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# Antiserum prodction and reverse transcription polymeraes chain reaction (RT-PCR) for detection of bean leaf roll virus

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**Bean leaf roll virus (BLRV) is a recently isolated virus from *Vicia fabae* beans which was collected from eight different locations at Ismailia governorate, Egypt. This virus is belonging to the genus *luteovirus* and resembles its members in mode of transmission, symptoms and morphological appearance. In this study, BLRV was identified using multi techniques, direct enzyme linked immunosorbent assay (ELISA), antiserum with purified antigen, insect transmission and reverse transcription-polymerase chain reaction (RT-PCR). All plants collected from El- Tall El-Caber farms gave positive reactions with specific BLRV antibodies. Moreover, typical symptoms of BLRV, stunting, leaf roll, mosaic, chlorosis and yellowing, appeared on all plant cultivars under test (Giza 714, Giza 429, Giza461, Giza717 and Giza3). Six aphid species, *Acyrthosiphon pisum*, *Aphis craccivora*, *Aphis fabae*, *Aphis gossypii*, *Macrosiphum euphorbiae* and *Myzus persicae*, have obligatory transmitted BLRV in a circulative persistent manner. The percentages of transmission, acquisition and inoculation feeding period have been determined. RT-PCR technique was used for the detection and identification of the isolated virus particles from the infected faba bean plants. Using specific oligonucleotide primers, the coat protein gene, *cp*, was amplified from BLRV genome producing 400 bp fragments.**

**Key words:** Faba bean plant, *Aphis gossypii*, *Myzus persicae*, BLRV, ELISA, RT-PCR.

## INTRODUCTION

Bean leaf roll virus (BLRV) which is also known as pea leaf roll virus, is distributed worldwide (Ashby, 1984). Faba bean, *Vicia faba* L, is considered as one of the most important food legumes in Egypt. The species is considered a major staple food crop that is important for human and animal nutrition in developing countries (Bond, 1987). BLRV is endemic in white clover in the Southeastern United States (Damsteegt et al., 1995). Like other members of the *Luteoviridae*, BLRV is transmitted obligately by aphids in a persistent manner (Ashby, 1984). Cool-season food legumes (faba bean, lentil, chickpea and pea) and cereals (bread and durum wheat and barley) are the most important and widely cultivated crops in West Asia and North Africa (WANA), where they are the main source of carbohydrates and protein for the majority of the population. (Makkouk and Kumari, 2009).

The family *Luteoviridae* consists of eight viruses assigned

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to three different genera, *Luteovirus*, *Polerovirus* and *Enamovirus*; the complete genomic se-quences of pea enation mosaic virus (genus *Enamovirus*) and bean leaf roll virus (genus *Luteovirus*) from the Pacific Northwest, USA (Vemulapati et al., 2010).

Broad bean plants showing symptoms suggestive of viral infection such as stunting, leaf roll, mosaic, chlorosis, necrosis, and yellowing, were observed in the Andalucia, Baleares, Cataluna, and Murcia regions of Spain. A four-year field survey showed the presence of five different viruses, bean leaf roll virus (BLRV), beet western yellows virus (BWYV), bean yellow mosaic virus (BYMV), tomato spotted wilt virus (TSWV), and cucumber mosaic virus (CMV), in these regions (Fresno et al., 1997).

Persistently transmitted aphid-borne viruses pose a significant limitation to legume and cereal production worldwide. Surveys conducted in many countries in Wana during the last three decades established that the most important viruses are: faba bean necrotic yellows virus (FBNYV: genus *Nanovirus*; family *Nanoviridae*), bean leafroll virus (BLRV: genus *Luteovirus*; family *Luteoviridae*), beet western yellows virus (BWYV: genus *Polerovirus*; family *Luteoviridae*), soybean dwarf virus (SbDV: genus *Luteovirus*; family *Luteoviridae*) and chickpea chlorotic stunt virus (CpCSV: genus *Polerovirus*; family *Luteoviridae*) which affect legume crops, barley yellow dwarf virus-PAV and barley yellow dwarf virus-MAV: genus *Luteovirus*; family *Luteoviridae*).

On the other hand, cereal yellow dwarf virus-RPV (CYDV-RPV: genus *Polerovirus*; family *Luteoviridae*) affects cereal crops. Loss in yield caused by these viruses is usually high when infection occurs early in the growing season (Smith et al., 1999; D'Arcy et al., 2000; Leslie et al., 2002).

