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Studies On *Bean Yellow* Mosaic Virus Infecting Some Leguminous Crops In Egypt

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ABSTRACT

Bean yellow mosaic virus (BYMV) was isolated from naturally infected bean (*phaseolus valgaris*, L. cv. Karnk) and faba bean (*Vicia faba*, L cv. Balady) plants were collected from different districts in menoufia governorate, Egypt; showing vein chlorosis followed by green and/or yellow mosaic symptoms that may be due to virus infection. Diseased samples were subjected to identification of isolated virus based on symptomatology host range, serological tests (DAS- ELISA, Tissue blot immunoassay TBIA and electron microscopy). Histological studies of bean infected leaves by BYMV cleared that chloroplasts contained starch grain turned to spherical in shape and with lost of their envelopes totally or partially. Chlorophyll a, b and carotenoids as well as oxidative enzymes (peroxidase and polyphenol oxidase) were determined in viral infected leaves. Molecular detection of BYMV using specific primers was detected using RT-PCR; its products showed a single amplified fragment of ~ 900 bp in electrophoresis analysis.

Key words: *Bean yellow mosaic virus* (BYMV), faba bean, bean, symptoms, serological tests, DAS-ELISA, TBIA electron microscopy, chlorophyll, enzymes and RT-PCR.

INTRODUCTION

It is well known that many viral diseases can infect and affect leguminous crops and consider a serious problem in worldwide countries, because the significant yield reduction and economic losses. Many investigators mentioned that more that fifteen viruses have been identified to infect legumes; *Bean yellow mosaic virus* (BYMV) genus Potyvirus; one of the most important viruses. It is the type member of the genus Potyvirus in the Potyviridae family of plant viruses with cryptogram (*/*: */*: E/E: S/Ap) (Pierce, 1934 and Bos, 1970).

The BYMV infection on bean fields causing about 33% reduction in the number of pods/plants and 41% reduction in seed yield (Hampton, 1975). The same virus infects faba bean plants (*Vicia faba* L.) in most countries worldwide and cause a huge losses in field bean yield both in total yield and quality (Badr, 1987).

Ultra structural studies in disease tissues of bean and faba bean plants (size of organelles especially chloroplast). The reaction of virus infected in chloroplast inner structures as decrease in area of thylakoid systems, enhanced stromal area as well as starch storage and induction of plastoglobuli were noticed also (Zechmann *et al*., 2003).

Plant virus infection affects physiological processes such as photosynthesis, i.e. decreasing photosynthesis rate, pigment contents (Mojca *et al*., 2001), total soluble sugar contents, starch accumulation and respiration rate increasing (Shalitin and Wolf, 2000).

Oxidative enzymes (peroxidase and polyphenoloxidase) which catalyze lignin and other phenols that play important role

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in defense barriers for reinforcing the cell structure (Avdiushko *et al*., 1993).

Reverse transcription-polymerase chain reaction (RT-PCR) has been applied successfully to enhance detection sensitivity of plant viruses and other pathogens and made a pronounced impact in the area of detection and identification, Hadidi *et al*., (1995). Finally, this work aimed to isolate, define and examine whether BYMV can induce changes in infected leguminous leaves

MATERIALS AND METHODS

I. Isolation of the virus:

The virus was isolated from naturally infected faba bean plants growing under field condition at different location in Menoufia governorate, Egypt. Leaf samples showing mosaic and leaf roll symptoms (doubted to be due to virus infection) were collected and homogenized in a mortar and pestle, after adding phosphate buffer (1:5 w/v, 0.1 M, 0.1 ML, pH 7.2, Mahdy *et al.,* 2007), then the extracted sap was passed through a double layer of cheesecloth. The virus was purified biologically thought, the single local lesion technique as described by Khan and Monroe (1963), was followed *Ch. amaranticolor* L, plants were used as a local lesion host. Single local lesion was isolated, grinded in phosphate buffer pH 7.2 and back inoculated onto *Ch. amaranticolor* L. Finally the resulting local concentric lesions were singly back inoculated onto faba bean plants. Inoculated potato plants were kept in the greenhouse and used as a source of infection in the following experiments.

