Egyptian J. Virol. 1, 251-258 (2004)

Faba bean necrotic yellows virus (FBNYV) in Egypt: Characterization and Virus-Vector Relationship

A. E. Aboul-Ata, Mohga, M. El-Tahlawey, M. A. Amer and A.M. Mandour

Virus and Phytoplasma Research Deprtment, Plant Pathology Research Institute, Agricultural Research Center, Egypt

Faba bean necrotic yellows virus (FBNYV) has been isolated and identified as an isolate of FBNYV from Egypt. FBNYV characterization was proved according to the following criteria: Sypmtomatology, insect transmission (*Aphis craccivora*), serological tests (TAS-ELISA), PCR product for amplified replicase gene of FBNYV (920 bp). Virus-vector relationship was recognized as follows: AAP is 2 hr, IAP is 0.5 hr. Latent period, in the aphid vector, is 18 hr and retention period is 18 days. FBNYV was tested for incidence in the FBNYV-virulefirous aphids (*A. craccivora*) using PCR products of amplified replicase gene of FBNYV (290 bp). It is not trans-ovarially transmitted through new progenies of *A. craccivora*. The virus has been able to be transmitted by five different aphid species i.e. *A. craccivora* (93.84), *A. fabae* (89.09), *A. pisum* (77.50), *A. sesbaniea* (56.25), and *A. gossypii* (38.46). The virus transmissibility by aphid is higher parallel to number of insects allowed to acquire the virus. FBNYV is transmitted by Nymphal stage more than adult stage (78.7% and 34.3% respectively). FBNYV is being able to be acquired by *A. craccivora* insects after 4-day post-inoculation period. The virus is not being able to be transmitted throughout newly hatched nymphs without acquiring virus.

INTRODUCTION

Faba bean necrotic yellows virus (FBNYV) was the causal agent of such serious disease. Preliminary studies have shown that the disease etiology was an aphid-transmitted single stranded DNA virus in a persistent manner. (Makkouk *et al.*, 1992 and Katul *et al.*, 1993). FBNYV is one of devastating and endemic-causing diseases in Egypt (Salama, 1998). The major diseases-causing loss in Egyptian legume is FBNYV. Losses caused by FBNYV are more than 90% during the severe infection incidence (*Makkouk et al.*, 1992). It causes plant stunting, leaf yellowing and necrosis allover leaves, stems and pods. Pods are very small with no seeds or few small seeds, which are not marketable. FBNYV has been invading Egyptian faba bean fields since 1992 which severe epidemic was occurred. Seasonaliy, FBNYV infects many faba bean fields with different degrees of severity in Middle Egypt. Faba bean cultivated area has been decreased up to 75% because of FBNYV invasion. Cultivated area of faba bean has still as it is and farmers are afraid of FBNYV infection (Makkouk *et al.*, 1992).

This study aims to characterize FBNYV and to uncover some epidemic-causing factors of FBNYV so that correct integrated disease management (IDM) program can be manipulated.

MATERIALS AND METHODS

1.Isolation and Serological Detection

Three different samples of those have FBNYV-like symptom were collected from Fayium, Qualubia (Moshtohur) and Beni Suef. Those samples had different degrees of severity i.e. samples of Qualubia and Beni Suef have severe symptoms and the samples of Fayium had moderate symptoms. They were maintained separately, as virus sources of infection using insect inoculation (*Aphis*

craccivora), on 3-leaf stage seedlings of Misr 1 faba bean variety. The three FBNYV sources of infection were kept in 40 cm x 60 cm x 80 cm plastic cage in Dept. of Plant Virus and Phytoplasma Res. for further studies and detection tests (serological, PCR amplification...)

Isolated FBNYV-infected plant samples were tested for virus detection using ELISA as serological test. Method of Makkouk and Comeau (1994) were used. Non-infected samples were used as negative control. Infected plant samples with FBNYV were extracted in PBS pH 7.0 for triple-antibody sandwich indirect ELISA (TAS-ELISA) test carrying out. Monoclonal antibodies of FBNYV, that was kindly supplied by ICARDA, were used as attractive and detective antibodies. The goat-ant mouse antiserum conjugated with alkaline phosphates was used. Yellow color represents positive reaction. ELISA reader was used at 405 nm wavelength for determination of positive reaction. Twice as much of ELISA-reading value for negative control was considered positive.

