tables

Table **page**

List of Figures

Figure **page**

List of plates

Plate **page**

List of Appendices

Appendix **Page**

Abstract

Purification and Partial Characterization of Zucchini Yellow Mosaic Virus in Jordan

By

Nasser Mohammed Ahmed Abdullah

Supervised by

Prof. Dr. Abdullah M. Al-Musa

A study was conducted for purification and partial characterization of zucchini yellow mosaic virus (ZYMV) and to investigate the occurrence of other strains and variants of the virus in Jordan. Different preliminary experiments were conducted to develop a procedure to purify the virus. The infected squash tissue harvested four weeks after inoculation and precooled overnight at 4 °C were homogenized with 1:3 (w/v) ratio of phosphate buffer 0.01M, pH 8.5 containing 0.02M Na_2SO_3, 0.005M EDTA and 0.01M DIECA. The homogenate was emulsified with 1:1 (v/v) of chloroform; low speed and high speed centrifugation were used to break the emulsion and to precipitate the virus respectively. Density gradient centrifugation was used for further purification of ZYMV-M.

A locally bred rabbit was injected with the partially purified virus to obtain homologous antiserum containing specific antibodies for ZYMV-M inorder to be used for a rapid detection of the virus from the diseased plants and for further identification and differentiation between the other strains or variants of the virus involved in this study.

Ninety virus isolates were recovered from diseased cucurbits showing mosaic symptoms and symptomless *Molucella leavis* collected during 1989 were identified as ZYMV on the basis of host range and serology. Isolates (46-89, 51-89, 57-89 and 65-89) were chosen to represent the remaining isolates on the basis of their reaction on the second true leaf of Cucurbita pepo cv. Victoria. The study extended information on response of selected

host plants to infection by these isolates, efficiency of certain aphid species to transmit them and to compare the four isolates serologically in SDS-immunodiffusion test and in indirect ELISA using ZYMV-M specific antiserum and antiserum for the cylindrical inclusions of ZYMV-CT.

Introduction

Cucurbits are profitable vegetable crops cultivated on large area. During the 1990 growing season; sixty one thousand dunums were planted to cucurbits, with squash covering 30% of this area (1).

Several viral diseases attack cucurbits to the extent that they became a limiting factor of production (2). One of these is the zucchini yellow mosaic virus (ZYMV) which is reported to attack cultivated cucurbits (3, 4). Infected plants show pronounced reduction in growth, yellowing, mosaic and blistering of leaves, while fruits show distortion and malformation (5).

ZYMV, a potyvirus, is aphid transmitted in the nonpersistant manner (2, 4, 5, 6, 7, 8, 9, 10, 11). The virus has several strains and variants that differ in symptoms, host range, aphid transmissibility and virulence towards resistant genes in some crops (3, 6, 7, 12).

The Jordanian isolate of ZYMV (ZYMV- M) was recovered originally from melon and was found in all other cucurbits (6). The melon isolate (ZYMV-M) is of the same biotype of the French isolate (ZYMV- ES) but differs from the Connecticut isolate (ZYMV- CT) (6). No attempts were made to study occurrence of other strains and variants of ZYMV in Jordan. Moreover further characterization of ZYMV- M was hampered by the fact that the virus was not purified. Hence this study was initiated to extend knowledge on ZYMV- M and to investigate the possibility of occurrence of other strains or variants in Jordan. Isolates recovered will be investigated with regard to their symptoms, host range, aphid transmissibility and serology.

ZYMV is one of the most wide spread and destructive viruses affecting cucurbits. Symptoms of infected cucurbits include mosaic and distortion of leaves and fruits resulting in significant yield losses especially when infection occurs at early stages of plant growth.(2, 3, 4, 5, 6)

Potyvirus group members like ZYMV are difficult to manipulate *in vitro* because of their tendency to aggregate during purification(21). Different procedures were developed for purification and chemical characterization of zucchini yellow mosaic virus. Fresh leaves from infected zucchini squash *Cucurbita pepo* cv's. Elite and Senator, cucumber *Cucumis sativus* harvested 2-3 weeks after inoculation (5, 9, 12, 22) or melon *Cucumis melo* cv. Vedrantais harvested 3-4 weeks after inoculation (10) served as virus sources for purification of ZYMV in different countries. The tissue is then frozen or cooled prior to extraction (22). Homogenization of ZYMV infected tissue was done using several buffers that include 0.5M or 0.3M phosphate buffer pH 8.5 at rate 1:3 to 1:4 (5, 10), and 0.2M borate buffer pH 7.5 (11). Additives added to the homogenization buffers included ethylene diamine tetra acetic acid (EDTA) in 0.005 - 0.01M (5,9), sodium diethyl dithiocarbamate (Na-DIECA) in 0.01M and 0.2% (w/v) (5, 10), sodium sulfite (Na_2SO_3) in 0.02M (5), ascorbic acid in 0.2% (w/v) (11) or 2-mercaptoethanol in 0.1% (10).

A wide variety of procedures have been used to partially clarify tissue extracts. 1:1 (v/v) Freon 113 (Fluorocarbon: 1,1,2-diflouro-1,2,2-trichloethane) (5,10), 20% (v/v) chloroform (11) or a mixture of chloroform and carbon tetra chloride (1:1 v/v) (9) were used in clarification of ZYMV in different countries .

ZYMV was partially purified and concentrated in the aqueous phase by high speed centrifugation at 60,000 g for four hours. The high speed pellet was collected and the supernatant was discarded (5,10). Polyethylene glycol

(PEG) 6000 added at 6% (w/v) was used to precipitate the virus (9,12). Both high speed centrifugation and PEG precipitation may be combined(11).

Several buffers were used for suspending the high speed pellets including 0.02 M phosphate buffer pH 7.4 (10), 0.05 M phosphate buffer pH 7.5 (12), 0.05 M borate buffer pH 7.5 (11) or 0.05 M sodium citrate buffer pH 7.5 containing 0.02 M Na_2SO_3 (5). Urea and mercaptoethanol seemed to reduce virus particle aggregation during low speed centrifugation by weakening hydrophobic and disulfide bond respectively (23).

Further purification of ZYMV could be achieved using 10 - 40 % (11,22) or 10 - 50 % (5) sucrose density gradient centrifugation in which virus suspension is layered on top of sucrose gradients and centrifuged at 104,000 g or 70,000 g respectively for two hours in swinging pocket rotor (5,22). The use of other procedures such as equilibrium density gradient centrifugation has been reported (5,9,10).

Antiserum to ZYMV had been obtained with titers of 1:1024 and 1:1048 using the slide precipitin test (5), 1:8 using crude virus preparations in SDS-immunodiffusion test (9). 1:64 in microprecipitin test using purified antigens (22). and 1:1000 using the decoration test (24).

Although plant viruses remain like parent virus during replication, occasionally changes through a process of mutation resulted in the appearance of new mutants or strains (25). ZYMV has already displayed a remarkable variability suggesting that it has existed for a long time(3,4).

The French isolates of ZYMV could be represented by two distinct strains, the first strain was ZYMV - E9 that induced cracks on the fruits of melon and wilting reaction on melon cv. Doublon, while the second strain was ZYMV- E15 that induced mottling and hardening of the fruit flesh of

infected melon plants, furthermore ZYMV- E15 is not able to induce wilting reaction on melon cv. Doublon(26).

According to the behavior of melon line PI 414723, which shows resistance towards certain strains of ZYMV like ZYMV- E15, the French isolates fell into two pathotypes: the first pathotype includes isolates that induce no symptoms on melon line PI 414723 plants represented by ZYMV- E15 strain. These will be referred to as pathotype 0. while the second pathotype includes isolates that induced systemic chloronecrotic spotting on all inoculated plants of melon line PI 414723 represented by ZYMV- E9 strain. these isolates will be referred to as pathotype 1(7).

As a result of changes in the environmental conditions during the growth of melon line PI 414723 plants inoculated with ZYMV - E15 and ZYMV- E9, all PI 414723 plants developed systemic yellowing, stunting, mosaic, and leaf deformation symptoms. So two variants (D41 and B02) were obtained from ZYMV- E15 and ZYMV- E9 strains respectively. These variants could be differentiated from the oriby their ability to induce yellowing, stunting, mosaic and leaf deformation on inoculated PI 414723 plants, while they could not be differentiated from the original strains by aphid transmissibility or serology. Therefore they are placed in a third pathotype called pathotype 2(7).

In SDS immunodiffusion tests, strains E9 and E15 and their variants B031 and D41 respectively reacted with homologous antisera prepared against strain E9 and E15 by forming single precipitin line which fused without spuring indicating that the two strains and their variants are very similar if not identical(7).

A variant of ZYMV which is poorly aphid transmissible (ZYMV-E15 PAT) was obtained from the efficiently aphid transmissible strain (ZYMV-

E15). Efficiency of *Myzus persicae* in transmission of the poorly aphid transmissible variant (ZYMV-E15 PAT) is lower than the efficiency of *Myzus persicae* in transmission of the original strain (ZYMV-E15). The two isolates ZYMV-E15 PAT and ZYMV-E15 could not be differentiated by symptomology, host range and serology(27).

A mild variant of ZYMV (ZYMV-WK) was recovered from melon inducing mild mottle on the leaves of melon and squash plants, while no symptoms were observed on the fruits. The origin of ZYMV-WK was the poorly aphid transmissible variant (ZYMV-E15 PAT) which was originated from ZYMV-E15 strain. ZYMV-WK induced systemic mild mottle on cucurbits and chlorotic local lesions on *Chenopodium amaranticolor*, while ZYMV-E15PAT and ZYMV-E15 induced severe systemic symptoms on cucurbits and a well defined necrotic local lesions on *C. amaranticolor*. The aphid transmissibility of ZYMV-WK was tested using *Myzus persicae* and *Aphis gossypii*, it appeared that ZYMV-WK is poorly aphid transmissible similar to its origin ZYMV-E15 PAT(3).

In SDS- immunodiffusion tests, ZYMV-WK was compared with its originating isolates ZYMV-E15 PAT and ZYMV-E15 using specific antiserum for ZYMV-E15 . All three isolates reacted with fused precipitin line without spur formation, indicating that ZYMV-WK is closely related if not identical to its originating isolates(3).

The differentiation between the American isolates of ZYMV was based mainly on symptomology and host range. So the American isolates of ZYMV could be represented by two distinct strains, ZYMV- CT (Connecticut strain) which induced yellowing symptoms similar to those described in France and Italy, and ZYMV- FL (Florida strain) which induced milder symptoms on squash compared with ZYMV- CT and the symptoms appearance was delayed 3- 5 days(9,28).

In SDS immunodiffusion test, it appeared that ZYMV- CT and ZYMV- FL reacted with specific antisera for both strains forming fused percipitin lines without spur formation indicating that the two strains are closely related if not identical to each other(28).

ZYMV isolates from Turkey and Egypt were compared biologically and serologically with ZYMV- CT and ZYMV- FL strains. It appeared that the Egyptian isolate and the Turkish isolate of ZYMV were similar to the ZYMV- CT but are different from ZYMV- FL in time needed for symptoms appearance and the severity of symptoms in squash. In SDS immunodiffusion test, it appeared that all isolates of ZYMV from Egypt and Turkey compared with the American strains ZYMV- CT and ZYMV- FL reacted by forming fused precipitin lines without spur formation indicating that they are closely related if not identical(22).

