Molecular Analysis of Cucumber Mosaic Cucumovirus Symptoms Development on Squash Plants

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Abstract: A wide range of severe symptoms were appeared on inoculated squash plants (Cucmus pepo cv. El-Skandrani) with CMV under greenhouse condition. One of the first signs of systemic CMV infection, is vein clearing in the youngest leaves (about 7 days), the veins become translucent and leave produced subsequently showed a mosaic (about 10 days) severe mosaic and mottling (about 15 days). Then changed in leaves growth form i.e. little, malformation and no-Lamina giving shoestring the so-called fern leaf (about 20-25 days). The virus was transferred from each symptom to squash and chenopodium amaranticolor plants by sap mechanical inoculation then conformed by DBIA-assay. The change in chlorophyll contents to ensure that all these symptoms resulted from CMV-s EG. SDS-PAGE, peroxidase isozyme separation and RAPD-PCR to molecular analyze of S-CMV-EG symptoms development on squash plant leaves. SDS-PAGE of protein separation showed variability protein pattern and contents of healthy and S-CMV symptoms infected squash leaves (m-mosaic, S-mosaic, crinkling and malformation) with 24, 26, 28, 25, 20 and 22, 21, 20, 22, 16 soluble and unsoluble polypeptides respectively. As well as. DISC-PAGE isozyme showed 6, 7, 8 and 8 peroxidase isozymes respectively. RAPD-analysis revealed DNA polymorphic among CMV-symptom development on squash plants. RAPD analysis using two random primers revealed 8 polymorphic of total 15 amplified fragments with 53% under CMV infection. Crinkle symptoms revealed the highest number with 18 markers followed by S-mosaic 17 and malformation 16 and mild mosaic with 15 bands. [Journal of American Science 2010;6(8):94-103]. (ISSN: 1545-1003).

Key words: Squash plants, CMV symptoms development, SDS-PAGE, DISC-PAGE, PAPD PCR.

1. Introduction

Cucumber mosaic cucumorius is widely distributed in different crops in Egypt and has been reported as a main problem for squash crop production in open field and protected agriculture besides causing severe losses in cucurbit crops (Staniulus et al., 2000). The virus showed several types of symptoms including, vein clearing, mild and severe mosaic blisters, green vein banding and chlorotic local lesion followed by vein netting, yellow often necrosis and plant death, leaf distortion, crinkle and malformation, and filformshape (Gomaa, 2008). Therefore, it was important to define the etiology of this virus by symptomatology, biology, serology and protein related index tests.

Leal and Lastra (1984) revealed that the reduction in chlorophylls, soluble proteins and nitrogen contents was evident in leaves infected by tomato yellow mosaic virus that causes severe symptoms. The subcelular distraction of virus stimulates the synthesis of soluble proteins which were detected by SDS-PAGE. The most prominent as judged by the intensity of their staining, is the enhancement of 14.3, 20, 39, 58 and 97 kDa proteins (Hadidi, 1988; El-Dougdoug, 1996 and Sherif and El-Habbaa, 2000). Certain host proteins were increased dramatically as part of a general physiological response in infection by viruses and other pathoens (Comacho-Henriquez and Sanger, 1982). Viral infection with yellow leaf carl, leaf roll, mosaic, curl, crinkle and malformation leaves of tomato and squash exhibited higher activity of peroxidase polyphenol oxidase and esterase (Tag El-Din et al., 2006).

The present study aims to the study the chemical and molecular variability of CMV symptoms development in infected squash plants, using, SDS-PAGE, DISC-PAGE and RAPD-PCR.

2. Material and Methods

Virus source:

Cucumber mosaic cucumoviridae CMV-s EG isolate was obtained from the Virology Lab, (Megahed, 2008). Agric. Microbiology Dept., Fac. of Agric., Ain Shams Univ. The virus isolate maintance on Nicotiana glutinosa.

Virus detection : In order to determine the presence of CMV in inoculated and symptoms squash plants, the goat antirabbit immunoglobuline-alkaline phosphtase conjugate (Sigma A 4503) was used by Dot blot immunoassay as described by Lin et al. (1995).