2. Molecular Characterization

Total nucleic acid was extracted from FBNYV-infected and healthy plant materials (Faba bean) and FBNYV-viruleferous aphids (*A. craccivora*) according to Roja *et al.* (1993) with some modifications. Total DNA was prepared by mixing 300 mg of soft leaf tissue and 3, 5, 10 and 20 viruleferous insects in a 1.5 ml micro centrifuge tube with one ml of (100 mM Tris-HCl, 100 mM EDTA and 2.5 M NH_3- acetate, pH 8.0) and incubated at 60 °C for 15 min. The mixture was then emulsified with an equal volume of phenol/chloroform (1:1). Mixture was vortexed for 3 min. and then centrifuged for 10 min. at 5 rpm. Aqueous phase was re-extracted with an equal volume of chloroform. Nucleic acids were precipitated by centrifugation at 10 rpm for 5 min. and pellets were washed using 70% ethanol and re-suspended in sterile distilled water. Nucleic acid preparations were incubated at 37 °C for 1 h with RNAse. (20 ug/µl) in water.

Amplification was performed in thin walled PCR tubes. Each tube contained the following reaction mixture: 5 ul of 10 X PCR buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, and 0.001 gelatin); 3 ul of 25 mM $MgCl_2$; 1 ul of 10 mM dNTPs; 1 ul of each 10 pmol complementary (5`- ttt ccc gct tcg cta agt tta a -3`) and homologous (5- aca ccc tcc ttg gaa ctg gta taa-3`) specific primer for replicase gene of FBNYV (Salama, 1998); 2.5 units of Taq DNA polymerase and sterile water to a volume of 45µl. Five µl of the DNA extraction was added to the PCR tube reaction and amplified with the following cycling parameters: denaturation at 94°C for 1 min; primer annealing at 62°C for 2 min and extension at 72 °C for 1 min for 35 cycles with a final extension at 72 °C for 7 min. Aliquots of 10ul of PCR amplified products were analyzed on 1 % agarose gel stained with ethedium bromide in TBE buffer (89 mM Tris-HCL; 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.3 at 100 volt for 1 h. PCR marker (Promega Co.) was used to determine the size of PCR amplified, viewed over a UV light source and photography with gel Documentation (Sambrook *et al* .1989)

3. Virus-Vector Relationship

Virus-free colonies of *A.craccivor*, *A.fabea*, *A.pisum*, *A.sesbaniea*, *A.gossypii*, *A.nerii*, and *M.persicae* were allowed to acquire FBNYV from the three FBNYV sources of infection (Fayium, Qualubia and Beni Suef). Previous aphid species

were allowed to inoculate healthy Misr 1 faba bean indicator seedlings to determine virus-transmission ability by adult age of each aphis species. *A. craccivor* insects were used for determination of both acquisition access period (AAP) and inoculation access period (IAP) using different periods (5 min – 48 hr). (Table 5). Also, *A. craccivor* insects were used to determine latent period (Table 6). retention period in the insect vector and post-inoculation period (Fig 3) using daily transmission of virulefirous insects for 18 continuous days onto faba bean indicator seedlings. *A. craccivora* insects did ability of FBNYV transmission by different aphid stages. Also, effect of number of insects per plant on transmission ability of FBNYV was studied by the same aphid species (Table 3, 4, 5 and 6). Three replicates were used all time. Trans-ovarial transmission ability of FBNYV was also studied using *A. craccivera* as well.

RESULTS AND DISCUSSIONS

Characterization of *Faba bean necrotic yellows virus* (FBNYV)

Virus identification and characterization was based, in this work, on different items: symptoms caused by isolated virus, serological reactions, insect transmission, and molecular biological characterization using PCR.