In Jordan, the melon isolate of ZYMV (ZYMV- M) recovered from naturally infected melon was identified as a new strain of ZYMV. Identification was based on symptomology, host range, aphid transmission, electron microscopy and serology.The Jordanian strain of ZYMV (ZYMV- M) was compared with the French strain ZYMV- ES and the Connecticut strain ZYMV- CT. Comparison among the three strains was done by using differential host range that includes *Cucurbita pepo* cv. Victoria and some bean *Phaseolus vulgaris* cultivars, such as Black turtle 2, Widusa, Tormine and Serbo, and serologically in indirect ELISA using three different kinds of antisera, ZYMV- B (Berlin isolate) specific antiserum, ZYMV- ES specific antiserum and antisera produced against the cylindrical inclusions of ZYMV- CT (6).

ZYMV- M produced vein clearing, raised green blisters , ZYMV- ES produced vein clearing, green vein banding while ZYMV- CT produced vein clearing, interveinal chlorosis and reduction in the leaf size on the

second true leaf of *Cucurbita pepo* cv.Victoria. On the other hand, ZYMV-M and ZYMV- ES were able to infect locally Serbo and Tornine, while ZYMV- CT failed to infect these two bean cultivars(6).

From the results of the comparative host range tests, it appeared that ZYMV- M and ZYMV-ES belonged to the same biotype which differs from that of ZYMV- CT. This suggestion was confirmed by the results of indirect ELISA, as indicated by the high absorbtion value of ZYMV- CT when the antisera for the cylindrical inclusions of ZYMV- CT was used in comparison with those of ZYMV- ES and ZYMV-M when specific antisera ZYMV-ES and ZYMV- B were used(6).

Materials and Methods

Purification of Zucchini Yellow Mosaic Virus (ZYMV-melon isolate)

The melon isolate of ZYMV (ZYMV-M) recovered originally from melon *Cucumis melo* in Jordan (6) was passed through *Ranunculus sardous* and maintained in *Cucurbita pepo* cv. Victoria for purification and subsequent studies. Squash seedlings at the cotylodinary stage previously dusted with 6000 mesh carborundum were mechanically inoculated with ZYMV-melon isolate. Inoculum used in all experiments, unless otherwise stated, was prepared by triturating infected squash leaves in sterilized mortor and pestile using 0.01M neutral phosphate buffer containing 0.01M sodium diethyl dithiocarbamate and 0.01M cystine hydrochloride (D+C). Infectivity of tissue extracts or purified preparations was assayed on *Chenopodium amaranticolor* using the half leaf comparison method. In this method equal number of half leaves was inoculated with treatments in a uniform manner and the opposite half leaves were inoculated with the control. Chlorotic local lesions were counted 10-15 days later. Relative infectivity of the treatments was expressed as percent ratio of the total number of local lesions produced by the control treatment applied on the opposite half leaves of each treatment. To establish that the culture had not become contaminated with Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV) and Watermelon mosaic virus (WMV-2), a differential host range including *Nicotiana glutinosa* and *Lavatera trimestris* plants were inoculated periodically with inoculum from ZYMV-melon isolate infected squash plants as described earlier (16,20).

Determination of the proper time of harvesting infected tissue:

An experiment was conducted to determine the time at which the highest virus concentration was reached in infected squash leaves, in order to determine the proper time of harvesting the tissue for purification.

Five sets, each of five squash seedlings were mechanically inoculated at the cotyledonary stage in the same day. The uppermost leaves of the inoculated plants were harvested at different dates: one, two, three, four and five weeks after inoculation. Inoculum was prepared by triturating infected squash leaves in 0.01M neutral phosphate buffer containing 0.01 D+C (1:1 w/v) ratio at 4-5°C. Extracts were then strained through cheese cloth and assayed on *Chenopodium amaranticolor* using the half leaf comparison method. The control was the extract of fresh leaves harvested two weeks after inoculation.

Effect of precooling and freezing on infectivity of ZYMV-M

An experiment was conducted to determine the effect of pre-cooloing and freezing of the infected squash leaves on the infectivity of ZYMV-melon isolate. Ten grams of infected squash leaves were harvested 4 weeks after inoculation . Leaves were divided into three equal samples: one sample was triturated immediately after harvesting in 0.01M neutral phosphate buffer containing 0.01M D+C (1:1 w/v) at 4-5°C. The remaining samples were processed differently before they were triturated as in the first sample. One was precooled overnight at 4-5°C, and the other was frozen at -15°C for one week. Extracts were strained through cheese cloth and assayed separately on *C. amaranticolor* using the half leaf comparison method. The control was the extract of fresh leaves harvested 4 weeks after inoculation, each treatment was replicated eight times in a completely randomized design.

Buffer experiments:

The effect of different buffers on the infectivity of ZYMV-melon isolate was studied. Potassium phosphate buffer with 0.01, 0.05 and 0.5M; pH 7.5 or 8.5, citrate buffer with 0.01M; pH 5.6 and borate buffer with 0.01M; pH 8.5 were included in this test. Potassium phosphate buffers were tested with or without the addition of 0.005M ethylene diamine tetra-acetic acid disodium salt (Na-EDTA), 0.01M sodium diethyl dithiocarbamate (DIECA) and 0.02M sodium sulfite (Na_2SO_3). Citrate and borate buffers were tested with or without the addition of 0.01M D+C.

Infected squash leaves were harvested 4 weeks after inoculation and precooled overnight at 4°C. Leaves were triturated in equal amount of distilled water (1:1 w/v) at 4°C. Extract was strained through cheese cloth and divided into 1ml aliquots. Each aliquot was then diluted with 1ml of the appropriate buffer to give the desired molarity and pH, the control was prepared by diluting 1ml aliquot with 1ml distilled water. All treatments, including the control, were stored overnight at room temperature and then assayed separately on *C. amaranticolor* using the half leaf comparison method, each treatment was replicated four times in a completely randomized design.

Clarification experiments:

Different clarification treatments that would change the solubility of the host material without changing the solubility of the virus, or vice versa, were tested as follows:

Infected squash leaves were harvested 4 weeks after inoculation and precooled overnight at 4°C. Leaves were triturated in 0.01M neutral phosphate buffer containing 0.01M D+C. The extract was strained through chessecloth and divided into 20ml samples treated as follows:

الصفحة غير موجودة من أصل المصدر

Determination of the best buffer for suspending high speed pellet

several buffers were tested to determine the buffer that retains maximum infectivity of high speed centrifugation pellets. Precooled infected squash leaves harvested 4 weeks after inoculation were triturated in 0.01M phosphate buffer containing 0.005M EDTA, 0.02M Na_2SO_3 and 0.01M DIECA. The extract was strained through cheesecloth, emulsified with chloroform for 30 min. at 4°C, and then centrifuged for 30 min. at 3000 g. The aqueous supernatant was divided into equal volume of aliquots and centrifuged at 92,000 g for 2.5 hrs. Each pellet was then suspended 2-4 hrs at 4°C in 2 ml of 0.01M phosphate buffer pH 8.5, 0.05M citrate buffer pH 7.5, or 0.01M borate buffer pH 8.5. All buffers were tested with or without the addition of 0.5M urea, 0.1% mercaptoethanol. Citrate buffer was tested with the addition of 0.02M Na_2SO_3 . Suspensions were centrifuged at 11700 g for 10 minutes and supernatants were separately assayed on *C. amaranticolor* using the half leaf comparison method, each treatment was replicated four times in a completely randomized design.

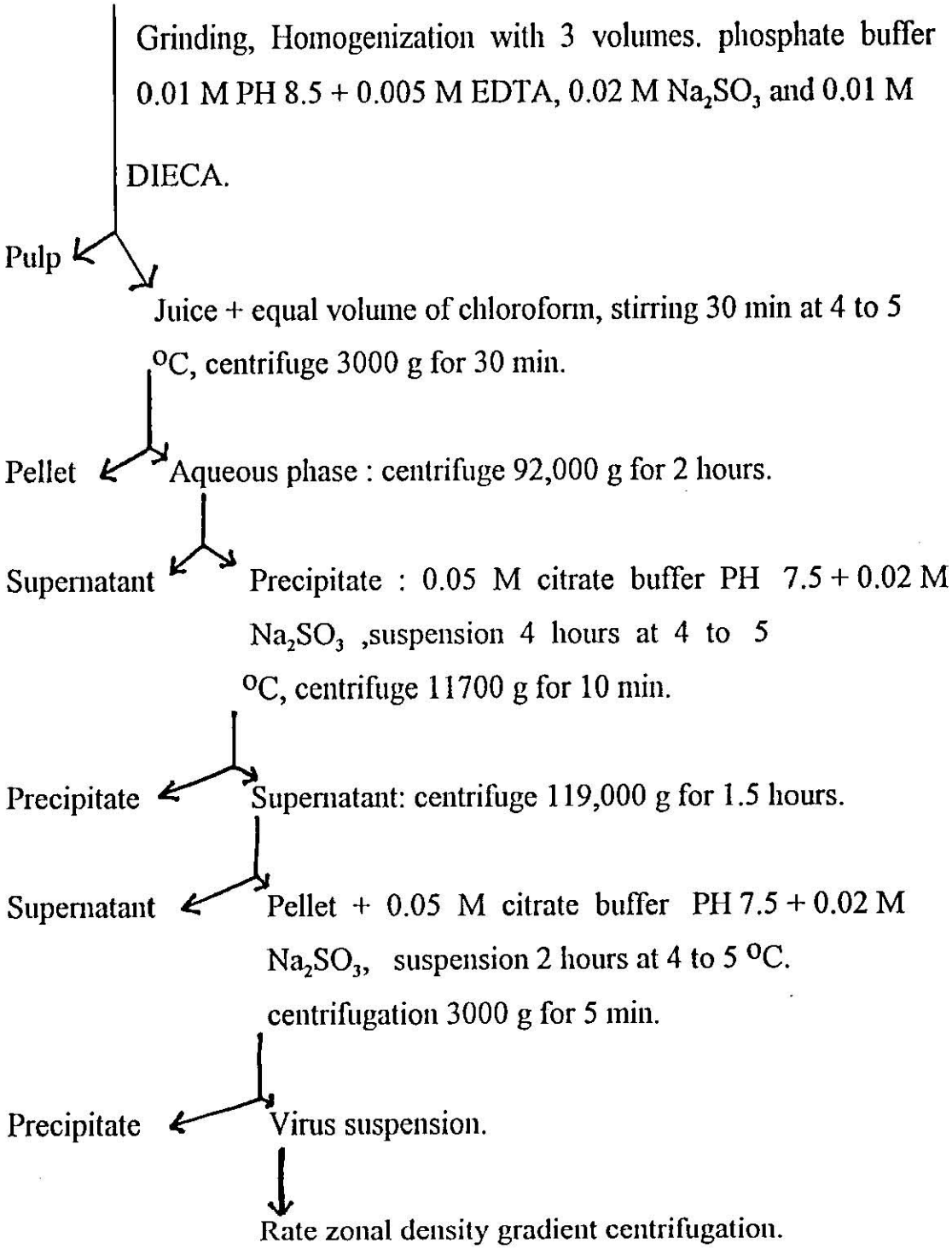
Purification procedure:

Fourty to sixty grams of infected squash leaves were harvested 4 weeks after inoculation and precooled overnight at 4 °C. Leaves were homogenized for 3 to 5 minutes in a precooled waring blender with 3 volumes (w/v) of 0.01M phosphate buffer pH 8.5 containing 0.005M EDTA, 0.02M Na_2SO_3, and 0.01M DIECA. The extract was then expressed through cheesecloth. Equal volume of chloroform was added and stirred for 30 min. at 4 to 5°C, then centrifuged at 3000 g for 30 min. Supernatant was collected and centrifuged at 92,000 g for 2.5 hrs . The pellets were suspended in 2ml of 0.05M citrate buffer pH 7.5 containing 0.02M Na_2SO_3 for 4hrs at 4 to 5°C. Suspensions were pooled and given a second cycle of differential centrifugation. The final high speed pellet was suspended in

0.05M citrate buffer pH 7.5 containing 0.02M Na_2SO_3 at 4 to 5°C for 2hrs. Following a low speed centrifugation (3000 g for 5 min.), 2 ml virus suspension was layered on 10-40% sucrose density gradient. Rate zonal density gradient centrifugation was done according to Brakke 1967 (23) in columns prepared by layering 7, 7, 7 and 4 ml of sucrose solutions containing 400, 300, 200 and 100 grams of sucrose/ liter, respectively, dissolved in 0.01 M neutral phosphate buffer. The sucrose columns were allowed to stand overnight at 4 to 5 °C before use. The gradients over layered with virus suspension were centrifuged at 24,000 round per minute (rpm) in the swinging bucket number 27 rotor in Beckman L5-50 ultracentrifuge for 2 hrs. Density gradient columns were fractionated at 254 nm wavelength in 1.5 ml fractions using an ISCO density gradient fractionater. Fractions were dialyzed at 4 to 5 °C against 0.01 neutral phosphate buffer, scanned in Beckman D-25 spectrophotometer, and the absorbance of 260 nm of each fraction was recorded. Each fraction was then assayed for infectivity on *C. amaranticolor*. An equal amount of leaf tissue taken from non-infected squash plants of comparable age was processed as in the infected tissue included as a control. The purification procedure is illustrated in the following flow scheme:

Infected squash leaves (cv.Victoria) harvested 4 weeks after inoculation.

Precooled overnight at 4 - 5 °C



Serology and Antisera Production

A locally bred rabbit was used for the production of ZYMV- M specific antiserum. Normal serum was collected before the first injection, 0.25% sodium azide was added to the serum before it was stored at -15 °C.

The virus was dialyzed in 0.01M neutral phosphate buffer saline and emulsified with Freunds incomplete adjuvent in 1:1 v/v ratio . The rabbit received three 2ml intramuscular injections of a purified virus suspension over a 15 days period. One week after the last injection, the animal was bled every 3- 4 days for a peof 45days. 10 ml of blood per bleeding was obtained and stored at room temperature for 1-2 hours to clot, which was then removed by a spatula. Antisera was obtained by centrifugation at 3000 g for 15 minutes after which serum was pipetted and precipitated red blood cells were discarded. Antisera were mixed with 0.25 % sodium azide as a biocide and stored at - 15 ^{O}C.

Antisera titer was determined using the ouchterlony agar double diffusion test, Antisera was diluted 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024 using normal saline solution . Ouchterolony agar double diffusion tests were done as described by Purcifull (14) in autoclaved Noble agar solution solidified in plastic Petri dishes. Media was made of 0.8% purified agar and 0.5% sodium lauryl sulfate. The mixture was dissolved in boiling disttled water. After the addition of 0.1% sodium azide, hot solution was poured in the Petri dish and allowed to cool.

Squash leaves infected with the melon isolate of ZYMV and healthy antigens were tested against sera to ZYMV-melon isolate. Crude antigens were prepared from freshly harvested leaves by grinding 1gm/ml of 0.01M neutral phosphate buffer followed by the addition of 1ml of 3% SDS. The samples were pressed through cheesecloth and placed in the peripheral wells

surrounding the antiserum central well. Serology plates were stored at room temperature under humid conditions.

Sample collection and virus isolation

Leaf samples of different cultivated cucurbits showing mosaic symptoms and of symptomless *Molucella leavis* were collected from different locations representing all cultivated areas in Jordan at different dates during 1989 and 1990. All samples were tested for the presence of virus(es) using the following differential host range described previously by Al-Musa 1989 (20) including: *C. pepo* cv. victoria, *Ranunculus sardous, Lavatera trimestris* and *Nicotiana glutinosa.* Indicator plants were dusted with 6000 mesh carborundum. Inoculum was prepared by grinding infected leaves using sterilized mortor and pestile, the grinding medium was 0.01M neutral phosphate buffer containing 0.01M DIECA and Cystein (D+C). Inoculation of test plants was done by forefinger at the cotyledonary stage. The plants were observed 3-4 weeks for symptom development. All tested plants were back indexed on *C. pepo* cv. victoria 30 days after inoculation.

Virus isolates: Ninety virus isolates were identified as ZYMV according to the differential host range mentioned previously. Initial differentiation of the isolates was done based on the symptoms produced on the second true leaf of *C. pepo* cv. Victoria as described by Al-Musa 1989 (6). Four isolates (65-89, 57-89, 51-89 and 46-89) were selected to represent the remaining isolates. The selected isolates were passed through *Ranunculus sardous* and maintained in squash *C. pepo* cv. Victoria for subsequent studies.

Response of selected host plants:

One to several plant introductions in eight plant species belonging to cucurbitaceae, in addition to two *Chenopodium* spp. were inoculated with 46-89, 51-89, 57-89 and 65-89 isolates to investigate their response to them. Plants to be tested were grown in plastic pots under green house conditions. At the cotyledonary or the first true leaves stage, plants were dusted with 6000 mesh carborundum and mechanically inoculated. Samples from inoculated tip leaves of all test plants were separately back indexed on *C. pepo* cv. Victoria.

Aphid transmission: The tested aphids were collected from various host plants in different locations. Cultures of *Myzus persicae*, *Aphis gossypii*, *Aphis fabae* and *Aphis craccivora* were reared on radish, squash, black night shade and fababean plants respectively, in the green house.

In all aphid transmission experiments, non-virulefirous apterous adult aphids were transferred with camel's hair brush to a Petri-dish, starved for 1 hour and given an aquisition feeding period of 10 minutes. Five aphids were transferred to each of 10 healthy plants of squash seedlings. After inoculation feeding period of five minutes, the aphids were killed with an insecticide and the plants were placed in the green house for observation of symptoms.

Serology: The ZYMV isolates were tested serologically against ZYMV-melon isolate antiserum using ouchterolony agar double diffusion tests. Plates for these tests were prepared by dissolving 0.8% purified agar in distilled water containing 1% sodium azide and 0.5% SDS, as described by Purcifull, 1979 (14). Virus antigens were prepared from squash seedlings infected with the four isolates by grinding the tissue with 0.01M neutral phosphate buffer (1 g/ ml) diluted with an equal volume of 3% SDS.

The indirect enzyme linked immunosorbent assay (ELISA) was employed in serological tests for the differentiation between the four isolates of ZYMV. Antisera for the melon isolate of ZYMV and antisera for the cylindrical inclusion bodies of ZYMV-CT were used in ELISA tests.

Indirect ELISA was done as modified by Al-Musa, 1989 (6). The infected squash leaves inoculated with isolates 65-89, 57-89, 51-89 and 46-89 were macerated at 1:10 (w/v) dilution in grinding buffer PEP with 0.45% diethyl dithiocarbamate (DIECA). ELISA plate wells were separately charged with 0.25 ml sap extract. Healthy extract and phosphate buffer saline-Tween (PBS-Tween) were used as controls. The plate was then incubated for 3 hours at room temperature. After the sap extract was discarded, the plate was washed with PBS-Tween three times each for 3 minutes and the wells were then charged with 0.25 ml antiserum diluted in PEP buffer to 1:1600. The plate was incubated 3 hours at room temperature, after which the antisera was discarded and the plate was washed 3 times with PBS-Tween. 0.25 ml of Goat-anti rabbit conjugate diluted to 1:5000 in PEP buffer were added to wells that had been coated with specific antisera for ZYMV-melon isolate or the cylindrical inclusion bodies of ZYMV-CT. respectively. The plate was incubated 3 hours at 37^{o}C, wells were washed with PBS-Tween and 0.2 ml of substrate was added. The substrate was prepared by dissolving 0.1 gm. of p-nitrophenyl phosphate disodium in 100ml of substrate buffer. Results of the reaction were taken 30 minutes after adding the substrate visually or by using ELISA reader EIA-TEK model 308. The results were considered possitive if the substrate color turn to yellow as compared to control wells, or if the absorbance values at 405nm were twice more than that of the values in control wells under similar conditions.

Results

Purification of Zucchini yellow mosaic virus (ZYMV-M).

Determination of the proper time of harvesting infected squash tissue:

The relative infectivity of infected tissue was greatest in leaves harvested four weeks after inoculation and decreased slowly thereafter (Figure 1). Leaves harvested five weeks after inoculation contained about 90% of virus concentration present in leaves harvested four weeks after inoculation. Squash leaves harvested four weeks after inoculation were to be used for purification of ZYMV-melon isolate.

Comparison of infectivity of fresh, precooled and frozen tissue:

The relative infectivity was significantly higher when infected squash tissue was precooled overnight at 4 to 5C compared to that of the control treatment (Table 1). Only 54% of the control relative infectivity was retained after a one week freezing period. Therefore precooled infected tissue was used in the purification of ZYMV-melon isolate.

Effect of buffers on infectivity of ZYMV-melon isolate:

The relative infectivity was significantly higher when infected squash leaves were triturated in 0.01M phosphate buffer, pH 8.5 containing 0.005M EDTA, 0.02M Na_2SO_3 and 0.01M DIECA, compared to that of other treatments (Table 2). Consequently, this buffer was used for extraction of squash infected tissue. Addition of 0.005M EDTA, 0.02M Na_2SO_3 and 0.01M DIECA and cysteine, resulted in a significant increase in the relative infectivity of ZYMV-melon isolate in most cases.