The seeds of squash (Cucurbita pepo cv, Eskandarani) and indicator plants for CMV-s EG

detection were kindly provided from Virology Lab., Fac. Agic., Ain Sham Univ., Cairo, Egypt.

The squash plants were grown into steam sterilized soil in pots under greenhouse conditions until the seedling stage (cotyledon leaf). The squash leaves were mechanically inoculated with CMV-s EG isolate prepared in 0.1 M phosphate buffer pH 7.4. The symptoms were recorded at 2-6 weeks and photographed after mechanical inoculation. The plants showed symptoms were indexed by Chenopodium amaranticolor as indicator host and also by DBIA assay.

Chlorophyll content :

Chlorophyll a, b and carotenoids were extracted and estimated according to Wettstein (1957) as mg/L.

Biochemical analysis :

Protein content was determined by Bradford (1976) using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis :

S odium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Extraction of soluble and non-soluble proteins were done according to Studier (1973).

Peroxidase (PRX) isozymes electrophoresis: Gel electrophoretic isozymes were performed according to Stegemann et al. (1985) among four symptoms leaves using one enzyme staining systems. Peroxidase revealed the most variables with good determined banding patterns.

Molecular analysis :

DNA extraction was performed by the method of Wulff et al. (2002).

DND amplification:

Random amplified polymorphic DNA (RAPD) analysis was applied according to Williams et al. (1990) using 10 mers of four random oligonucleotide primers obtained from (Metobion AG. Lena Christ Strasse, Martinsried, Deutschland) as shown in Table (1). The gels analysis was applied by programme (UVI geltec version 12.4, 1444-2005 USA).

Table 1. The four random primers used in DNA amplification

Primer name	Sequence (5`-3)
OPD-11	5`AGCGCCATTG3`
OPT-20	5`GACCAATGCC3`
2-19	5`GCACGGCGTT3`
2	5`AACGCGCAAC3`

Agarose gel electrophoresis :

PCR amplified products were analyzed using 1.2% agarose gel electrophoresis staining with ethidium bromate. The amplified DNA bands were visualized under UV light and the sizes of the fragments were estimated based on a DNA ladder of 100 to 2000 bp (manufactured by Bloron).

3. Results

Symptoms development :

Sequency of symptoms development on CMV infected squash plants (Cucurbita pepo cv. Eskandarani) showing signs of viral symptoms a wide range of severe symptoms were appeared on squash plants. One of the first signs of systemic infection is vein clearing in the youngest leaves (at 7 days), the vein become translucent and leaves produced subsequently showed a mosaic (10 days), severe mosaic yellowing, mottling (chlorosis) (20 days). Then changed in leave growth forms i.e. little, blisters, crikle, malformation and no-lamina giving shoestring (at 25-35 days). The so-called fern leaf. The symptoms were than developed into four main features on cultivated plants and thus they were classified into the following four categories:

1- The leaves were mild mosaic (Fig. 1-a); 2- The leaves were severe mosaic and blisters (Fig. 1-B). 3- The leaves were curling and narrow (Fig. 1-C) and 4- leaves showed reduction in blade and filiform or shoestring shapes (Fig. 1-D).

A number of eight plants infected squash plants showing viral different symptoms types were examined by dot blot immunoassays assay using specific polyclonal antibodies by DBIA. A purplish blue color was developed infected plants in positive reaction, whereas extracted from healthy plants remain green in the negative reactions (Fig. 2).

Another assay for viral detection and variability of symptom via local lesion diversity on Ch. amaranticolor by characteristic of morphological local lesions. It was observed variation in local lesions i.e. size, shape, colour without center and halo; chlorotic necrotic, fine and large (Fig. 1B).

Chloropyll and carotenoids :

Data presented in Table (2) showed that chlorophyll a and b were significantly decreased in infected plants showed mild and severe mosaic, where as curling and deformation plants didn't show significantly different from healthy plants. On the other hand carotenoids were not significantly changed.

Protein contents: was determined in squash leaves with individual symptoms elated to BSA (Table 2), it was revealed that, all the symptoms types due to increasing in total protein content with different values compared with healthy squash leaves.