*Luteoviridae* also consists of 12 unclassified and 20 tentative members of non-enveloped, 25 to 30 nm icosahedral *Luteovirus* virions that contain a single molecule of positive-sense ssRNA of 5.7 to 5.9 kb (Hull, 2002). These viruses are transmitted in a persistent manner by aphid vectors and are mostly restricted to the phloem of infected hosts (Casper, 1998). Bean leaf roll virus (BLRV) belongs to the genus *Luteovirus* (family *Luteoviridae*). It occurs in Europe, the Middle East, India, and the USA. It infects legumes including French bean, faba bean (*Vicia faba*) and pea (*Pisum sativum*). BLRV is phloem-limited and present at very low concentrations. Besides, it is transmitted by aphids in a persistent manner (Ashby, 1984).

The complete nucleotide sequence of the bean leaf roll virus (BLRV) genomic RNA and the termini of its smallest subgenomic RNAs were determined to better understand its mechanisms of gene expression and replication and its phylogenetic position within the *Luteoviridae*. The number and placement of open reading frames (ORFs) within the BLRV genome was *Luteovirus*-like. The nucleotide and predicted amino acid sequences of BLRV were most similar to those of soybean dwarf virus (SbDV). Phylogenetic analyses employing the neighbour-joining method and sisterscanning analysis indicated that the BLRV nonstructural proteins were closely related to those of Barley yellow dwarf virus-PAV (BYDV-PAV), a *Luteovirus*. (Domier et al., 2002).

The relationship of BLRV to other members of the *Luteoviridae* has been examined based on biological, immunological, and nucleotide sequence data. Serological data (D'Arcy et al., 1988; van den Heuvel et al., 1990; Smith et al., 1996) suggest that BLRV is related most closely to soybean dwarf virus (SbDV). Sequences of the BLRV coat protein gene have been reported (Prill et al., 1990; Cavileer and Berger, 1994; Chomic et al., 2011) and predicted that BLRV was most closely related to poleroviruses.

As mentioned above, SbDV has a genome organization similar to members of the genus *Luteovirus*, but may have arisen through recombination between a *Luteovirus* and a *Polerovirus* (Rathjen et al., 1994; Terauchi et al., 2001).

The main purpose of this study was isolation and detection of BLRV using ELISA technique, symptomato-logy, aphid transmission, purification, antiserum produc-tion. RT-PCR technique was used for the identification of the new BLRV strain isolated from the infected faba bean plants at Ismailia, Egypt.

## MATERIALS AND METHODS

### Source of isolation

Naturally infected broad bean samples were collected from eight locations in Ismailia governorate, Egypt. Three farms per location and 10 plants were collected from all locations. The bean leaf roll virus (BLRV) isolated was obtained from faba bean plants showing the identical symptoms suggestive of viral infection such as stunting, leaf roll, mosaic, chlorosis, necrosis and yellowing.

### Identification of the isolated virus

Samples were inspected for BLRV symptoms and then marked for further detection assay by direct ELISA test, according to the method described by Clark and Adams (1977). Faba bean plants showing the identical symptoms of leaf roll were grown and reacting positively with BLRV antiserum and healthy faba bean plants were used for further experiments.

### Aphid transmission

The six pea aphid (*Acyrthosiphon pisum*), cowpea aphid (*Aphis craccivora*), black bean aphid (*Aphis fabae*), cotton aphid (*Aphis gossypii*), potato aphid (*Macrosiphum euphorbiae*), and the green peach aphid (*Myzus persicae*) were collected from the faba bean, vetch and lentil field and identified in plant protection department, faculty of agriculture, suez canal university, Ismailia, Egypt) and finally transferred to healthy Chinese cabbage seedlings in the green house. The upper leaves of faba bean plant with identical symptoms of BLRV were removed from the stem with petioles and placed in Petri dishes covered with wet filter papers. Six non-viruliferous aphids were fasted for one hour and then transferred to

**Table 1.** The rabbit immunization scheme used for antiserum production against BLRV.

| Injection no. | Route of injection | Dosage mg/ml purified virus |
|---|---|---|
| 1 | Intramuscular | 1 |
| 2 | Intravenous | 1 |
| 3 | Intramuscular | 1 |
| 4 | Intravenous | 1 |
| 5 | Intramuscular | 1 |
| 6 | Intravenous | 1 |

diseased leaves. All types of non-viruliferous aphids received a 48-h acquisition access period on symptomatic leaves. Aphids were then transferred to different cultivars from healthy faba bean plants (Giza3, Giza461, Giza717, Giza 429 and Giza 714) for a 72-h inoculation access period by 10 aphids per plant in the green house. Seedlings were sprayed by contact insecticide, transferred to air conditioned chamber at 20 to 22°C and kept for observation. Some plants were kept free from insects and were subjected to healthy non-viruliferous insects from stock cultured as control. Four replicates were used for this test and 20 plants per replicate. The inoculated seedlings were kept in an insect proof cage till symptoms appearance. The same procedure was conducted to prepare the control except those insects which were fed on virus free seedlings.