Furthermore, a significant increase in relative infectivity was observed when the pH of phosphate buffewith 0.01Mand 0.05M was increased

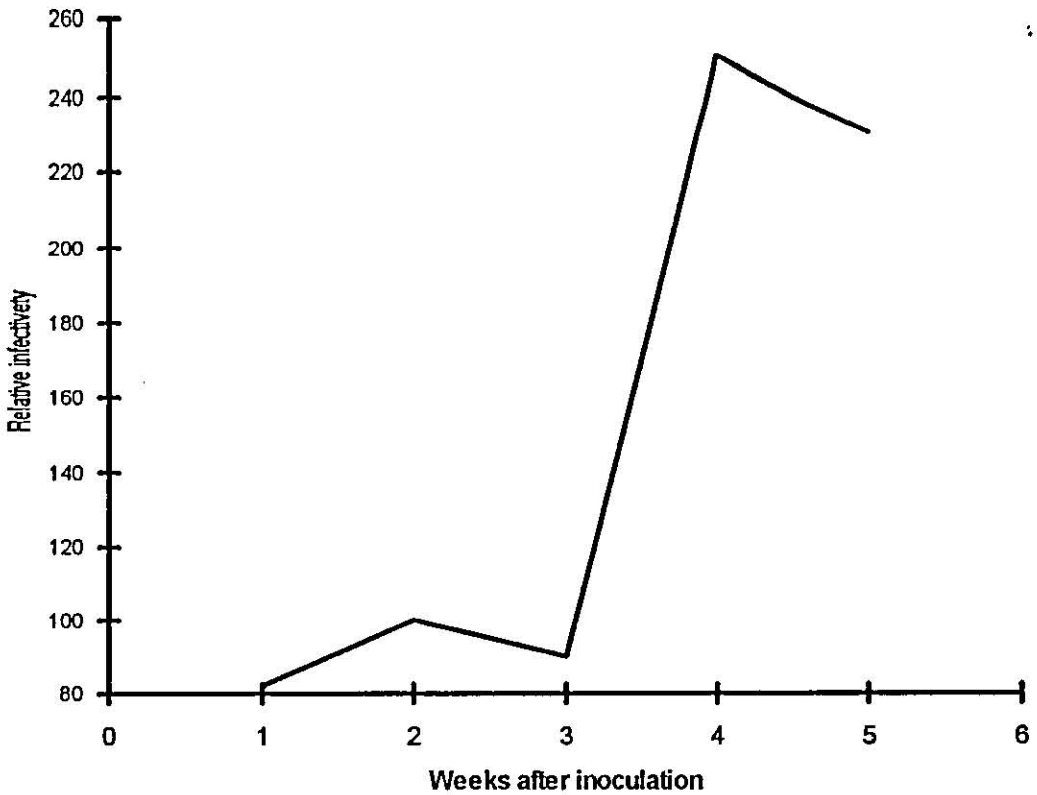


Fig 1: Relative infectivity of ZYMV-M infected squash leaves harvested at different times after inoculation.

Table 1: Relative infectivity of ZYMV-M in sap extracted from fresh, precooled and frozen infected squash tissues harvested four weeks after inoculation.

Treatment	Relative infectivity (a)
Precooled tissue	185a (b)
Frozen tissue	54b
Fresh tissue (Control)	100b

(a) : Relative Infectivity was expressed as a percent ratio of the total number of local lesions produced by the control treatment applied on the opposite half leaves of each treatment.

(b) : Means followed by same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

Table 2: Effect of different buffers on infectivity of ZYMV-M in infected squash crude sap.

Buffer	Relative infectivety(a)
Phosphate buffer 0.01M pH 7.5	110.5d (b)
Phosphate buffer 0.01M pH 7.5 containing 0.02M Na_2SO_3 +0.01M DIECA + 0.005M EDTA	91.7f
Phosphate buffer 0.01M pH 8.5	120.2bc
Phosphate buffer 0.01M pH 8.5 containing 0.02M Na_2SO_3 + 0.01M DIECA + 0.005M EDTA	150.2a
Phosphate buffer 0.05M pH 7.5	88.4f
Phosphate buffer 0.05M pH 7.5 containing 0.02M Na_2SO_3 + 0.01M DIECA + 0.005M EDTA	118.0c
Phosphate buffer 0.05M pH 8.5	120.8bc
Phosphate buffer 0.05M pH 8.5 containing 0.02M Na_2SO_3 + 0.01M DIECA + 0.005M EDTA	125.3b
Phosphate buffer 0.5M pH 7.5	00.0i
Phosphate buffer 0.5M pH 7.5 containing 0.02M Na_2SO_3 + 0.01M DIECA + 0.005M EDTA	1.44i
Phosphate buffer 0.5M pH 8.5	00.0i
Phosphate buffer 0.5M pH 8.5 containing 0.02M Na_2SO_3 + 0.01M DIECA + 0.005M EDTA	5.3i
Citrate buffer 0.01M pH 5.6	52.7h
Citrate buffer 0.01M pH 5.6 containing 0.01M (D+C)	108.7d
Borate buffer 0.01M pH 8.5	81.0g
Borate buffer 0.01M pH 8.5 containing 0.01M (D+C)	122.0bc
Control (Distilled water)	100.0e

(a): Relative infectivity was expressed as a percent ratio of the total number of local lesions produced by the control treatment applied on the opposite half leaves of each treatment.

(b): Means followed by same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

from 7.5 to 8.5, regardless of the presence of 0.005M EDTA, 0.02M Na_2SO_3 and 0.01M DIECA. On the other hand, relative infectivity was decreased significantly when the ionic strength of phosphate buffer increased to 0.5M (Table 2).

Using borate buffer with 0.01M, pH 8.5 and citrate buffer with 0.01M, pH 5.6 with or without additives significantly reduced the relative infectivity compared with 0.01M phosphate buffer, pH 8.5 containing 0.005M EDTA, 0.02M Na_2SO_3 and 0.01M DIECA (Table 2).

Effect of clarification treatments:

The relative infectivity was significantly higher when the extract of infected squash leaves was emulsified with 1:1 (v/v) ratio of chloroform, compared to other treatments (Table 3). Furthermore, this treatment resulted in the clearest supernatant collected after the first low speed centrifugation (3000 g for 30 minutes) and the smallest pellet of the first high speed centrifugation (92,000 g for 2.5 hours). Consequently this treatment was used for clarification of tissue extracts. High relative infectivity was obtained when the tissue extract was emulsified with a mixture of chloroform and carbontetrachloride. Chloroform was used for clarification of ZYMV-melon isolate because it gives the highest relative infectivity and it resulted in removal of large amount of host material.

Freezing of the infected tissue, adjustment of tissue extract to pH 4.9, use of celite and butanol significantly decreased the relative infectivity (Table 3).

Selection of buffer for suspending high speed centrifugation pellets:

The highly significant relative infectivity of the supernatant collected from the first differential centrifugation was retained when high speed

Table 3: Effect of different clarification procedures on infectivity of ZYMV-M, low speed centrifugation (LSC) supernatant color, and high speed centrifugation (HSC) pellet size and color.

Treatment	LSC Supernatant Color	HSC Pellet Size	HSC Pellet Color	Relative[a] Infectivity
Freezing	green	large	dark green	46g [b]
Adjustment to pH 4.9	off white	small	off white	11.5h
Charcoal	green	large	green	179d
Celite	green	small	light green	7.3h
Chloroform	yellowish	small	yellow	600a
Butanol	light green	small	green	35g
Carbon tetrachloride	light green	small	light green	295c
Chloroform + Butanol	light green	small	light green	115e
Chloroform + Crabontetracloride	light green	small	light green	503b
Control	green	large	green	100f

(a) Relative infectivity was expressed as a percent ratio of the total number of local lesions produced by the control treatment applied on the opposite half leaves of each treatment.

(b) Means followed by same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

pellets were suspended in 0.05M citrate buffer, pH 7.5 containing 0.02M Na_2SO_3 (Table 4). Consequently this buffer was used as a pellet suspension medium. Addition of 0.1% mercaptoethanol to citrate and borate buffers seemed to decrease significantly the relative infectivity as compared with that of the same buffers but containing no mercaptoethanol. No significant differences were obtained when 0.5M urea was added to the mentioned buffers. Relative infectivity was significantly decreased when 0.5M urea was added to the phosphate buffer with 0.01M, pH 8.5 (Table 4).

Rate zonal density gradient centrifugation:

Rate zonal density gradient centrifugation of the concentrated clarified virus suspension gave four visible bands 12-15, 17-20, 27-30 and 32-35 mm below the miniscus of density gradient columns (Plate 1). There were only two visible bands 12-15 and 17-20 mm below the miniscus of density gradient columns apparently containing concentrated extracts from healthy squash tissue processed similarly (Plate 2).

An ISCO scanning pattern of a gradient containing purified ZYMV-melon isolate reveals the presence of four absorbtion peaks, wherease only two absorbtion peaks were observed in the scanning pattern of a gradient containing concentrated preparations from healthy squash tissue (Fig. 2). The first two peaks located close to the miniscus are likely to be host proteins and/or ribosomes as indicated by high 260/280 ratio and lack of infectivity. 260/280 ratio and infectivity indicated the third and fourth peaks contained virus particles.

Association of infectivity with the visible density gradient zones:

The optical density at 260nm and the infectivity of each fraction collected from sucrose density gradient columns containing purified virus are plotted in (Fig. 3). Fraction 11 corresponding to the lower visible band

Table 4: Relative infectivity of the first differential centrifugation supernatant using different pellet suspending buffers.

Suspending buffer	Relative infectivity (a)
Citrate buffer 0.05M pH 7.5 containing 0.02M Na_2SO_3	428.2a (b)
Citrate buffer 0.05M pH 7.5 containing 0.02M Na_2SO_3 and 0.1% mercaptoethanol	201.0bc
Citrate buffer 0.05M pH 7.5 containing 0.02M Na_2SO_3 and 0.5M Urea	284.6abc
Borate buffer 0.01M pH 8.5	371.0ab
Borate buffer 0.01M pH 8.5 containing 0.1% mercaptoethanol	109.0c
Borate buffer 0.01M pH 8.5 containing 0.5M Urea	254.2abc
Phosphate buffer 0.01M pH 8.5	417.0a
Phosphate buffer 0.01M pH 8.5 containing 0.1% mercaptoethanol	220.6abc
Phosphate buffer 0.01M pH 8.5 containing 0.5M Urea	179.6bc
Control (Distilled water)	100.0c

(a): Relative infectivity was expressed as percent ratio of the total number of local lesions produced by the control treatment applied on the opposite half leaves of each treatment.

(b): Means followed by the same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

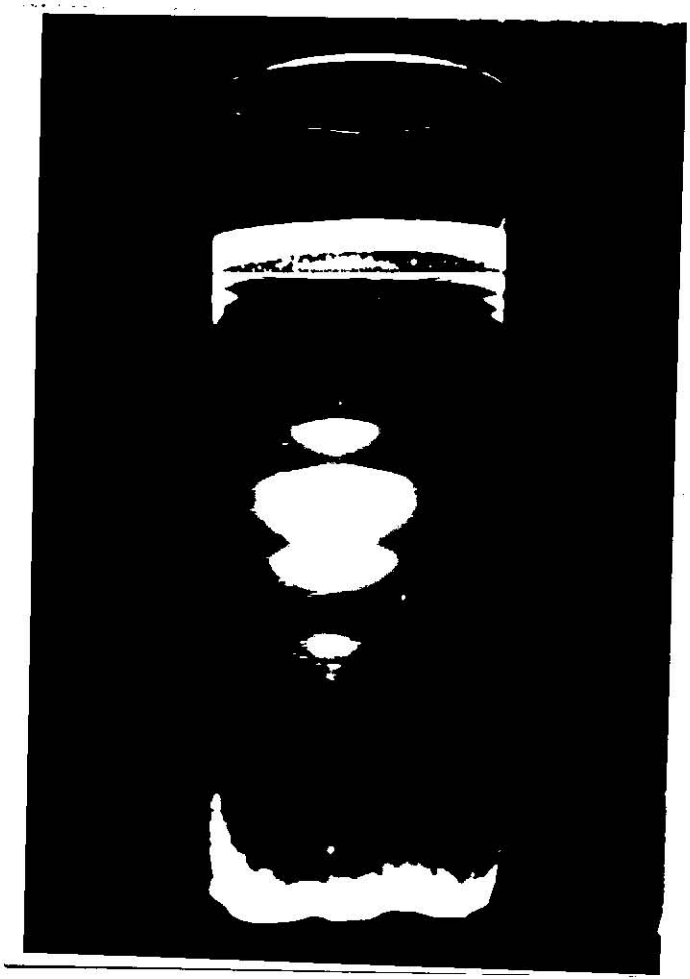


Plate 1: Rate-zonal sucrose density gradient tube containing purified ZYMV-melon isolate preparation.