Table 2. chlorophyll; carotenoids and protein contents in squash leaves at different periods of symptoms developments

Symptoms development / Chlorophyll* and protein contents	Symptoms development				
	Healthy	**m-Mosaic**	**S- Mosaic**	**Crinkling**	**Malformat-ion**
Chlorophyll-a	1.015	0.234	0.095	0.695	0.792
Chlorophyll-b	0.625	0.185	0.047	0.375	0.410
Carotenoids	0.412	0.425	0.445	0.495	0.485
Proteins **	7.87	4.76	5.44	6.37	6.23

* Photopigment : mg/g fresh.
** Protein content: mg/ml

SDS-PAGE proteins analysis :

SDS-PAGE profile of soluble proteins extracted from infected and uninfected squash leaves presented in Table (3) and Fig. (3). SDS-PAGE analysis revealed 32 bands with different molecular weights ranged from 275 to 10 as shown in Fig. (3).

The soluble protein bands of the four plants which revealed different symptom types were varied in number and density of bands, whereas mild mosaic, severe mosaic, crinkle and malformation were revealed the highest total number with 24, 26, 26, 25 respectively bands while healthy displayed the lower number with 20 bands.

The variability analysis of symptoms development showed some proteins bands disappeared in healthy for example 9, 7, 8 and 9 band for mild, severe mosaic, crinkling and malformed respectively (Table 2). The present of variability between symptoms development was 53% related to total bands.

The protein bands were appeared in both healthy and in under all symptoms types with 32 bands. Some other protein 17 bands were appeared under symptoms types and displayed in healthy. Some of these bands were appeared in both healthy and symptoms types such as bands number 15, 19, 18 and 16 protein bands respectively (Table 3).

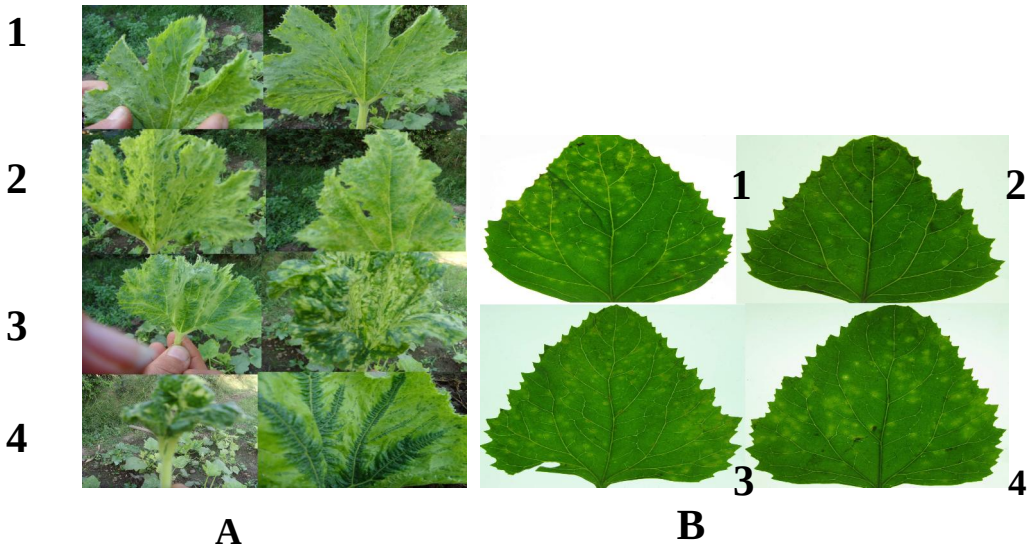

Fig. (1): Symptoms development on infected squash leaves by CMV, A. Systemic symptoms, B. Variability of local lesion on Ch. amaranticolor 1- mild mosaic; 2- severe mosaic, 3- crinkle and 4- malformation symptoms.