Isolation and Serological Assays

FBNYV-like symptoms in the field were mostly stunting deformation, twisting, yellowing and necrosis of leaves. Flowering in FBNYV infected plants was reduced with little or no pod formation. Seeds from infected plants were often small. Described symptoms were in agreement with the finding of Katul *et al.* (1993). Samples have FBNYV-like symptoms were collected from Beni Suef, Fayium and Qualubia. Those samples have shown difference in symptom severity i.e. Fayium Isolate had moderate FBNYV symptom. However, both FBNYV isolates collected from Beni Suef and Qualubia had severe symptoms. Those FBNYV isolates were isolated separately using *Aphis craccivora* insects on Misr 1 faba bean seedlings. Inoculated plants showed symptoms same as those shown in the field. Symptoms are leaf yellowing, up rolling, smaller in size, necrotic lesions and plant stunting (Fig 1). Monoclonal antibody of FBNYV was used as attractive and detective antibody in triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) technique (Franz, 1996). As shown in Table (1). The three FBNYV isolates reacted positively with FBNYV antiserum with different readings. ELISA readings represent antigen-antibody reaction. So, virus antigen of studied samples could have different reactions with the antiserum used. Those studied-sample antigens could have different concentrations and or different isolates (Katul *et al.*, 1993)

Molecular Characterization

PCR was used to identify of the FBNYV from infected FBNYV-plants using specific primers for the replicase gene of FBNYV. The PCR reaction in Fig (1) showed that the specific primer pairs for PCR product amplified from infected faba bean with *Faba bean necrotic yellows virus* (Lane1 = isolate brought from Fayium, 2 = Isolate brought from Beni Suef and 3 = Isolate brought from Moshtohur, Qualubia). Non- amplified PCR product was observed from uninfected tissue (lane 4). Whereas the amplified FBNYV fragments were as expected (920 bp) comparing to the DNA marker (lane M). The three studied samples those FBNYV-like symptoms were

confirmed, using PCR amplification, that they are belonging to FBNYV. As shown in (Fig 1) bands representing amplification have different intensity. This difference could be related with the variability, among different isolates (Katul *et al.*, 1993). Healthy *A. craccivora* insects were allowed to feed on Moshtohour isolate to acquire FBNYV. Extraction of those virulefirous insects has been amplified using complementary and homologous primers of FBNYV-replicas gene. The results shown in (Fig.2), whereas the amplified FBNYV fragment were as expected (920 bp) comparing to the DNA marker (lane M), these fragments were obtained from 3,5,10 and 20 viruliferous aphids (lane 1,2,3,and 4). The obtained PCR product with 20 aphids was denser than 3 or 5 aphid. No fragment from non-viruliferous aphids was obtained (lane 5).

Results showed that the first component of the FBNYV was represented the replicase gene which confirmed by PCR amplification using specific primers. This was in agreement with findings of Katul *et al.* (1995) and Salama (1998). PCR has been adapted to the detection of virus in the vectors i.e. aphids (Lopez-Moya *et al.*, 1992; Hadidi *et al.*, 1993; Levy and Hadidi, 1994; Canning *et al.*, 1996 and Amer, 1999), whiteflies (Navot *et al.*, 1992 and Deng *et al*, 1994), thrips (Tsuda *et al.*, 1994) and leafhopper (Takahashi *et al.*, 1993). In addition, successful detection of viruses in their nematode vector has been also reported (Van der Wilk *et al.*, 1994)