II. Identification:

A-Host range and symptomatology:

Seedling of eleven plants species and cultivars belong to three different families, were mechanically inoculated with the isolated virus. The inoculated seedling were grown under greenhouse condition (25 ± 5ºC) and observed daily for 30 days for any virus symptoms. Inoculated plants showing visible or no visible symptoms were checked by a serological test.

B- Serological typing:

The isolated virus was detected by enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clark and Adams (1977) .ELISA kits (completely ready to use) were supplied by LOEWE® Biochema GmbH, Germany. Tissue blot immunoassay was used to detect the isolated virus as described by Lin *et al*., (1990) .

C- Electron microscopy:

Ultra histopathological change due to virus infection were studied using faba bean leaves, the work was done in TEM lab FARP. Faculty of Agriculture Research Park- Cairo University**-** Giza.

D. Determination of photosynthesis pigments:

For determination of photosynthetic pigments, i.e. chlorophyll a, b and carotenoids. After 21 days of inoculation with virus, the leaves of healthy and infected faba bean plants, were taken and the pigments were extracted by 85% aqueous acetone according to **Fadeel's method (1962)**. The absorbance was determined using Carl- Zeis Spectrocolourimeter at the wave lengths of 440, 644, 662 nm.

E. Determination of antioxidant enzymes activities:

●Peroxidase enzyme activity:

For determination of peroxidase activity the method described by Fehrman and Dimond (1967). Aliquots of the supernatants were assayed for measuring the peroxidase activity using SPEKOL spectrophotometer at 470 nm.

●Polyphenol xidase enzyme activity:

Polyphenol oxidase activity was determined by the method described by (Coseteng and Lee, 1978). Aliquots of the supernatants were assayed for measuring the peroxidase activity using SPEKOL spectrophotometer at 495 nm. Three replicates were used in each treatment. This work was done in Central Lab, Faculty of Agriculture, Menoufia University.

F- RT-PCR test:

F.1. Extraction of total RNA from plant tissues:

Total RNA was isolated from the infected faba bean leaves plants using (RNA Purification Kit obtained from gene jet ™ RNA) according to manufacturer's instructions.

F.2. Design and synthesis of the primers:

The primer set was designed by Mohamad *et al*., (2008) for RT-PCR amplification of BYMV RNA based on the alignment of coat protein (CP) gene sequences of known BYMV strains obtained from the NCBI website (GenBank). The primer set of BYMV-CPU and BYMV-CPD, was designed to amplify the entire CP gene of BYMV RNA 3 (Table 2). The expected band size of the PCR product using the designed primers was 907 bp.

Table (1): Primer pairs designed for RT-PCR based on coat protein gene sequence BYMV RNA 3.

Primer pairs	Primer sequence	Product size (bp)
BYMV-CPU	5′-GTCGATTTCAATCCGAACAAG-3′	907
BYMV-CPD	5′-GGAGGTGAAACCTCACTAATAC-3′	

F.3. cDNA synthesis:

2 µl of RNA, and 1.5 µl of 10 µM of the complementary primers (BYMV) were mixed in a sterile RNase-free microcentrifuge tube with nuclease-free water to a final volume of 15 µl. The tubes were heated to 70°C for 5 min, then cooled immediately on ice and spun briefly to collect the solution at the bottom of the tube. The following components were added to the annealed primer/template: 5 µl of 5X M-MLV reaction buffer [250 mM of Tris-HCl (pH 8.3), 375 mM of KCl, 15 mM of MgCl2, and 50 mM of DTT (dithiothretol)], 2 µl of 10 mM deoxynucleoside triphosphates (dNTPs, 25 units of RNasin® ribonuclease inhibitor, and 200 units of M-MLV RT enzyme (Promega) and nuclease-free water to final volume of 25 µl. the tubes were mixed gently by flicking the tubes and incubated for 60 min at 37°C **(Soliman, 2002)**.