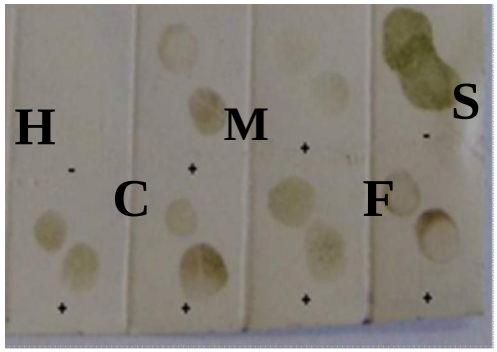

Fig. (2): Dot blot immunoassay for CMV detection in symptoms types on squash leaves against specific IgG-CMV polyclonal, H = healthy leaves, M = mild mosaic, S= severe mosaic, C = crinkle and F = malformation.

Table (3): SDS-PAGE analysis of soluble protein patterns of the variable the symptoms development related to CMV-squash inoculated.

Band no.	Healthy	Mild mosaic	Severe mosaic	crinkling	Malformation
275	-	+	+	-	+
195	-	+	+	-	+
161	-	+	-	-	-
104	+	+	+	+	+
92	+	+	+	+	+
85	+	+	+	+	-
74	-	-	-	+	+
68	-	+	+	+	+
63	+	+	+	+	-
59	+	-	+	+	+
58	+	+	-	-	-
55	+	-	+	+	+
52	+	+	+	+	+
50	-	+	+	+	+
46	+	+	+	+	+
40	-	+	-	+	-
38	+	-	+	+	+
36	+	+	+	+	+
34	+	+	+	+	+
33	-	-	+	+	+
31	+	+	+	+	+
28	+	+	+	+	+
26	-	+	+	-	-
24	+	-	+	+	-
22	+	-	+	+	+
20	+	+	+	-	+
19	+	+	+	+	+
18	+	+	+	+	+
17	-	+	+	+	+
14	-	-	-	+	+
11	+	+	+	+	+
10	-	+	-	+	+
Total bands	20	24	26	26	25

SDS-PAGE analysis revealed 26 bands with different molecular weight ranged from 238 to 20 kDa as shown in Fig. (3). The non-soluble protein of symptoms development were varied in number and density whereas 22, 21, 20, 22 bands of mild mosaic, severe mosaic, crinkling and malformation respectively, while healthy plant displayed the lower 16 bands.

SDS- profile of non-soluble proteins extracted from the infected plants with CMV and non-infected presented in Table (4). They were varied between symptoms types in numbers and density where as symptom types plants revealed increasing in protein profiles their healthy plants. The number increase was 6, 5, 4 and 6 bands for mild mosaic severe mosaic, crinkle and malformation symptom types respectively (Table 4 and Fig. 3).

On the other hand the newly induced bands under CMV infection 9 bands in symptoms types plants where disappeared in healthy plants.

The variability analysis of symptoms showed some bands disappeared in healthy and between symptoms plants. The percent of variability between symptoms plants was 48% related to total bands.

Peroxidase isozymes : Results of peroxidase isozymes are shown in Table (5) and Fig. (4). Each symptom types and healthy plants could be characterized by unique set. of isozymes. The total number of peroxidase isozymes shown in all symptoms types were 11 isozymes while those of each type 7, 8, 8 and 9 for mild mosaic severe mosaic, crinkling and malformation respectively as well as 7 bands for healthy ones. The variability between symptoms types was 60% related total peroxidase isozymes (Table 5).

Peroxidase isozyme (PRX) analysis displayed a total of 11 band whereas 5 of them were variable (polymorphic) and 5 (monomorphic) other bands among the different symptoms types of CMV-squash inoculated. As well as the control as presented in the zymogram (Table 5) and Fig. (4). The effect of CMV-EG strain infection could be observed among of 4 peroxidase bands, whereas bands No. 2, 5, 6 and 7 disappeared in the control (healthy) plants and appeared in some symptom types, on the other hand polymorphic band No. 1 appeared in healthy and symptom type 3; polymorphic band No. 2 appeared in symptoms type 1 and 4; polymorphic band No. 5 appeared in four symptoms types; polymorphic band No. 4 appeared in symptoms types 1, 2 and 4 and polymorphic band No. 6 appeared in symptoms types 3 and 4. However, 7 bands were appeared in healthy plants , 3 polymorphic bands No. 1 with symptom type 3 and No. 7 with symptoms types 3 and 4, as well as 3 monomorphic bands No. 3, 8 and 9 (Table 5 and Fig. 4).