Plate 2: Rate-zonal sucrose density gradient tube containing purified preparation from healthy tissue.

27-30 below the miniscus, infectious. produced typical systemic symptoms of ZYMV-melon isolate on squash and typical chlorotic spots on *C. amaranticolor*. Some infectivity was obtained from zones below the 27-30 mm zone (Fig. 3), which indicated virus particle aggregation upon purification.

The virus suspension obtained from the 27-30 mm band was dialyzed against 0.01M phosphate buffer saline and used for production of ZYMV-melon isolate specific antiserum.

Light absorbtion properties:

The purified virus suspended in 0.01M neutral phosphate buffer had an ultraviolet absorbtion spectrum typical of a nucleoprotein, with a maximum and minimum at 260 and 245 nm, respectively (Fig. 4). The 260/280 ratio (uncorrected for light scattering) ranged from 1.17 to 1.35. A value of 1.28 (the average of ten readings) was obtained, suggestive of a low nucleic acid content, around 6% as expected for an elongated viruses.

Serology:

Homologous titer of ZYMV-melon isolate specific antiserum and heterologous (against host components) titer were 1:16 and 1:4, respectively, as determined by the SDS-double diffusion test. The maximum homologous titer was obtained 6 weeks after the initial injection, no reaction was observed in periferal wells containing normal serum. Precipitin lines were observed between the ZYMV-melon isolate specific antiserum and crude virus preparations (Plate 3). On the otherhand, no precipitin lines were observed between the normal serum and virus preparations.

A faint precipitin line was observed between the ZYMV-melon isolate specific antiserum and host components (Plate 3). When the specific

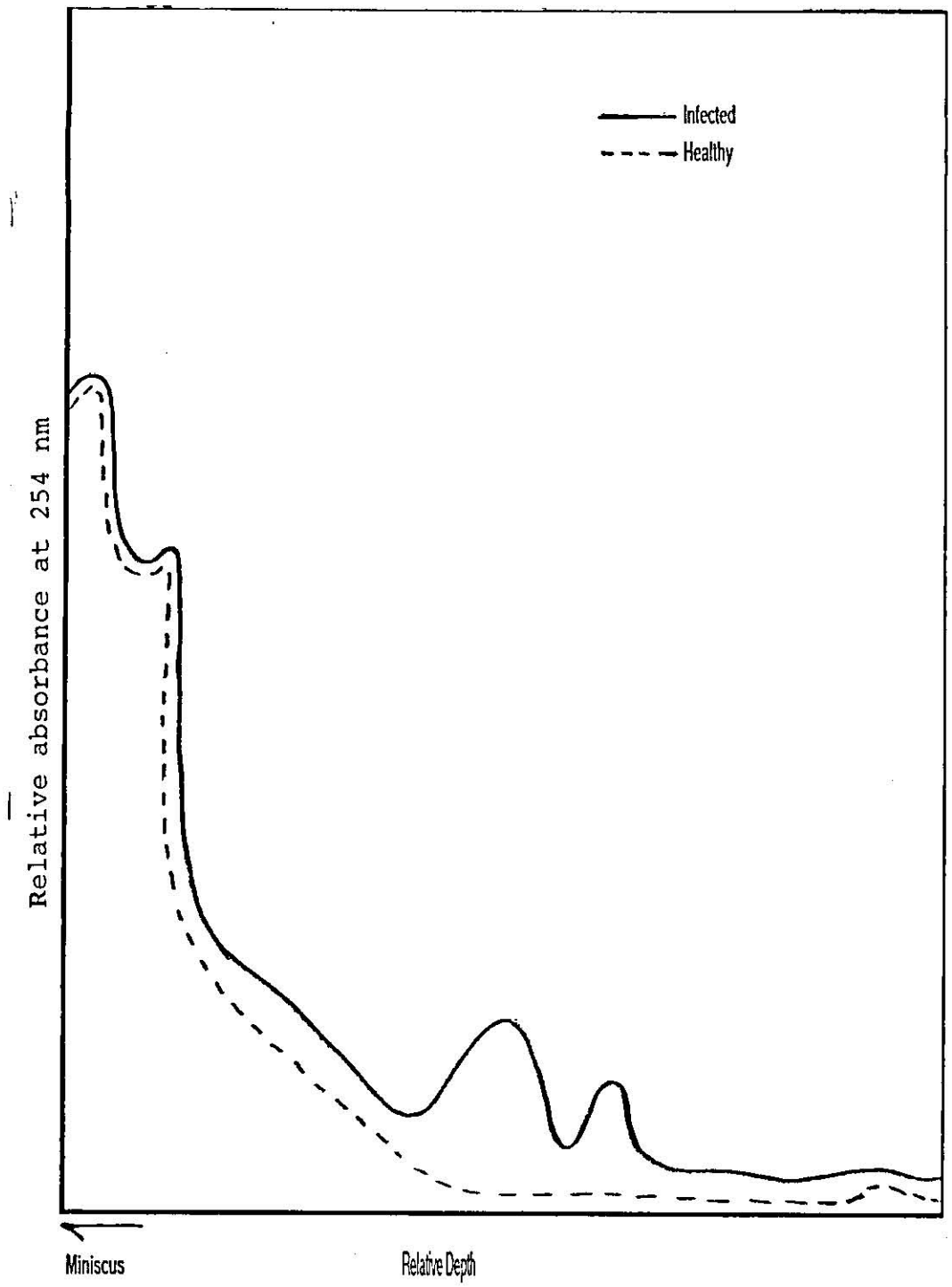


Figure 2: ISCO scanning of a typical rate zonal sucrose density gradient tube on which partially purified ZYMV-melon isolate or concentrated healthy preparation had been centrifuged at 103,000 g for 2 hours.

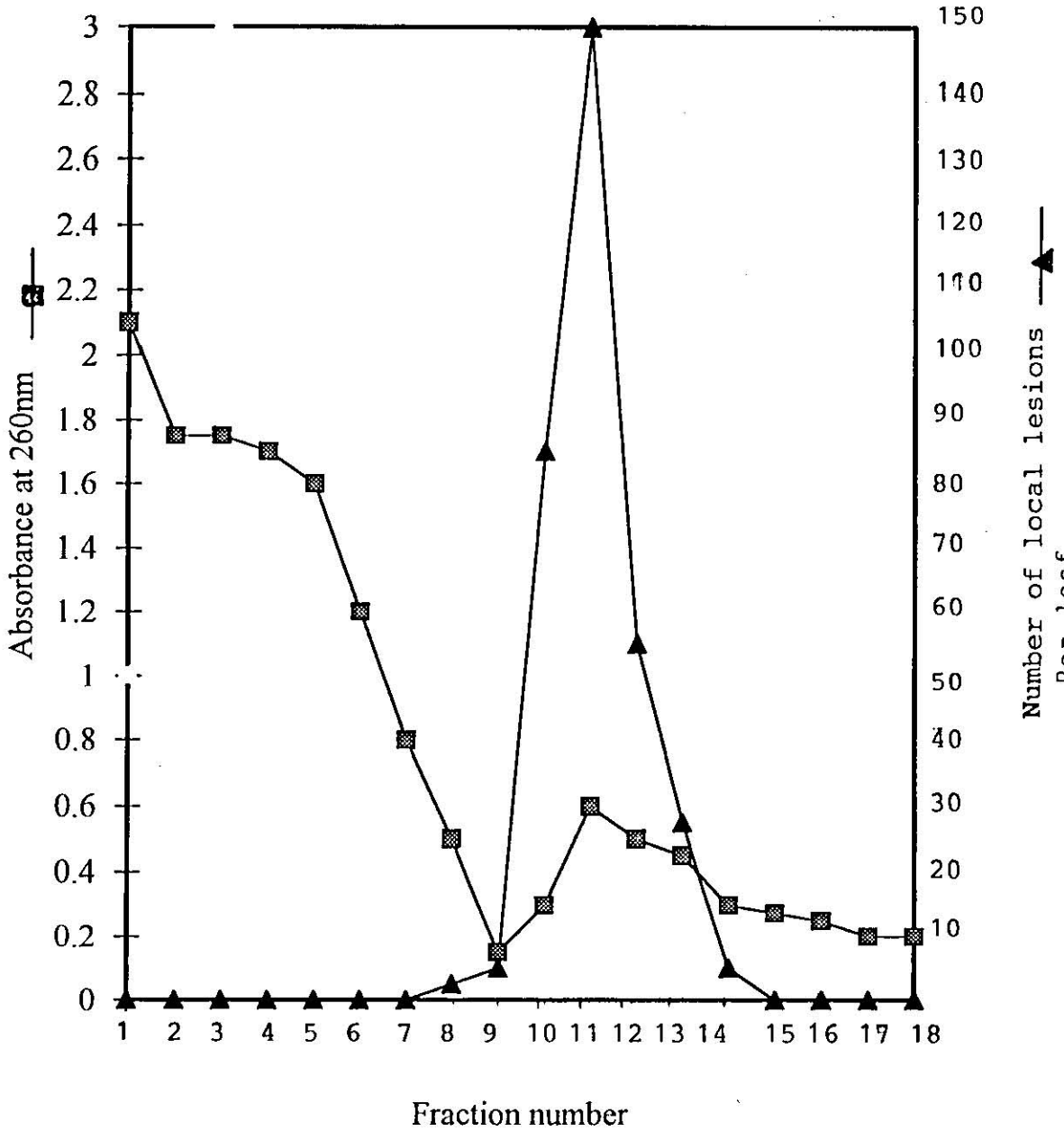


Figure (3): Association of infectivity with the visible bands of ZYMV-M which had been centrifuged on rate zonal sucrose density gradient.