**Acquisition period and inoculation period**

Non-viruliferous *Acyrthosiphon pisum* was allowed to feed on infected faba bean cv. Giza 3 plant for different periods, 5, 10, 15, 30 and 45 min,1, 4, 6 h, and, one, two, three, four, five, six and seven days. Aphids were then transferred to healthy seedlings of faba bean cv. Giza 3 by means of 10 aphids per plant and then they were left to be fed for three days. Viruliferous *Acyrthosiphon pisum* were left to feed on the infected faba bean cv. Giza 3 plant for two days then transferred to feed on healthy seedlings of faba bean cv. Giza 3 for different intervals, 5, 10, 15, 30, 45, 60, 240, and 360 min. Longer time intervals were also applied, one, two, three, four, five, six and seven days after which plants were sprayed with a 0.2% of malathion spray. Ten (10) plants were used in every trail.

**Virus purification**

The methodology of Ashby and Huttinga (1979) was used for bean leaf roll virus (BLRV). Purification from infected broad bean plants is performed using polyethylene glycol precipitation of crude extracts followed by clarification with chloroform-butanol, differential centrifugation and density-gradient centrifugation. Purified virus suspension was fractionated in vials and stored at -20°C for further use (Ashby and Kyriakou, 1982).

**Antiserum production of BLRV**

***Rabbit immunization and bleeding***

New Zealand rabbit, about 4.5 kg, was used for production of antiserum. A total of 6 mg of the purified virus were used for injection. Two routes of injection were applied. For intramuscular injections, virus was diluted with 0.85% NaCl in distilled water (W/V) and mixed with an equal volume of freund's incomplete adjuvant. Injection was performed in the right and the left hind thighs, respectively using a 5 ml - disposable syringe. For intravenous injection, virus was diluted with 0.85% solution of NaCl in distilled water (W/V). Injection was made in the left ear at the marginal vein using 1 ml insulin disposable syringes. The rabbit immunization scheme used for antiserum production against BLRV is illustrated in Table 1. A week after last injection, the rabbit was bled from the right ear. Blood was left to coagulate for 2 to 3 h at 37°C then kept at 4°C overnight. Antiserum was separated through centrifugation at 4000 rpm for 15 min.

**Determination of antiserum titer and antigen dilution end point using the micro-precipitation test**

Antiserum titer was measured with the micropricipitation test described by Van slogteren (1955). Clarified and purified sap of bean leaf roll virus were diluted with a two-fold dilution series, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512, respectively, using phosphate buffer of pH 7.6 containing 0.85% NaCl. On the other hand, prepared BLRV antiserum and imported BLRV antiserum (Institut National de la Recherche Agronomique de Tunisie (INRAT), Rue Hedi Karray, 2049 Ariana, Tunis, Tunisia) were diluted with the same buffer, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048, respectively. Controls of healthy faba bean sap and normal serum were involved. The reaction was done between BLRV purified sap and its induced and imported antisera, separately. Another two reactions were performed between BLRV purified extract and its imported and induced antisera, separately. The reaction was performed in plastic Petri dishes (9 cm diameter) at room temperature by mixing equal aliquots (7 µl) of the given dilution of the antigen with another dilution of the antiserum. The reaction mixture was covered with paraffin oil and plates were incubated at 37°C for 2 h before examination. Stereomicroscopy with descending light was used for detecting the reaction appearance. Plates were r e-examined each ½ h up to 6 h. Virus dilution end point and antiserum titer were also determined.

**BLRV identification by direct ELISA**

Eight samples were obtained from naturally infected broad bean plants which is grown in Ismailia Governorate (Srapum, Abo-Soltan, Sabaa-Abar,El-Mahsama, Abo-Swair station, Riad El-Blah and El-Tal El-kaber). At first, these samples were tested by direct ELISA test against three BLRV antibodies (imported from Institut National de la Recherche Agronomique de Tunisie (INRAT), Rue Hedi Karray, 2049 Ariana, Tunis, Tunisia), prepared antiserum, bean yellow mosaic virus (BYMV) and Faba bean necrotic yellows virus (FBNYV). One microtiter plate was used for only one antibody; each plate contained 80 samples with two replicates for each sample. This procedure was carried out following the standard methods of Clark and Adams (1977).