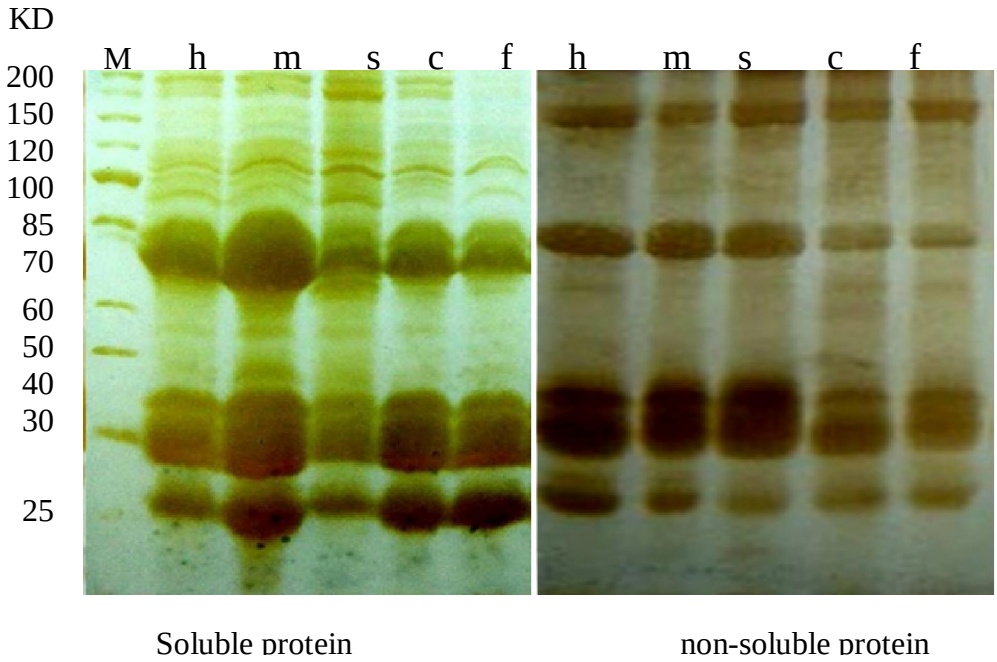

Fig. (3): SDS-PAGE (12%) of soluble protein (a) and non-soluble protein fractions extracted from the infected squash leaves with CMV. H = healthy leaves, m = mild mosaic, s = severe mosaic, c= crinkle and F = malformation.

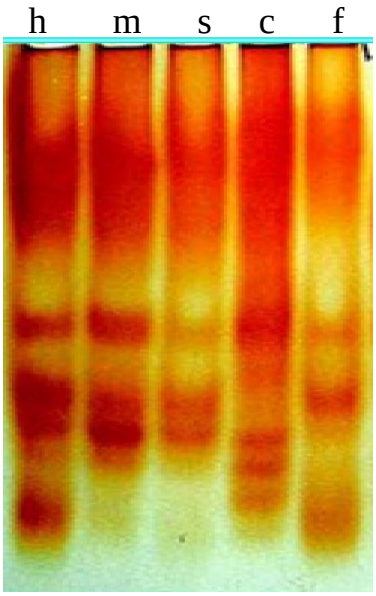

Fig. (4): Peroxidase (PRX) isozyme profiles of squash leaves infected with CMV. H = healthy leaves, m = mild mosaic, s = severe mosaic, c= crinkle and F = malformation.

Table (4):SDS-PAGE analysis of non-soluble proteins patterns of the variable the symptoms development related to CMV squash infection.