F.4. Reverse transcription–Polymerase Chain Reaction(RT- PCR):

RT-PCR amplification was performed in a final volume of 25 µl as the following : 2.5 µl of cDNA,2.5 µl of 10X buffer, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of d NTPS, 1µl of each forward and reverse sense primers at 10 µM, 0.2 µl Tag DNA polymerase and 11.5 µl of dH_2O. Amplification was performed in an automated thermal cycler (Applied Biosystems gene) programmed for the following thermo-cycling conditions: 47 ºC for 30 min for cDNA synthesis, 5 min at 94 ºC for reverse transcriptase inactivation and initial deneturation, followed by 35 cycles of 60 s at 94 ºC, 1min at 50 ºC and 2 min at 72 ºC, and final extension for 5 min at 72 ºC (Mohamad *et al*., 2008). RT-PCR amplified DNA fragments were separated by 1% a garose gel electrophoresis (Seakem LE, FMC, Bio Products, Cat. No. 50004) in 0.5x TBS buffer (90 mM Tris acetate, 90 mM boric acid, 2 mM EDTA, PH 8.0).

DNA ladder (1µg/µl in 10 mM Tris – HCL, PH 7.5 1mM EDTA), 100 bp DNA ladder, PROMEGA, Cat-No G210A.i.e., it consists of 11 double stranded DNA fragments with sizes of (100, 200, 300, 400, 500, 600, 700, 800, 900, 1.000 and 1.5000 bp) was used to determine the size of RT-PCR products. The gels were stained with 10µg/ml of ethidium bromide (10 µg dissolved in 1 ml of water) [2,7-Diamino-10-ethyl-9-phenyl phenanthridinium bromide; homidium bromide] for 10 min **(**Sambrook *et al.,*1989) and visualized with UV illumination using Gel Documentation System (Gel Doc 2000, BIO -RAD). The expected size of the RT-PCR product was 907 bp for BYMV.

RESULTS

I-Isolation of the virus:

Bean yellow mosaic virus was isolated from naturally infected faba beans (*Vicia fabae*, L. cv. Balady) and infected bean (*phaseolus valgaris*, L. cv. Karnk) plants growing under field conditions in Menoufia governorate, Egypt and showing yellow mosaic, leaf curling , malformation and often stunting symptoms (Fig 1,A). Inoculation buffer solution was prepared using diseased samples that collected from naturally infected plants.

Plant virus mechanical inoculation was done on healthy bean and faba bean plants as well as other indicators and host plants under greenhouse conditions.

II-Identification:

A- Host range and symptomatology:

Eleven plants species and cultivars belong to three different families, were inoculated mechanically with virus isolate. The reaction of different plants to BYMV was classified to different categories according to their reaction (Table2and Fig. 1, B and C).

B- Serological typing:

The virus was detected by DAS-ELISA, positive reaction obtained with the specific antiserum. BYMV readily detect immunologically using Tissue blot technique (TBIA) on nitrocellulose membrane which probed with polyclonal antisera diluted 1/1000 in TPS buffer and goat anti rabbit alkaline phosphates conjugate diluted 1/8000, was used as secondary antibody (Fig.1,D).

C- Electron microscopy:

Data in (Fig.1, E and F) showed that main identified differences between infected and healthy cells in infected tissues, i.e. cell organelles like chloroplast; that lost their normal arrangement and turned to spherical, longish and curved in shape. In heavy infection, the chloroplast totally or partially damaged envelopes, as a result of infection in stroma matrix exclusion the cytoplasm. Many starch grain and amorphous inclusion were observed.