Virus-Vector Relationships

Virus-vector relationship information is essential to access virus epidemiological manipulation and applied science applications. There is some factors were studied in this work: Acquisition access period (AAP) was determined as 2 hr (Table 4). Inoculation access period (IAP) was recognized as 0.5 hr (Table 4). Latent period (LP), in the aphid vector, is 18 hr (Table 6) and retention period (RP) is 18 days (Fig. 3). Moreover, FBNYV was tested for incidence in the FBNYV-virulefirous aphids (*A. craccivora*) using PCR products of amplified replicase gene of FBNYV (290 bp) (Fig. 2). The virus can not be transmitted through new progenies of *A. craccivora* without acquiring virus (Table 5). There is no transovarial transmission (Table 5). The virus could be transmitted by five different aphid species i.e. *A. craccivora* (93.84), *A. fabea* (89.09), *A. pisum* (77.50), *A. sesbaniea* (56.25), and *A. gossypii* (38.46) (Table 2). The virus can not be transmitted by two aphid species (*A. nerii* and *Myzus persicae*) (Table 2). The virus transmissibility by aphid insects is higher using higher number of insects allowed to acquire the virus (Table 3). FBNYV is transmitted by Nymphal stage more than adult stage (78.7% and 34.3% respectively) (Table 3). FBNYV is being able to be acquired by *A. craccivora* insects after 4-day as post-inoculation period (Fig. 3). *A. craccivora* needed longer AAP period than IAP period and that is typical behavior of persistent aphid-transmitted viruses (Sugawara *et al.*, 1977; Cohen *et al.*, 1983; Horn, 1994 and Salama, 1998)

The above mentioned studies indicated that it could be concluded that the three studied samples brought from Fayium, Beni Suef and from Moshtohour, Qualubia could be different FBNYV isolates. This finding is in agreement with Katul *et al.* (1993) who has proven, by serological and molecular biological approaches, that there could be more than 2 isolates of FBNYV. This finding could lead us to identify an appropriate IDM strategy and/or breeding for FBNYV resistance.

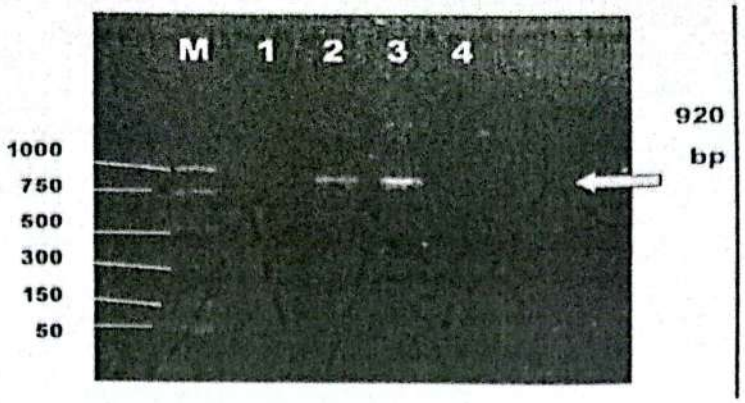

Fig. (1): Agarose gel electrophoresis 1 % for PCR amplified products from faba bean plants infected with *Faba bean necrotic yellows virus* (Lane1 = isolate brought from Fayium, 2 = Isolate brought from Beni Suef and 3 = Isolate brought from Moshtohur, Qualubia). Non- amplified PCR product from uninfected tissue (lane 4). The arrow indicates 920 bp . Lane M 50 bp PCR marker (Promega Co Cat. No. G3161)

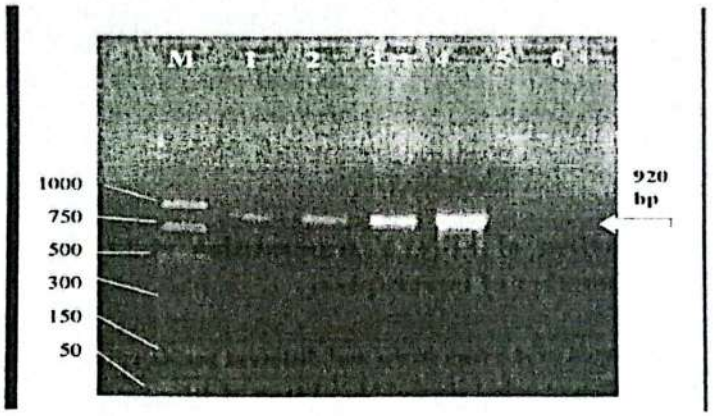

Fig. (2): 1% agarose gel electrophoresis for cDNA amplified products using the primer specific for replicase gene of FBNYV from 3, 5, 10 and 20 FBNYV-viruliferous aphids (lane 1,2,3and 4). Uninfected aphids (5 aphids) (lane 5). PCR-DNA marker (1000, 750, 500, 300, 150 and 50 bp) lane M.