Band no.	Healthy	Mild mosaic	Severe mosaic	crinkling	Malformation
238	+	+	+	+	+
161	-	+	+	-	-
109	+	+	+	+	+
92	+	+	+	+	+
85	+	+	+	+	+
74	+	-	-	-	+
68	-	+	-	-	+
63	-	+	+	+	+
51	+	+	+	+	+
58	-	+	+	+	+
57	+	+	+	+	+
55	+	+	+	+	-
52	-	+	+	+	+
50	-	+	+	+	+
46	+	+	+	+	+
40	+	+	+	+	+
38	-	-	-	-	+
36	+	+	+	+	+
34	+	+	+	+	+
33	+	+	+	+	+
31	+	+	+	+	+
28	+	+	+	+	+
26	+	+	-	+	+
24	-	+	-	-	+
22	-	-	+	-	-
20	+	+	+	+	+
Total bands	16	22	21	20	22

Table (5): Peroxidase (PRX) isozymes analysis of the variable bands of the variable the symptoms development related to CMV squash infection

Band no.	Healthy	Symptoms development				Polymorphism
		m-mosaic	s-mosair	Crinkle	malformation	
1	+	-	-	+	-	Polymorphic
2	-	+	-	-	+	polymorphic
3	+	+	+	++	+	monomorphic
4	+	+	+	-	+	polymorphic
5	-	+	+	+	+	polymorphic
6	-	-	+	+	+	polymorphic
7	-	-	+	+	+	monomorphic
8	+	+	+	+	+	monomorphic
9	+	+	+	+	+	monomorphic
10	+	+	+	+	+	monomorphic
11	+	+	-	-	-	Polymorphic
Total bands	7	8	8	8	9	

Such new peroxidase bands may related to CMV infection in mild and severe symptoms types. In general, results in table (5) which represent the peroxidase enzyme polymorphism among the 4 symptoms types could be served as a model for analyzing the gene action and different mild and severe symptoms types.

RAPD analysis of CMV infected squash plants :

Four random primers, OPT-20, OPD-11, 2-19 and 2 were used in random amplified polymorphic DNA (RAPD) analysis in infected squash plants with CMV-s EG.

Primers OPT-20 and OPD-1 revealed 12 amplified fragments with sizes ranged from 690 to 190 bp. Whereas 8 fragments were polymorphic and 4 commonly detected among the symptom type 5 , plants with molecular size 690, 590, 340 and 380 bp. As well as the healthy squash plants revealed 16 amplified fragment (Table 6 and Fig. 5).

The healthy squash plants was varied considerably in the presence of amplified fragments where 14 fragments, thus m-mosaic, s-mosaic crinkle and malformation were 15, 16 and 18 and 16 amplified fragments respectively. On the other hand 4 amplified fragments appeared in healthy plants only and disappeared in infected plants, Table (6).

Table (6):RAPD analysis of symptom development on CMV-infected squash plants using RAPD primers

Molecular weight (bp)	**OPT-20**					OPD-11					Polymorphism
	H	**M**	**S**	**C**	**F**	**H**	**M**	**S**	**C**	**F**	
1045	+										Unique
920	+										Unique
865	+										Unique
765	+										Unique
690	+	+	+	+	+	+	+	+	+	+	Monomorphic
630	+++	+	+	++	++	-	-	-	-	-	Polymorphic
590	-	+	++	+++	+++	+	+	+	+	+	Monomorphic
415	+	-	++	+++	++++	-	-	-	-	-	Polymorphic
440	-	++	+++	+++	++++	-	-	-	+	-	Polymorphic
340	+	++	+++	++++	++++	+	+	+	+	+	Monomorphic
331	-	+++	+++	++++	++++	+	+	-	-	-	Polymorphic
320	-	+++	+++	++	++	-	-	-	-	-	Polymorphic
380	+	+	+	+	+	+	+	+	+	+	Monomorphic
240	-	+	++	+	+	-	-	+	-	-	Polymorphic
210	-	-	-	+	-	-	+	-	+	++	Polymorphic
190	-	-	-	-	-	-	+	+	+	+	Polymorphic
Total bands	9	9	10	11	10	5	6	6	7	6	