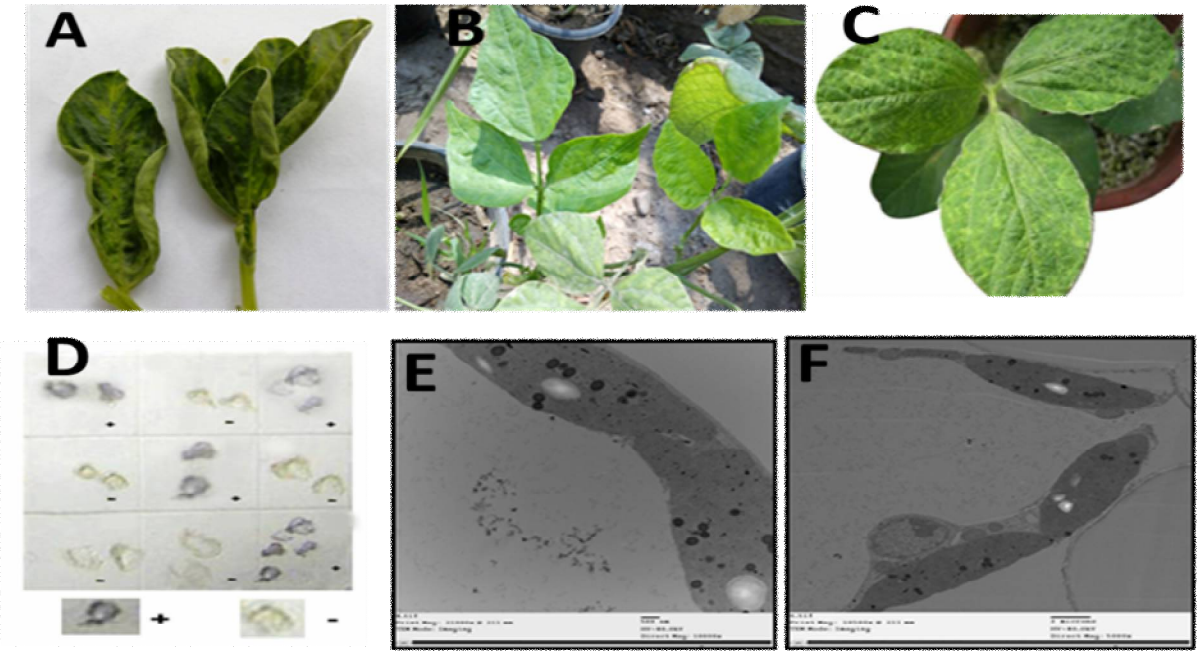

Fig (1): A: Green mosaic, leaf roll and malformation on infected faba bean leaves.
B: Systemic yellow mosaic, blisters and leaf deformations on *Phaseolus vulgaris* L. **C**: Mosaic and blisters on *Glycine max* L.
D: Tissue Blot Immunoassay for BYMV precipitation against specific IgG- BYMV polyclonal. + = Positive samples - = Negative samples.
E and F: Ultrastructural modifications of infected leaves, showing, chloroplasts (Ch) lost their normal arrangement and appeared spherical or longish and curved in shape and many starch grains (St).

D. Determination of photosynthesis pigments:

In *Vicia faba* plants infected with BYMV, there was a highly gradual decline in photosynthesis pigments. The obtained data in Table (3) showed that, Virus infection caused marked reduction in Chl a , Chl b and carotenoid contents (0.46 , 0.18 and 0.12 mg/gd.w) , compared with those (1.53 , 0.64 and 0.41 mg/gd.w) in healthy control for Chl a , Chl b and carotenoids respectively.

E. Determination of peroxidase and polyphenol oxidase enzymes activity:

The obtained results in Table (3) showing a great changes in peroxidase and polypheol oxidase enzyme activity between infected and healthy leaves of faba bean. It was 0.848 and 0.198 in peroxidase and polyphenol oxidase respectively in infected leaves.

Table (2): Susceptibility and reaction of different plants to mechanical inoculation with the isolated BYMV, under greenhouse conditions.