Table (1): Serological reaction against different *Faba bean necrotic yellows virus* (FBNYV) samples collacted from Moshtohur, Qualubia, Beni Suef and Fayium using monoclonal antibody of FBNYV (ICARDA supply)

FBNYV Isolate	ELISA read (Min.)	ELISA read (Max.)	ELISA read (Mean)
Moshtohur	0.222	0.283	0.244 (+++)
Beni Suef	0.163	0.285	0.195 (++)
Fayium	0.166	0.276	0.168 (+)
Negative	0.123	0.133	0.133

Table (2): Determination of FBNYV transmission ability by different aphid species

Exp. No	Different aphid species						
	A.craccivora	*A.fabea*	*A.pisum*	*A. sesbania*	*A.gossypii*	*A. nerii*	*M. persicae*
1	18*/20**	9/10	15/20	12/20	8/20	0/25	0/20
2	14/15	12/15	22/30	18/30	6/15	0/30	0/15
3	29/30	28/30	25/30	15/30	11/30	0/20	0/30
%	**93.84**	**89.09**	**77.50**	**56.25**	**38.46**	**0.0**	**0.0**

10 aphid insects per plant were used. * = Number of infected plants ** = Number of inoculated plants
Acquisition access period (AAP) and Inoculation access period (IAP) = 48 hr.

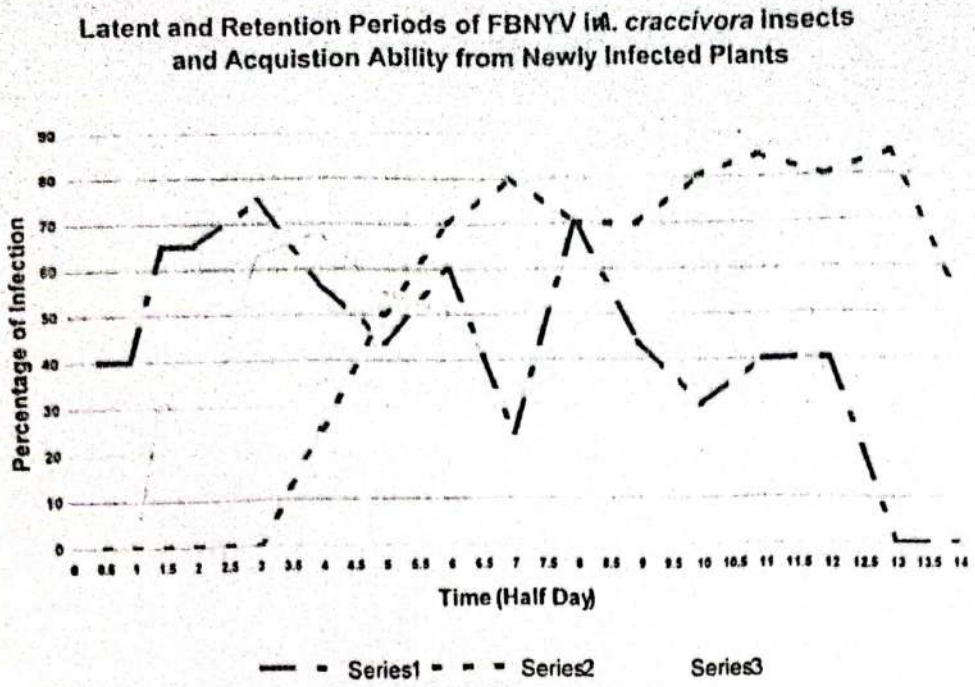

Fig. (3): Determination of latent (series 3) and retention periods (series 1) of FBNYV in *A. craccivora* aphid insects as well as ability of acquisition from newly infected plants (Post acquisition period) (series 2)

Table (3): Determination of FBNYV transmission ability by different aphid stages and different number of insects/plant

Exp. No	Different stages of *A. craccivora* and different numbers of insects/plant					
	Effect of different aphid stages***			Effect of different number of insects/plant		
	Nymph	Apterous Adult	Adult	1insect/plant	5insect/plant	10insect/plant
1	28*/30**	17/30	15/30	5/20	13/20	15/20
2	15/20	10/20	6/20	6/30	18/30	28/30
3	17/20	12/20	3/20	4/30	14/30	27/30
%	85.71	55.71	34.28	18.75	56.50	87.50

Acquisition access period (AAP) and Inoculation access period (IAP) = 48 hr.