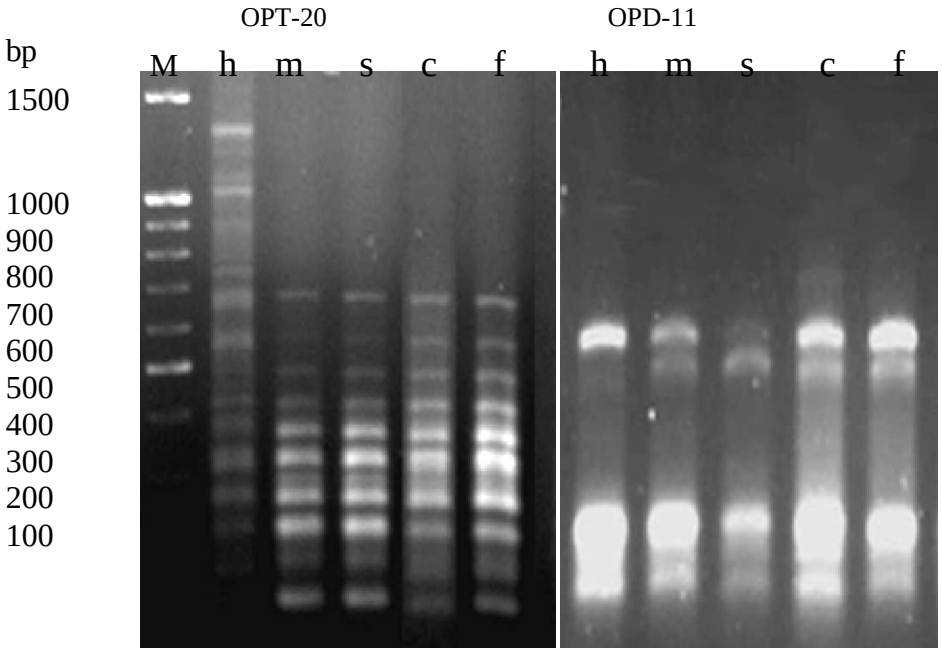

Fig. (5): RAPD amplified products of CMV-infected squash leaves using two primers. OPD-11 and OPT-20. H = healthy leaves, m = mild mosaic, s = severe mosaic, c= crinkle and F = malformation.

Moreover, the numbers of fragments of symptoms types were affected by different behaviour either by increase or by decrease compared with healthy plants. For contrast, the crinkle symptoms increased in 9 bands, S-mosaic (7) and malformation increased in 7 bands and m-mosaic increased 6 bands related healthy plants. Meanwhile, the crinkle symptoms type revealed induced amplified fragment that were nor existed either in the healthy or mild and severe mosaic and malformation symptoms (440 bp) and s-mosaic symptoms with 240 bp in OPD-11 RAPD primer. There bands resulted from RAPD-PCR amplified products could be used as RAPD markers for symptoms types against CMV infection in squash plants.

Table (7): Summarized results of biochemical and molecular variation among symptoms types of CMV-infected squash plants.

CMV symptom types	Photopigments			Proteins				RAPD primers		
	Chl a	Chl b	Carote-noid	Protein content	SP	NSP	PRX	OPD20	OPD11	Total M.M
Healthy plants	1.015	0.625	0.412	7.87	26	16	6	8	5	62
Mild-mosaic	0.234	0.185	0.425	4.76	24	22	7.	9	6	68
Severe mosaic	0.095	0.047	0.445	5.44	26	21	7	10	6	70
Crinkling	0.695	0.375	0.495	6.37	26	20	8	11	7	72
Malformation	0.792	0.410	0.485	6.23	25	22	8	10	6	71

Chl = chlorophyllNSP = non-soluble protein M.M = molecular markers SP = Soluble rotein
PRX = peroxidase isozymes

The results of biochemical and molecular variation for symptom types of CMV-infected squash plants were summarized in Table (7). Consequently the genetic analysis of CMV symptom types showed that crinkle was the highest biochemical and molecular markers (72) followed by malformation (71) severe mosaic (70) and m-mosaic (68) compared healthy with 62 molecular markers.

4. Discussion

The effects of viruses on plants are multifarious, practically and function of a plant may be disturbed. Symptoms will be named and briefly described. They may be grouped according to their effect on overall growth, colour, water content, tissue life span, shape of organs, plant anatomy and plant physiology. Most plant

virus diseases and viruses are named after their main symptoms in a particular host.