Host plant tested				Symptoms induced	ELISA test
Family	Scientific name	English name	Variety		
1.Chenopodiaceae	*Chenopodium album*	Ouares	—	CLL	+
	Ch. amaranticolor.	Lamb'S	—	CLL	+
	Ch. quinoa.	Gooses Foot	—	CLL	+
2- Fabaceae	*Glycine max* L.	Soya bean	Lee	YM	+
	Phaseolus vulgaris L.	Common bean	Giza karnk	SYM &St LC&Bl & LD	+ +
	Pisum sativum L.	Garden pea	Mister B	VC& M & BY	+
	Trifolium alexadrium L.	Egyptian clover	—	GM	+
	Vicia fabae L.	Broad bean	Balady	Cl & YM & LR	+
	Vigna unguiculata L.	Cowpea	Black eye	M	+
3- Solanaceae	*Nicotiana tabacum* L.	Tobacco	White-Burly	O	-
	Nicotiana glutinosa L.	Wirginia plant		O	-

Abbreviation of symptoms:

CLL = Chlorotic local lesion
LD = Leaf deformation
YM = Yellowing Mosaic
BL = Blisters
O = No symptoms
LC = Leaf curling
M = Mosaic
GM=Green mosaic
Cl = Chlorosis
LR = Leaf roll
VC = Vein clorosis
SYM: Systemic yellow mosaic
St= Stunting
BY= Bright yellow
+ = Positive reaction
- = Negative reaction

Table (3): Effect of *Bean yellow mosaic virus* on photosynthesis pigments and enzymes activity in infected *Vicia faba* L. cv. Balady. They are means of three replications.

Samples / Measurements	Healthy	Infected
Chlorophyll a	1.53	0.46
Chlorophyll b	0.64	0.18
Carotinoids	0.41	0.14
Peroxidase	0.531	0.848
Polyphenol oxidase	0.108	0.198

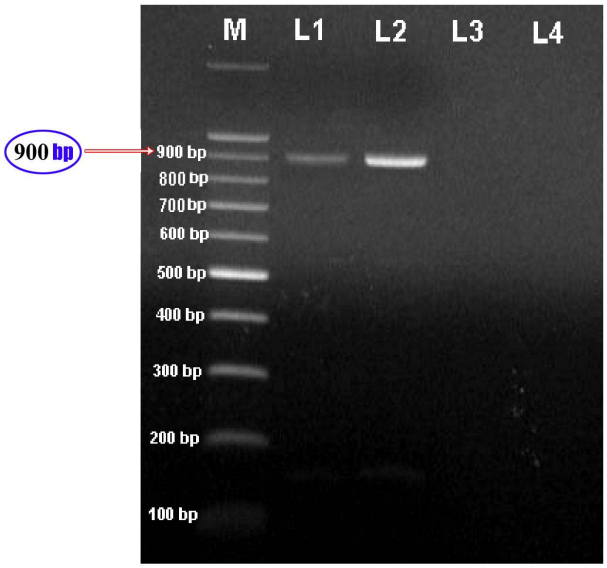

Fig (2): Agarose gel electrophoresis analysis of RT-PCR amplified products. M: 100 bp DNA ladder; L1, L2: two faba bean infected with BYMV; L3, L4: healthy plant sample as –be control.

DISCUSSION

BYMV was isolated from isolated from naturally infected faba bean (*Vicia fabae*, L.cv. Balady) plants were collected from from different locations in Menoufia Governorate, Egypt.. The BYMV was isolated before from faba bean in Egypt by Badr, (1978); Khatab Eman, (2002); Hemida, (2005); Sameh, (2005) Mahdy *et al*., (2007) and Deya Eldeen *et al*., (2008).

Bean yellow mosaic virus (BYMV) infects and severely affects many legume crops including bean and faba bean, resulting huge yield loss. Sever mosaic, deformation, malformation, blisters and leaf curling were noticed as a visually detected symptoms. Similar symptoms were reported with the same virus (BYMV) infection in other legumes such as (*Phaseolus vulgaris* L.), *Pisum sativum*, *Trifolium alexadrium* and other legumes. *Chenopodium amaranticolor* was used as local lesion host in all assayed trials, while *Phaseolus vulgaris* was used as systemically host. Similar results were also reported by Zechmann *et al*., (2003); Sameh, (2005); Ali *et al*.,(2006) and Deya-Eldeen *et al*., (2008).