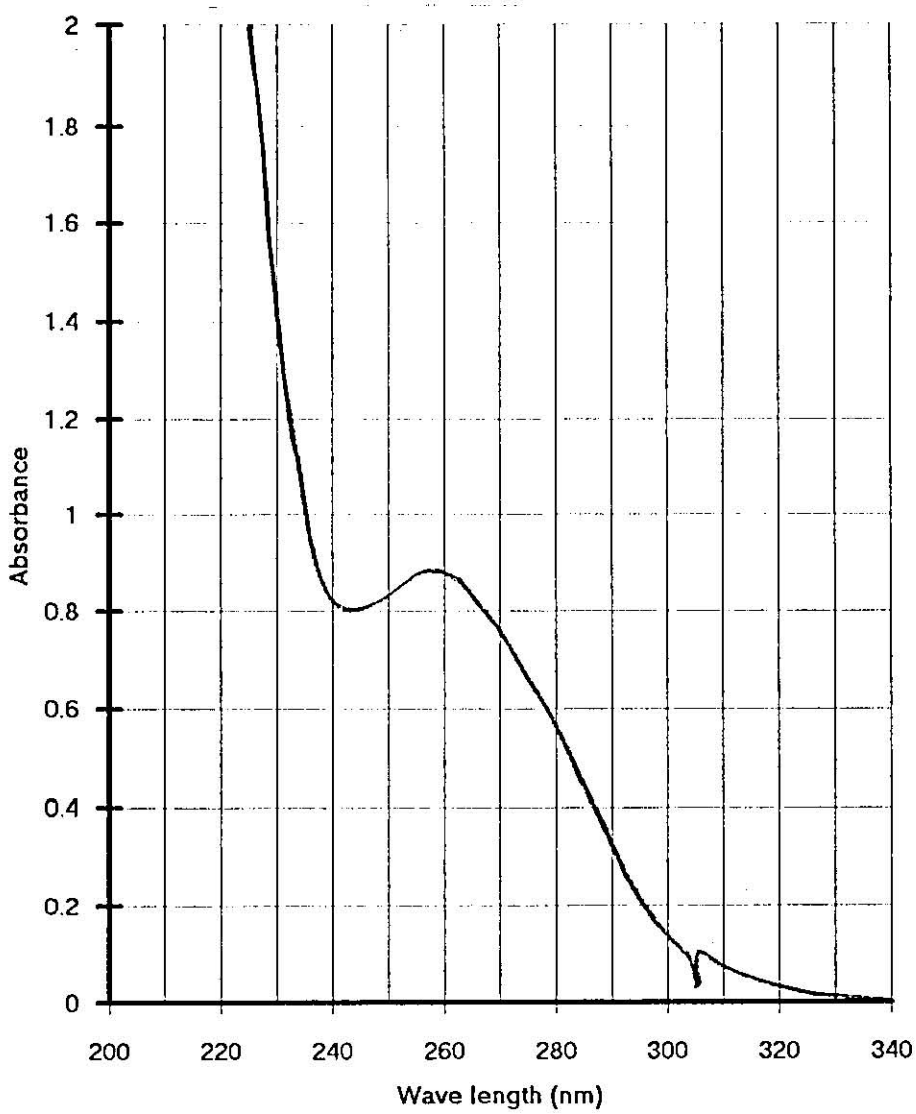


Figure 4 : Absorbtion spectrum of purified preparation of ZYMV-M .

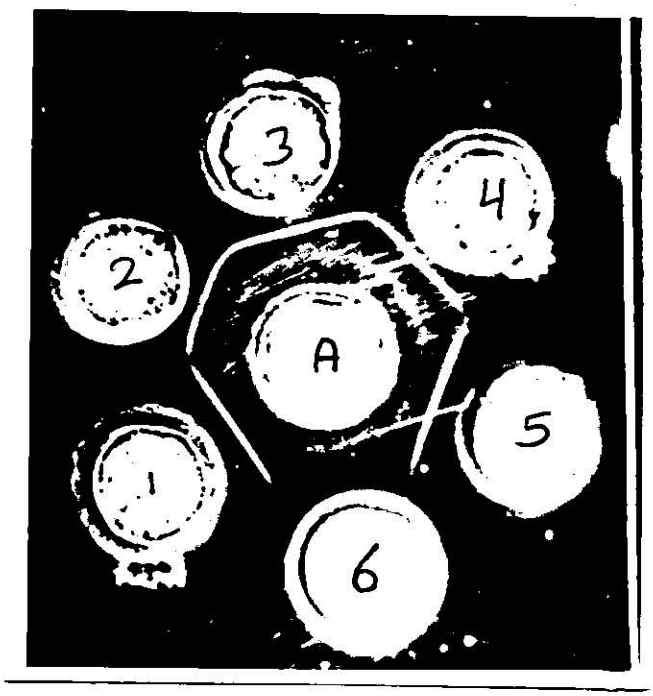


Plate 3: Serological test in agar ouchterlony agar-gel diffusion test.

A: central well contain homologous antisera for ZYMV-melon isolate.

Peripheral wells 1, 2, 3, 4 and 5 contain sap from ZYMV-melon isolate antigen, 6 contain healthy squash.

الصفحة غير موجودة من أصل المصدر

Table 5: Origin, symptoms description on the origin plant and reaction of four isolates of ZYMV on *Cucurbita pepo* cv. Victoria.

Isolate number	Origin of the isolate	Symptoms description	Symptoms on the second true leaf of *C. pepo* cv. Victoria				Notes
			First isolation	Second isolation	Third isolation	Fourth isolation	
46-89	Pumpkin	Mosaic and motling	M, GVB	M, GVB	M, GVB	M, GVB	
51-89	Snake cucumber	Yellowing, Green blisters	M	M	M	MM	*
57-89	*Moluccella leavis*	Symptomless carrier	Mo, LD	Mo, LD	Mo, LD	Mo, LD	
65-89	Snake cucumber	Mosaic, Green blisters	M, GB	M, GB	M, GB	M, GB	

M: Mosaic, GVB: Green vein banding, MM: Mild mosaic, Mo: Motling, LD: Leaf deformation, GB: Green blisters.

***: Mild mosaic symptoms appeared on the second true leaf of *Cucurbita pepo* cv. Victoria after three times of subculturing, suggestive of accidental contamination with mild mutant of ZYMV induced by nitrous acid in the virology laboratory by Dr. A. Al-Musa. The nitrous acid mild isolate was active at the time of the work.**

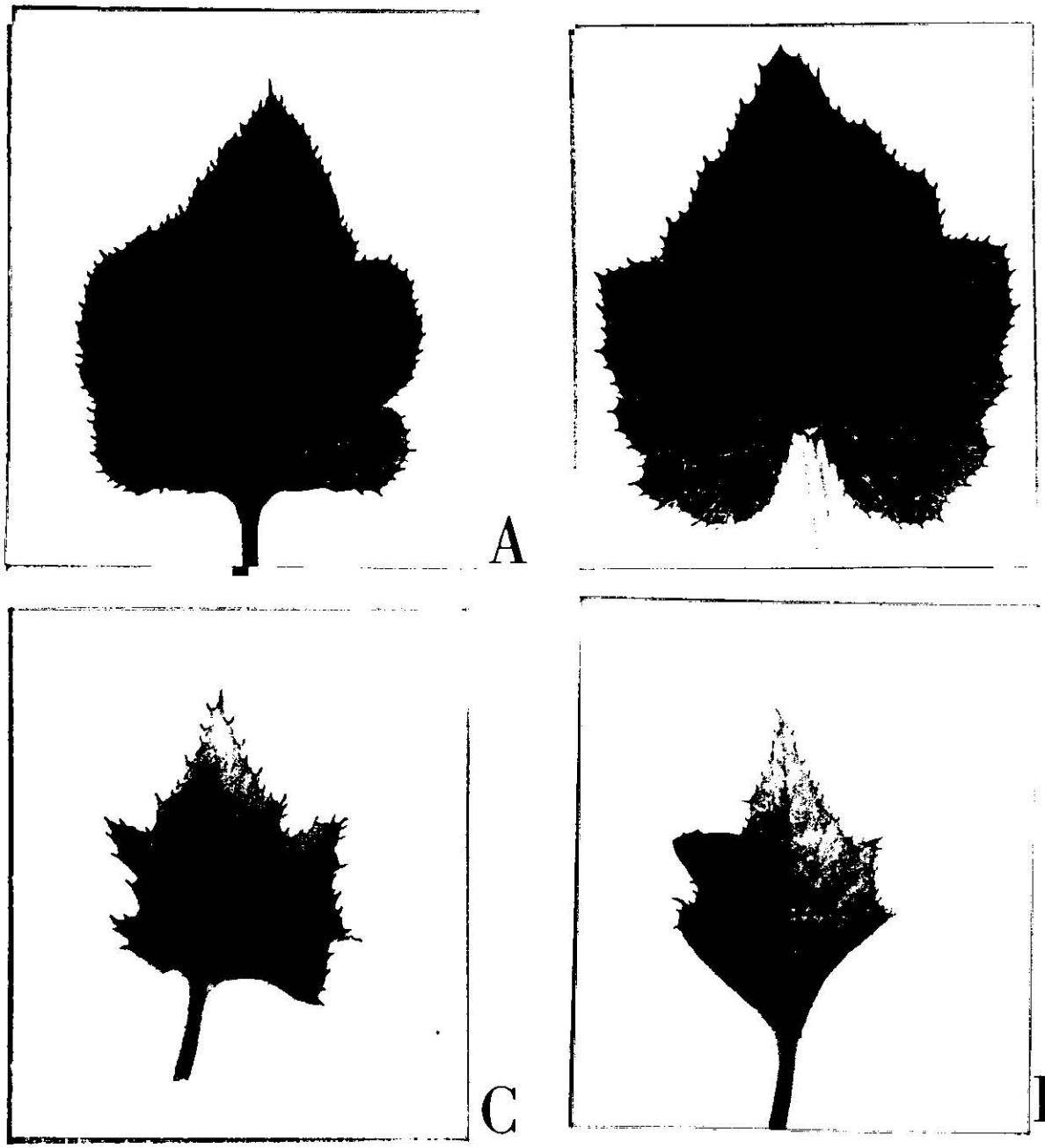


Plate 4: Symptoms of the four isolates of ZYMV on the second true leaf of *Cucurbita pepo* cv. Victoria. A: 46-89 isolate, B: 51-89 isolate, C: 57-89 isolate and D: 65-89 isolate.