- = Number of infected plants
- ** = Number of inoculated plants

*** = Five insects/plant were used

Table (4): Determination of acquisition access period (AAP) and inoculation access period (IAP) of FBNYV using A. craccivora insects

Time tested	AAP and IAP of FBNYV							
	Acquisition access Period (AAP)*				Inoculation Access Period (IAP)**			
	Rep. 1	Rep. 2	Rep. 3	%	Rep. 1	Rep. 2	Rep. 3	%
5 min	0/10	0/10	0/10	0.0	0/10	0/10	0/10	0.0
30 min	0/10	0/10	0/10	0.0	1/10	0/10	0/10	2.5
2 hr	0/10	1/10	0/10	3.3	2/10	3/10	5/10	33.3
6 hr	2/10	1/10	2/10	16.6	6/10	8/10	9/20	57.5
12 hr	1/10	2/10	3/10	20.0	9/10	9/10	9/20	67.5
24 hr	6/10	7/10	7/10	66.6	9/10	9/10	13/20	82.5
48 hr	10/10	10/10	9/10	93.3	10/10	10/10	17/20	9. 5

5 insects/plant were used * = IAP = 48 hr ** = AAP = 48 hr

Table (5): Determination of ability of FBNYV transmission by *A. craccivora* insects through newly borne progeny

Percentage of FBNYV transmission through newly-borne progeny				
Status of insect tested	Exp. number			%
	1	2	3	
FBNYV-virulefirous mother insects	5*/10**	7/10	5/10	56.6
Newly-borne progeny	0/10	0/10	0/10	0.0

5 insects/plant were used
* = Number of infected plants
** = Number of inoculated plants

Table (6): Determination of latent period of FBNYV in *A. craccivora* insects those were allowed to acquire the virus for 12 hrs

Time (hours) tested just after AAP of FBNYV-virulefirous *A. craccivora* insects										
Exp.	12hr	18hr	24hr	2day	3day	4day	5day	6day	7day	8day
1	0*/10**	0/10	1/10	7/10	7/10	6/10	6/10	4/10	2/5	1/10
2	0/10	0/10	2/10	6/10	8/10	2/5	3/5	1/5	0/5	0/5
3	0/10	1/10	3/10	6/10	2/5	3/5	1/5	2/5	2/10	1/5
%	0.0	3.3	20	63.0	68.0	55.0	50.0	35.0	20.0	10.0

AAP = 12 hr
• = Number of infected plants
** = Number of inoculated plants
5 insects/plant were used

REFRENCES

Amer, M. A. (1999). Comparative studies on the diagnostic methods for the *Potato virus Y* disease. M.Sc. Thesis, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Canning, E. S.G.; Penrose, M.J.; Barker, I. and Coates, D. (1996). Improved detection of *Barley yellow dwarf virus* in single aphids using RT-PCR. J.Virol. Methods 56: 191-197.

Cohen, S.; Duffus, J. E.; Larson, R. C.; Hiu, H. Y. and Flock, R. A. (1983). Purification, serology and vector relationships of *Squash leaf curl virus*, a whitefly transmitted geminivirus. Phytopathology, 73: 1669-1673.

Deng, D.; McGrath, P. F.; Robinson, D. J. and Harrison, B. D. (1994). Detection and identification of whitefly-transmitted *Geminiviruses* in plants and vector insects by the polymerase chain reaction with degenerate primers. Ann. Appl. Biol. 125: 327-336.

Franze, A.; Makkouk, K. M.; Katul, L. and Vetten, H. J. (1996). Monoclonal antibodies for the detection and differentiation of *Faba bean necrotic yellows virus* isolates. Annals of Applied Biology, 128: 255-268.