More obvious and better known are colour changes, especially of leaves. Mosaic disease has long been synonymous with virus disease. CMV-s EG mosaic of squash is one type of variegation as mentioned of tobacco and abutilon (Bos, 1983 and Mathews, 1991). Diffusely bordered variegation is often called mottling but there are various intermediates. The term vein mosaic (vein clearing) denotes an irregular mosaic along veins and vein-banding a regular one.

Actually decrease in photosynthetic rate of the infected leaves is often associated with development of the symptoms (Plat et al. 1979).

The effects are, of may be, related to changes within organs or entere plants, symptoms are variable and greatly depend on the host plant and how long it has been infected, the virus strain and the environmental conditions. For example, symptoms that are particularly noticeable in plants that have been growing in cool bright conditions since infection may become masked in host poorly-lit conditions. The time after infection before symptoms appear depends on the virus, the plant and the environmental conditions too; it is commonly a few days or weeks in herbaceous plants through it may be one or more years in woody ones.

Under greenhouse conditions the symptoms could be more clear and restricted. It should be independent of the virus and should reflect the host genetics. Symptoms of CMV disease on squash differed depending on the isolates as well as the environmental conditions (Makkouk et al., 1979 and Aref and El-Dougdoug, 1996). By comparing the data presented in this study of the viral symptoms development and mechanically transmitted with the results. CMV-s EG was detected biological and serological in infected squash plaints (Megahed, 2008), Abo El-Nasr et al. (2004), it was concluded that the three viral symptoms had ZYMV viral complex of one or more strains. Similar results were obtained for TYLCV-E (Aref and El-Dougdoug, 1996). Local lesions diversity of CMV produced n Ch. amaranticolor were used CMV identification and variability as well as isolation of CMV strains (Megahed 2008).

Protein isozymes, and DNA analysis should be independent of host genetics under virus inducer. Whereas, amino acid sequence of polypeptides are dependent on nucleotide sequence of their coding genes; therefore on electrophoretic analysis of protein and isozymes of CMV infected leaves which showing different symptoms approximates the analysis of there genetic variation. The variation of CMV symptom types was detected via determination of polypeptides by using SDS-PAGE and peroxidase isozymes using DISC-PAGE. Data presented in Table (2) indicated that the number of protein fractions increased in infected plants as recorded in severe mosaic followed crinkle and malformation than mild mosaic. Also this results indicated the CMV due to increasing protein content or certain protein fraction or both together. For example severe mosaic showed decrease in protein content but increase in protein fractions. This clearing that despite increasing the biodegradation of protein content new protein types were detected due to virus infection. The same observation was reported by (Hadidi 1988 and El-Dougdoug, 1996). The synthesis of new proteins are due to the host-virus interaction previous results also indicated that protein contents could be increased dramatically (Camacho-Henriquez and Sanger 1982) or decreased (Lead and Lastra, 1984).

The enzymatic pools and their metabolic pathways are the most important factors affecting pathogenicity especially with viruses. The results showed that the level of peroxidase in infects leaves of crinkle and severe mosaic were higher than those in healthy leaves. This high level play an important role in the defense mechanism. Increase in this enzyme activity and isozymes has been detected after infection by pathogens in different host pathogen combination (Hammeschmidt et al. 1982 and Sherif and El-Habbaa, 2000).

DNA prepared was found crucial for RAPD-PCR. The yields of DNA were determined of spectrophotometrically as, 11, 14, 15 and 13 µg/0.05 g fresh tissues. The PCR conditions for DNA analysis were optimized by investigated each factor individually, the optimized conditions were detailed in material and methods section. A total of scoroble amplified DNA fragments ranging in size 1045 to 190 bp were observed using the two primers were as polymorphic with 8 and 4 bp monomorphic fragments with detected among symptoms types. Interesting to note that, the four symptoms types were varied in there formed and developed. Where 15, 16, 18 and 16 for m-mosaic, S-mosaic, crinkle and malformed symptom, respectively compared with DNA fragments in healthy leaves. The genetic variability among symptoms types has yet 16 to be established rapid and unambiguous of genetic variability among symptoms types has greatly benefied from recent advances in DNA fragments based on the PCR and random amplified polymorphic DNA (El-Dougdoug et al. 2007 and Sharma, 2003).

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