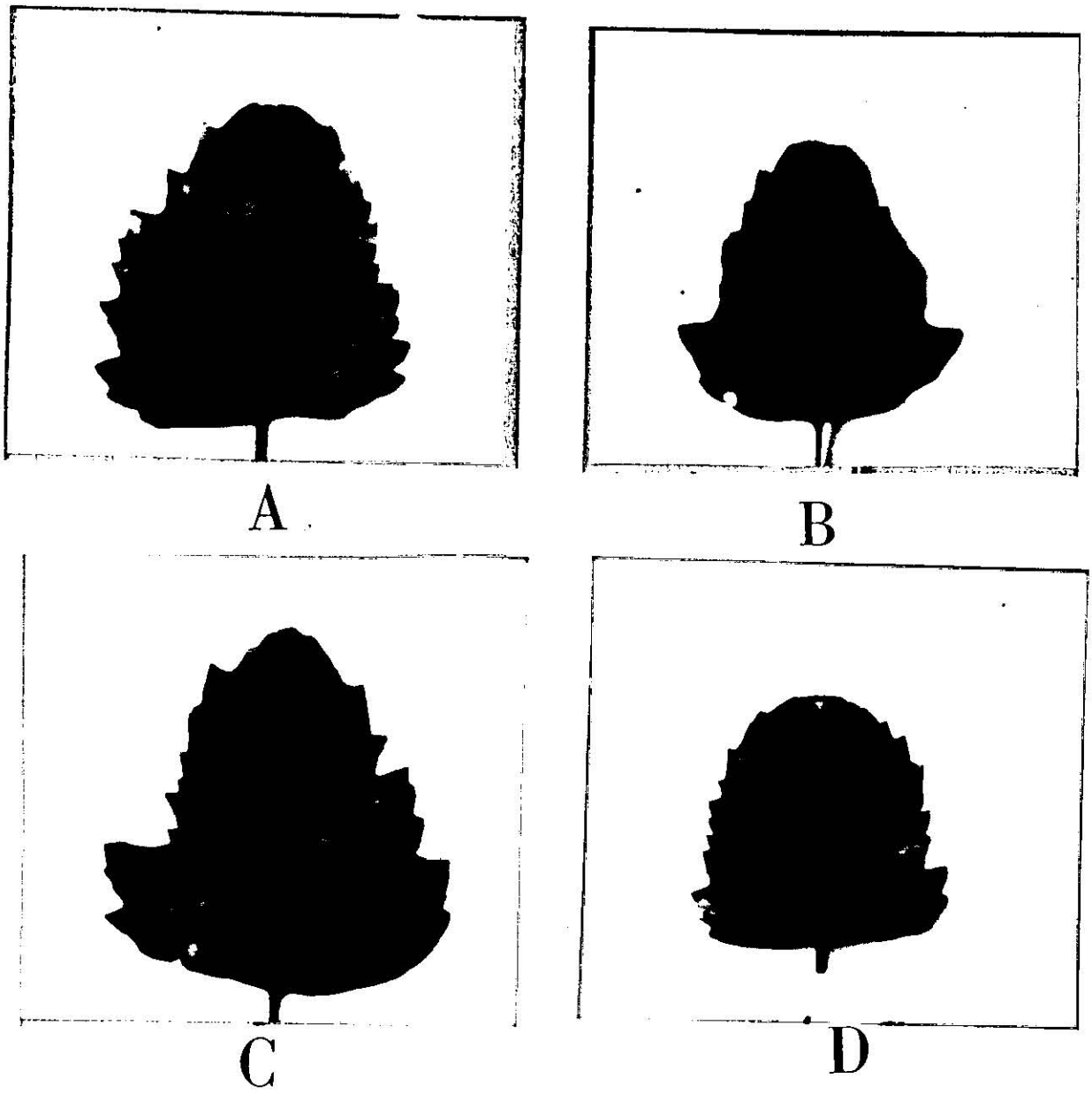


Plate 5: Symptoms of the four isolates of ZYMV on *Chenopodium quinoa*.
A: 46-89 isolate, B: 51-89 isolate, C: 57-89 isolate and D: 65-89 isolate.

and 438543, *Cucurbita martinizii* PI's 513099 and 512103, *Cucurbita moschata* PI 211993, *Cucurbita sorroria* PI's 512211, and 512227, and *Cucurbita texana* PI 7932 reacted with mosaic symptoms. (Table 6).

Plant species which were not infected and virus could not be recovered from both inoculated and tip leaves, included *Cucurbita ecuadorensis* PI 432445, *Cucurbita martinizii* PI's 438698 and 512106, *Cucurbita moschata* PI 438764 and *Cucurbita sorroria* PI 512218 of cucurbitaceae family (Table 6).

51-89 isolate: *C. quinoa* (Plate 5,Table 6) developed faint chlorotic local lesions on inoculated leaves 7 to 10 days after inoculation with no virus recovered from tip leaves. The isolate produced systemic mosaic on the second true leaf of inoculated *C. pepo* cv. Victoria plants (Table5), after three times of subculturing of this isolate on *C. pepo* cv. Victoria , infected plants showed systemic mild mosaic symptoms on the second true leaf of that host (Plate 4, Table 5). It produced systemic mild mosaic on tip leaves of inoculated *C. argyrosperma* PI's 442207, 442223, *C. moschata* PI 381818 and *C. texana* PI 7932, symptoms appeared 10 to 15 days after inoculation (Table 6). No visible symptoms were observed on *C. argyrosperma* PI's 442236 and 512115, *Cucurbita moschata* PI's 165575, 196925 and 211993 but upon back indexing of both inoculated and tip leaves they proved to be infected and virus was isolated (Table 6).

Plant species which were not infected and virus could not be recovered from both inoculated and tip leaves, included *C. amaranticolor* from chenopodiaceae family, *C. ecuadorensis* PI 432445, *C. lundelliana* PI's 438542 and 438543, *C. martinizii* PI's 438698, 512099, 512103, and 512106, *C. moschata* PI 438764, *C. sororria* PI's 512199, 512211, 512212, 512218 and 512227 from cucurbitaceae family (Table 6).

57- 89 isolate: *C. amaranticolor* and *C. quinoa* developed chlorotic local lesions on inoculated leaves and virus could not be recovered from tip leaves in these plants (Plate 5,Table 6). The isolate produced systemic motling and leaf deformation on the second true leaf of inoculated *C. pepo* cv. Victoria (Plate 4,Table5). It produced systemic motling and leaf deformation on *C. argyrosperma* PI's 442207, 442223 and 512115, *C. moschata* PI's 165575, 196925, 211993, and 381818 . Symptoms appeared 10 to 15 days after inoculation (Table 6). *Cucurbita argyrosperma* PI 442236, *Cucurbita martinizii* PI's 512103 and 512106, *Cucurbita moschata* PI 438764, *Cucurbita sororia* PI's 512211, 512212, 512218 and 512227, and *Cucurbita texana* PI 7932 produced motling 10 to 15 days after inoculation (Table 6). *C. sororria* PI 512199 was symptomless carrier whereas *Cucurbita martinizii* PI 512099, reacted with systemic chlorotic spots about two weeks after inoculation (Table 6).

Plant species which were not infected and virus could not be recovered from both inoculated and tip leaves included *C. ecuadorensis* PI 432445, *C. lundelliana* PI's 438542 and 438543, and *C. martinizii* PI 438698 from cucurbitaceae family (Table 6).

65-89 isolate: *C. amaranticolor* and *C. quinoa* developed chlorotic local lesions on inoculated leaves and virus could not be recovered from tip leaves in these plants (Plate 5,Table 6) . The isolate produced systemic mosaic and raised green blisters on the second true leaf of the inoculated *C. pepo* cv. victoria (Plate 4,Table5). It produced systemic mosaic, green blisters and filiform leaves on *C. argyrosperma* PI's 442207, 442223, 442236 and 512115, and *C. moschata* PI's 165575, 211993, and 381818. Symptoms appeared 10 to 15 days after inoculation (Table 6). while only mosaic symptoms appeared on *Cucurbita martinizii* PI's 438698, 512099, 512103 and 512106, *Cucurbita moschata* PI's 196925 and 381818 and

Cucurbita texana PI 7932. *Cucurbita moschata* PI 438764 and *Cucurbita sororia* PI's 512199, 512211, 512212, 512218 and 512227 producsystemic and vein clearing. Symptoms appear 10 to 15 days after inoculation (Table6).

Plant species which were not infected and virus could not be recovered from both inoculated and tip leaves include *C. ecuadorensis* PI 432445 and *Cucurbita lundelliana* PI's 438542 and 438543 (Table 6).

Aphid transmission:

The four isolates of ZYMV (46-89, 51-89, 57-89 and 65-89) were efficiently transmitted by *Myzus persicae* and *Aphis gossypii* in the non-persistant manner (Table 7). However, the transmission rate of 65-89 isolate by the two aphid species was at least twice that of the other isolates(46-89, 51-89 and 57-89). Using *Aphis fabae* and *Aphis craccivora*, transmission rates of 46-89, 57-89 and 65-89 isolates were less than those of the same isolates when *Myzus persicae* and *Aphis gossypii* were used (Table 7).

Aphis fabae and *Aphis craccivora* failed to transmitt 51-89 isolate (Table 7).

Serology:

In SDS-immunodiffusin tests, isolates 46-89, 51-89, 57-89 and 65-89 reacted with ZYMV-melon isolate specific antiserum and antiserum to the cylindrical inclusions induced by ZYMV-CT. (Connecticut strain) by forming precipitin lines which fused without spuring.

In indirect ELISA tests, the four isolates 46-89, 51-89, 57-89 and 65-89 reacted with ZYMV-melon isolate specific antiserum. 65-89 isolate gave the highest absorbance value at 405 nm wave length compared with those of 57-89 and 46-89 isolates (Table 8).

Table 6: Symptoms on plant species infected with 46-89, 51-89, 57-89 and 65-89 isolates.

Plant species	Symptoms of plant species infected with:			
	46-89	51-89	57-89	65-89
Chenopodium amaranticolor	CLL	NI	CLL	CLL
Chenopodium quinoa	CLL	FCLL	CLL	CLL
Cucurbita argyrosperma				
PI 442207	M, GVB	MM	Mo, LD	M, GB, FL
PI 442223	M, GVB	MM	Mo, LD	M, GB, FL
PI 442236	M, GVB	SC	Mo	M, GB, FL
PI 512115	M, GVB	SC	Mo, LD	M, GB, FL
Cucurbita ecuadorensis				
PI 432445	NI	NI	NI	NI
Cucurbita lundelliana				
PI 438542	M	NI	NI	NI
PI 438543	M	NI	NI	NI
Cucurbita martinizii				
PI 438698	NI	NI	NI	M
PI 512099	M	NI	SCS	M
PI 512103	M	NI	Mo	M
PI 512106	NI	NI	Mo	M
Cucurbita moschata				
PI 165575	M, GVB	SC	M, LD	M,GB, FL
PI 196925	M, GVB	SC	M, LD	M
PI 211993	M	SC	M, LD	M,GB, FL
PI 381818	M, GVB	MM	M, LD	M,GB, FL
PI 438764	NI	NI	Mo	M, VC
Cucurbita pepo cv. Victoria	M, GVB	MM	Mo, LD	M, GB, FL
Cucurbita sororia				
PI 512199	M, GVB	NI	SC	M, VC
PI 512211	M	NI	Mo	M, VC
PI 512212	M, GVB	NI	Mo	M, VC
PI 512218	NI	NI	Mo	M, VC
PI 512227	M	NI	Mo	M, VC
Cucurbita texana PI 7932	M	MM	Mo	M

CLL: chlorotic local lesion. NI: not infected. FCLL: faint chlorotic local lesion. M: mosaic. GVB: green vein banding. MM: mild mosaic. GB: green blisters. Mo: motling. LD: leaf deformation. SC: symptomless carrier. VC: vein clearing. SCS: systemic chlorotic spots. FL: filiform leaves.

Table 7 : Transmission efficiency of 46-89, 51-89, 57-89 and 65-89 isolates by four aphid species.

Aphid species	46 89		51 89		57 89		65 89	
	(a)	(b)						
Myzus persicae	4/10	40%	3/10	30%	5/10	50%	8/10	80%
Aphis gossypii	2/10	20%	1/10	10%	3/10	30%	7/10	70%
Aphis fabae	1/10	10%	0/10	0 %	1/10	10%	4/10	40%
A. craccivora	2/10	20%	0/10	0 %	1/10	10%	2/10	20%

(a): Number of infected plants / Total number of tested plants.

(b): Percent ratio of infected plants.

Using antiserum to the cylindrical inclusions of ZYMV-CT, the absorbance values at 405 nm for 46-89 , 57-89 and 65-89 isolates were higher compared with those of the same isolates when ZYMV-melon isolate specific antiserum was used (Table 8).

No difference was noticed in the absorbance values of 51-89 isolate at 405 nm wave length when ZYMV-M specific antiserum and antiserum to the cylindrical inclusions of ZYMV-CT were used (Table 8).