#### Total RNA extraction

Total RNA was extracted by SV total RNA isolation system by spin protocol as recommend by the manufacturer instructions of Promega. Fifty milligram of BLRV-infected leaves of faba bean cv. (Giza3, Giza461, Giza717, Giza 429 and Giza 714) were ground in liquid nitrogen using a mortar and pestle. The purified RNA was stored at -20°C.

#### Reverse transcription - polymerase chain reaction (RT-PCR)

After total RNA extraction, RT-PCR detection was carried out using QIAGEN One Step RT-PCR Kit (Qiagene, Germany). To amplify the BLRV genome, a primer pair designed based on the sequence available in GenBank (AF441393) was used. The antisense primer, BLRV 5950C (5`-CTTTCGCCACCTTAACAACA-3`), which started from base 5,950 at the 30 end of the BLRV genome was used to synthesize cDNA from BLRV coat protein gene. The first-strand cDNA was then used in the PCR using BLRV5950C and the sense primer BLRV4F (5`-AAAGTTGACACCTTTACAAGAG-3`) which started at the fourth base of the 50 end of the viral genome. RT-PCR was performed for each primer pair in 20 µl mixture containing: 1 µg of total extracted RNA, 0.5 µM of each RT buffer (BRL), 1 µl RNasin (40 U; Promega) and 0.3 M ß- mercaptoethanol. Synthesis of cDNA was accomplished in water bath at 40°C for 1 h. Five microliters from the mixture were added to 45 µl of PCR mixture containing 10 X PCR buffer (Perkin Elmer), and 0.2 µM unit AmpliTaq™ (Perkin Elmer) by primer pairs. Samples were placed in a Perkin Elmer GeneAmpli thermal cycler 2400. PCR thermocycling conditions were as follows: RT at 45°C for 30 min, Initial denaturation at 95°C for 5 min, hot start at 95°C for 2 min, followed by 25 cycles of [denaturation at 92°C for 1 min, annealing for 1 min at special annealing primer 62°C depending on primer pairs in the mixture and extension for 1 min at 72˚C]. The final extension step was increased by 10 min at 72°C.The PCR products were analyzed by gel electrophoresis on 1% agarose gel (Sambrook et al., 1989).

## RESULTS AND DISCUSSION

Two hundred and forty (240) naturally infected faba bean plants were collected from, eight locations, Ismailia governorate, Egypt. The original BLRV isolate was obtained from faba bean plants (Collected from El- Tall El-Caber location) which showing the identical BLRV virus symptoms. Samples were inspected for BLRV symptoms and direct ELISA was used to confirm the presence of BLRV infections. All tested plants collected from El- Tall El-Caber farms were given positive reaction with specific BLRV antibodies.

This isolate was used for virus characterization and to study its effect of different cultivars of faba bean plants. Five plant cultivars belong to *Vicia faba* were inoculated with BLRV using infective pea aphid (*Acyrthosiphon pisum*) (Figure 1). Symptoms such as stunting, leaf roll, mosaic, chlorosis and yellowing appeared on tested plant cultivars (Giza 714, Giza 429, Giza 461, Giza717 and Giza 3) were appeared from 10 to 14 days after aphid transmissions. The same result was obtained by many scientists (Ashby and Huttinga, 1979; Reddy et al., 1979; Ashby, 1984; Schwinghamer et al., 1999; Leslie et al., 2002; Van Leur et al., 2002; Asma Najar et al., 2003. On the other hand, Larsen and Webster (1999) mentioned that during the 1998 growing season, affected pea plants exhibited mild chlorosis and mottling, leaf rolling, and stunting symptoms in Italy regions.

Six aphid species namely the pea aphid (*Acyrthosiphon pisum*), cowpea aphid (*Aphis craccivora*), black bean aphid (*Aphis fabae*), cotton aphid (*Aphis gossypii*), potato aphid (*Macrosiphum euphorbiae*), and the green peach aphid (*Myzus persicae*), were used to transmit BLRV. Results presented in (Table 2) show that all aphids species were obligatory aphid transmitted BLRV in a circulative persistent manner. The highest percentage of transmission was recorded (76.25 and 70%) by *A. pisum* and *A. craccivora*, respectively followed by *A. fabae* and *A. gossypii*, 33.75 and 28.75%, respectively.