DAS-ELISA test was used to confirm the identification of BYMV. However, this technique was mentioned by Hamed, (2006) to identify BYMV. In the tissue blotting technique, the specific antigen was immunologically localized with enzyme labeled antibody on nitrocellose membrane. This technique has been found to have much higher sensitivity for

detection of BYMV. These result in agreement with those recorded by Muthana *et al.*, (2001).

The advantage of TBIA technique proved to be able to detect BYMV, with small amount of antigen over standard ELISA and also provides simplicity, rapidity sensitivity and convenience for large numbers of samples. These results have already been declared by Hamed *et al*., (2012).

Ultrastrucural analysis revealed the formation of different sized and shaped abnormal chloroplasts. It became spherical in shape, while others appeared without envelopes and internal structures, e.g. grana and thylakoids were dilated. These chloroplasts ultrastructural changes in infected leaves by (BYMV) could have unfavorable effects on photosynthesis and could be partially decreasing chlorophyll concentrations during the viral infection (Lohaus *et al*., 2000) **.**Many starch grains were observed in BYMV-infected cells, virus infection inhibits decomposition of starch into dissolved sugars and thus prevent transmission to the outside of the leaf in the form of decomposed products accumulate inside the leaf , a result of its impact on enzymes analyzing starch (Fekry, 2006).

Physiological processes consider the main growth factor of plant; photosynthesis is the main factor in these processes and highly affected by viral infection (Arfan *et al*., 2007). Significant decreases in both photosynthesis rates and pigment contents as a result of (BYMV) infection that affecting growth inhibition. Many researchers recorded that viruses decrease the photosynthesis rate through inhibition of photosystem activation and decrease chlorophyll content (Zichman *et al*., 2003). In our investigation the decrease of photosynthesis pigments content could be explained by an extensive production of excitation energy that occurred by virus infection stress. The decrease in total pigment contents consider as response to (BYMV) infectin.

Concerning the peroxidase and polyphenol oxidase activity that estimated in infected and healthy leaves of faba bean. The infected leaves exhibited higher enzymatic activity compared with healthy ones. This result agree with data obtained by Hamed, *et al*., (2012) who reported that the rates of peroxidase activity were increased in infected cultivars in both leaves and bulbs of infected onion with *Tobacco rattle virus*. Peroxidase is one of the oxidative enzyme group, play an important role in different physiological reaction specially metabolism and anabolism. It hydrolyzes hydrogenperoxide resulted by dehydrogenase enzyme to water (Mengel, 1979).

Moreover Singh *et al*., (1989) recorded that virus infection increasing the polyphenol oxidase activity and resulting phenolic compounds accumulation. In addition, the content of polyphenol oxidase and peroxidase considered to play important roles in the resistance of chili pepper to *Cucumber mosaic virus* infection. Increasing phenols immediately after infection was much less marked in the resistant cultivars than the susceptible ones and was associated with increased activity of the enzymes.

Using of RT-PCR to detect (BYMV) in total nucleic acid extracts that prepared from infected faba bean plants, was adequate to virus detection; on day was enough for positive identification of (BYMV) from infected tissues. The amplified products of the virus were stable or stationary as: 1- The major product of (BYMV) infected tissue size was identical to ~ 900 bp from the cp gene of virus. 2- The specific primers did not amplify viral cDNA from extracts of healthy faba bean plants.

Two specific (BYMV) cp gene primers BYMV-CPU and BYMV-CPD; that used in our study; were designed and were more specific, sensitive and useful in RT-PCR for detection and amplifying BYMV cp gene sequence from total RNAs extracted from faba bean leaves. The two gene-specific primers were designed by Mohamad *et al*., (2008).

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