Table 8 : Serological comparison between 46- 89, 51- 89, 57- 89, and 65- 89 isolates, in indirect ELISA tests.

Isolates	Absorbance[a] at 405 (nm) ± (SE)[b]	
	ZYMV melon isolate antiserum	ZYMV-CT (conn. antiserum)
46- 89	0.892±0.016	1.272±0.028
51- 89	0.105±0.006	0.144±0.022
57- 89	1.206±0.105	1.520±0.072
65- 89	1.421±0.041	2.075±0.108
healthy control	0.089±0.006	0.048±0.002
buffer control	0.014±0.003	0.029±0.007

a: Mean absorbance (A_{405}) value of each isolate was obtained from five replicates.

b: Standard error of the mean.

Discussion

The highest infectivity of ZYMV-melon isolate was obtained in infected squash leaves harvested four weeks after inoculation then decreased slowly (Fig. 1).Concentration of unstable viruses rises to a peak, usually two to three weeks and some times four weeks after inoculation and then falls (10,29). This decrease in virus concentration may be due to decrease in virus replication in the old leaves as a result of a decrease in the enzymatic activity in these leaves (29).

The high infectivity level in extract of precooled squash leaves is likely to be due to the inhibition or decreased polyphenol oxidase activity at low temperature. Freezing of infected squash leaves before extraction had a harmful effect on the virus infectivity. The presence of high active virus inhibitors like polyphenol oxidase enzymes, which are known to inactivate viruses (23,30,31) explain the low relative infectivity of ZYMV-melon isolate in fresh leaves(Table 1).

Aggregation is a very critical problem in purification of most elongated viruses especially those in the potyvirus group (21,31). Purification of ZYMV-melon isolate was complicated by virus particle aggregation, as indicated by infectivity of zones below 27-30 mm zone (Fig. 3). Decreased infectivity of ZYMV-melon isolate by high ionic strength buffers, such as 0.5M phosphate buffer, is likely to be due to increased virus particle aggregation and decreased virus stability possibly due to decraesed net negative charge of virus particles or rapid replacement of polyvalent cations like Mg^{+2} associated with the virus particles by the monovalent cations in the buffer such as K^{+}. (23).

Differential centrifugation seemed efficient in partially purifying ZYMV-melon isolate. Rate zonal sucrose density gradient centrifugation

was tried initially to separate virus particles from contaminating host materials (5).

Rate zonal density gradient centrifugation at 24,000 rpm for tow hours gave a definite light scattering zone at 27-30 mm below the miniscus (Plate 1), which is likely to be a concentrated virus particles as indicated by the infectivity of fraction number 11 representing the mentioned zone (Figure 3), a lower degree of light scattering was visible at depths below that zone (Plate 1) combined with the infectivity of fractions 12, 13 and 14 (Figure 3), indicating a slight aggregation of virus particles. Fraction 11 containing the higher concentration of purified virus particles suspended in 0.01M neutral phosphate buffer had an ultraviolet absorbtion spectrum typical of a nucleoprotein, with a maximum and minimum at 260 and 245 nm , respectively (Figure 4). The 260/280 ratio (uncorrected for light scattering) has a value of 1.28 which suggested a low nucleic acid content, as expected for an elongated viruses (32).

About ninety virus isolates have been recovered from cultivated cucurbits showing mosaic symptoms and from symptomless *Molucella leaves*. The isolates were represented by four isolates (46-89, 51-89, 57-89, and 65-89) according to the symptoms produced on the second true leaf of *Cucurbita pepo* cv. Victoria (Table 5).

46-89 isolate produced mosaic and green vein banding, 51-89 isolate produced systemic mosaic, after three times of subculturing of the isolate on *C. pepo* cv. Victoria, the infected plants showed systemic mild mosaic symptoms on the second true leaf of that particular host (Table 5), suggestive of accidental contamination with mild mutant of ZYMV induced by nitrous acid in the virology laboratory by Dr. A. Al-Musa, that was active at the time. 57-89 isolate produced motling and leaf deformation while 65-89 isolate produced mosaic and raised green blisters (Plate 4). The

four isolates were identified as zucchini yellow mosaic virus (ZYMV) on the basis of symptomology and host range . The four isolates infect *Ranunculus sardous* with no visible symptoms and fail to infect *Nicotiana glutinosa,* unlike the cucumber mosaic virus (CMV) that infect *Nicotiana glutinosa* and *Ranunculus saradous* with no visible symptoms (20). They also failed to infect *Lavatera trimestris* opposite to watermelon mosaic virus (WMV-2) (20). Identification was further confirmed by serological test, the four isolates reacted with ZYMV- melon isolate antiserum and antisera produced for the cytoplasmic inclusions of ZYMV-CT using SDS-immunodiffusion test and indirect ELISA (Table 7).

Isolates 46-89 and 65-89 seemed to be closely related as results showed in (Table 6) whereby both isolates produced severe symptoms on *Cucurbita argyrosperma* PI's 442207, 442223, 442236 and 512115. This was also evident on *Cucurbita moschata* PI's 165575, 196925 and 211993 whereby isolate 46-89 and 65-89 showed severe symptoms on the same lines. While isolates 51-89 and 57-89 produced mild symptoms on the same lines in comparison with isolates 46-89 and 65-89. Isolate 46-89 can be differentiated on the host *Cucurbita lundelliana* PI 438543 since it produces leaf mosaic, while isolates 51-89, 57-89 and 65-89 can not infect this line. Isolate 51-89 can be differentiated using the host *Cucurbita martinizii* PI 512103 since it does not infect this host while the remaining isolates produce prominent mosaic on the same host. Isolate 57-89 can be differentiated using *Cucurbita martinizii* PI's 512103, 512106 and 438698 since it infects PI's 512103 and 512106 producing leaf mottling but does not infect PI 438698. Isolate 65-89 can be differentiated using the host *Cucurbita martinizi* PI 438698 since it infects this line producing leaf mosaic while isolates 46-89 51-89 and 57-89 do not infect this line (Table6).

It is known that ZYMV is transmitted by aphids in a nonpersistant manner (5). In this study 51- 89 had a low transmission rate (Table 7). The relatively poor transmission of 51- 89 isolate may have been attributed to lower titer in squash when the virus source plants were used two weeks after inoculation (12).

Previous reports indicated serological heterogeneity of ZYMV strains (6). In SDS- immunodiffusion tests, the four isolates of ZYMV tested appeared indistinguishable. however, the absorption values at 405 nm wave length of the four ZYMV isolates tested against antiserum for the cylendrical inclusions of ZYMV-CT were significantly different from those of the same isolates when ZYMV-M specific antiserum was used (Table 8).

ZYMV is one of the most destructive plant pathogens infecting cucurbit crops in many areas of the world. In this context, cross protection appeared to be an attractive alternative approach to control ZYMV (4). It has been shown that in squash, it can be effectively controlled in Taiwan (3) and France (4) using the mild strain ZYMV- WK.

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Appendix 1: Analysis of variance (ANOVA table) of data presented in table 1.

S.V	df	SS	MS	Fcal.	pr>F
Total	23	132927.9			
Treatment	2	71102.1	35551.0	12.08	0.0003
Error	21	61825.8	2944.0		

S.V: source of variation.

d.f : degrees of fredum

SS : sum of squares

MS : Mean square

Fcalc.: calculated F value

Appendix 2: Analysis of variance (ANOVA table) of data presented in table 2.

S.V	df	SS	MS	Fcal.	pr>F
Total	67	166069.2			
Treatment	16	165393.6	10337.1	780.32	0.0001
Error	51	675.6	13.2		

S.V: source of variation.

d.f : degrees of fredum

SS : sum of squares

MS : Mean square

Fcalc.: calculated F value

Appendix 3: Analysis of variance (ANOVA table) of data presented in table 3.

S.V	df	SS	MS	Fcal.	pr>F
Total	39	1604907.1			
Treatment	9	1602318.1	178035.3	2062.9	0.0001
Error	30	2589	86.3		

S.V: source of variation.

d.f : degrees of fredum

SS : sum of squares

MS : Mean square

Fcalc.: calculated F value

Appendix 4: Analysis of variance (ANOVA table) of data presented in table 4.

S.V	df	SS	MS	Fcal.	pr>F
Total	39	1003197.7			
Treatment	9	503765.6	55973.9	3.36	0.0059
Error	30	499432.1	16647.7		

S.V: source of variation.

d.f : degrees of fredum

SS : sum of squares

MS : Mean square

Fcalc.: calculated F value

الملخص

تنقية ودراسة جزئية لخصائص فيروس تبرقش واصفرار الكوسا في الأردن

إعداد

ناصر محمد أحمد عبد الله

إشراف

أ .د. عبد الله محمد الموسى

خصصت هذه الدراسة لتنقية فيروس تبرقش الكوسا واصفراره ودراسة خصائصة الجزئية لزيادة المعرفة حول امكانية وجود عزلات مرضية أخرى من الفيروس في الاردن. وأجريت عدة تجارب أولية لمعرفة الطريقة المخبرية المثلى للحصول على معلق نقي من هذا الفيروس. وبناءا على نتائج هذه التجارب وجد أن طحن أوراق الكوسا المصابة بمنظم الحموضة، فوسفات البوتاس (١غ:٣مل) ثم إضافة حجم متساو من الكلوروفورم بنسبة (١:١) حجم الى حجم الى ذلك المحلول لعمل مستحلب. وقد تم استخدام جهاز الطرد المركزي لكسر المستحلب وترسيب الفيروس، وتبع ذلك فصل الفيروس باستخدام طريقة التدريج الكثافي.

استخدم المعلق النقي من الفيروس لحقن أرنب محلي للحصول على مصل يحتوي على أجسام مضادة لهذا الفيروس، ويستخدم هذا المصل للكشف السريع عن وجود الفيروس سالف الذكر في النباتات المصابة لتعريف العزلات المرضية الاخرى من الفيروس وامكانية التفريق بينها باستخدام الفحوصات المصلية كما هو وارد في الدراسة.

وتم بهذه الدراسة تعريف ٩٠ عزلة شملت عينات من نباتات القرعيات المختلفة، التي تظهر أعراض التبرقش وعينات من عشبة أجراس بيت لحم *Molucella leavis* التي لا تظهر أعراضا للمرض وذلك في سنة ١٩٨٩ ، وعرفت جميع العزلات على أنها فيروس تبرقش واصفرار الكوسا بناءا على نتائج العدوى بمدى عائلي مبدئي معرف سابقا ودعمت نتائج التعريف بالفحوصات المصلية لتثبت ان جميع العزلات هي فيروس تبرقش واصفرار الكوسا، وقد امكن تمييز اربع مجموعات من الاعراض الظاهرة على الورقة الحقيقية الثانية من نبات الكوسا صنف فكتوريا، واخذت عزلة ممثلة عن كل مجموعة (٨٩/٤٦ ، ٨٩/٥١، ٨٩/٥٧ و ٨٩/٦٥). وأظهرت الدراسة وجود فروقات واضحة بين العزلات الاربعة من حيث استجابة أنواع مختلفة من النباتات للاصابة بهذه العزلات وكفاءت انتقال هذه العزلات بواسطة انواع محددة من حشرة المن إضافة إلى التفاعلات مع الامصال.