Hadidi, A.; Montasser, M. S.; Levy, L.; Goth, R. W.; Converse, R. H.; Madkour, M. A. and Skrzeckowski, L.J. (1993). Detection of *Potato leaf roll* and *Strawberry mild yellow edge Luteoviruses* by reverse transcription polymerase chain reaction amplification. Plant Dis. 77: 595-601.

Horn, N. M. (1994). Viruse involved in chickpea stunt. Ph.D. Thesis Agric. Univ. Wageningen, 137pp.

Katul, L.; Maiss, M. and Vetten, H. J. (1995). Sequence analysis of *Faba bean necrotic virus* DNA component containing a putative replicase gene. J. Gen. Virol. 76: 475-479.

Katul, L.; Vetten, H. J.; Maiss, M.; Makkouk, K.M.; D. E. Lesemann, D. E. and Casper, R. (1993). Characterization and serology of virus like particles associated with Faba bean necrotic yellows. Annals of Applied Biology 123: 629-647.

Levy L. and Hadidi, A. (1994). A simple and rapid method for processing tissue infected with *Plum pox Potyvirus* for use with specific 3-noncoading region RT-PCR assays. Bulletin OPPO/EPPO Bulletin 24: 595-604.

Lopez-Moya, J.J.; Gubero, J.; Ioez-Abella, D. and Diaz-Ruiz, J. R. (1992). Detection of *Cauliflower mosaic virus* (CaMV) in single aphids by the polymerase chain reaction (PCR). J.Virol.Methods 37 (2): 129-138.

Makkouk, K. and Comeau, A. (1994). Evaluation of various methods for the detection of *Barley yellow dwarf virus* by the tissue-blot immunoassay and its use for virus detection in cereal inoculated at different growth stages. Eur. J. Plant Path. 100: 71-80.

Makkouk, K. M.; Kumari, S. G. and Al-Daoud, R. (1992). Survey of virues affecting lentil in Syria. Phytopathology Medit., 31 :188-190.

Navot,N.; Zeidan, M.; Pichersky, R. and Czosnek, H. (1992). Use of polymerase chain reaction to amplify *Tomato yellow leaf curl virus* DNA infected plants and viruliferous whiteflies. Phytopathology 82: 1119-1202

Rojas, M. R.; Gilbertson, R. L.; Russell, D. R. and Maxwell, D.P. (1993). Use of degenerate primer in the polymerase chain reaction to detect whitefly- transmitted Gemini viruses. Plant Diseases. 77: 340-347.

Salama, M. I. M. (1998). Molecular and serological studies of some faba bean (*Vicia faba* L.) viruses. Ph.D. Thesis, Fac. Agric., Ain Shams Univ., Egypt.

Sambrook, J.; Fritsch, E. and T. A. Maniatis, T.A. (1989). Molecular cloning: A laboratory Manual. 2nd ed. Vol.1-3 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sugawara, M.; Kojima, M. and Murayama, D. (1977). Latent period and retention of *Potato leaf roll virus* in its vector, *Myzus persicae*. Ann. Phytopath. Soc. Japan 40 39-45.

Takahashi, Y.; Tiongco, E.R.; Cabauatan, P. Q.; Koganezawa, H.; hibino, H. and Omura, T. (1993). Detection of *Rice tungro bacilliform virus* by the polymerase chain reaction for assessing mild infection of plants and viruliferous vector leafhoppers. Phytopathology 83: 655-659.

Tsuda, S.; Fujisawa, I.; Hanada, K.; Hidaka, S.; Hogo, K.; Kameya-Iwaki, M. and Tomaru, K. (1994). Detection of *Tomato spotted wilt virus* S RNA in individual thrips by reverse transcription and plymerase chain reaction. Ann. Phytopathol. Soc. Japan 60: 99-103.

Van der Wilk, F.; Korsman, M. and Zoon, F. (1994). Detection of *Tobacco rattle virus* in nematodes by reverse transcription and polymerase chain reaction Europe Plant Pathol. 1: 109-122. .