On the other hand, the lowest percentage, 7.50%, was recorded by *Myzus persicae*. Similarly same results had already been observed by Ashby (1984); Damsteegt, et al. (1990); Damsteegt, et al (1992); Damsteegt et al (1995); Schwinghamer et al. (1999); and Van Leur et al. (2002). *Acyrthosiphon pisum* were fed on the infected plants cv. Giza 3 at different periods of 5, 10, 15, 30, 45, 60, 240, and 360 min. Longer time intervals were also used, 1, 2, 3, 4, 5, 6, and 7 days, and five aphids were used for each plant. Results recorded in (Table 3) showed that, *Acyrthosiphon pisum* adults became infective when they had 10 min feeding period on the infected plant. In addition, the highest percentage of plants infected by BLRV was observed with feeding period on the infected plants up to two and three days 100 and 90%, respectively. This percentage decreased from 50 to 60% with feeding period of four and five days, respectively. On the other hand, the percentage became increased again to 80% when 7 days feeding period were used. Five individuals of viruliferous *Acyrthosiphon pisum* were allowed to feed on healthy Giza 3 plant for 1, 4, and 6 h and one, two, three, four, five, six and seven days.

Results recorded in (Table 4) indicated that the highest percentage of infected plants (70%) was recorded when aphids had 10 and 15 min for different periods, 5, 10, 15, 30 and 45 min, of inoculation. On the other hand, percentage of the infected plants decreased from 70 to 50% when the feeding period was elongated to 6 h. Also, the percentage of infected plants was increased again to100% when the aphids feed on healthy plants for two and three days.

In addition, results indicated that aphids remain infective for seven days. Ashby, 1984; Schwinghamer et al., 1999; Larsen and Webster, 1999 had mentioned that the high aphid populations and diseased levels of nearly 100% were observed in susceptible varieties. Samples from affected fields were analyzed for the presence of Bean leaf roll virus (BLRV). Non viruliferous pea aphids (*Acyrthosiphon pisum* Harris) received a 48-h acquisition access period on symptomatic leaves. Aphids were then transferred to Puget pea and Diana faba bean for a 72-h

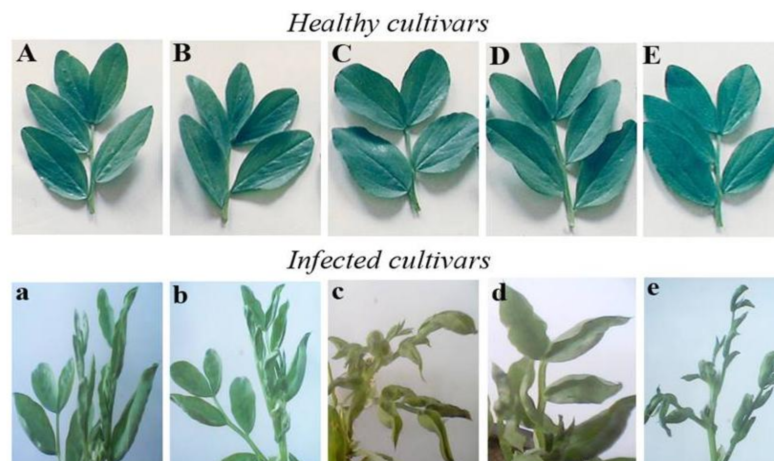


**Figure 1.** Symptoms caused by BLRV on faba bean plants. Healthy leaves; A: cv. Giza 714, B: cv. Giza429, C: cv. Giza 461, D: cv. Giza 717 and E: cv. Giza 3. Infected leaves; a, b, c, d and e showing stunting, leaf roll, mosaic, chlorosis and yellowing.

**Table 2.**Transmission of BLRV by *Acyrthosiphon pisum*, *Aphis craccivora*, *Aphis fabae*, *Aphis gossypii*, *Macrosiphum euphorbiae*, *and Myzus persicae*.

| Replicate | *Acyrthosiphon pisum* | | *Aphis craccivora* | | *Aphis fabae* | | *Aphis gossypii* | | *Macrosiphum euphorbiae* | | *Myzus persicae* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Infectivity* | % transmission | Infectivity* | % transmission | Infectivity* | % transmission | Infectivity* | % transmission | Infectivity* | % transmission | Infectivity* | % transmission |
| R1 | 16/20 | 80 | 15/20 | 75 | 6/20 | 30 | 4/20 | 20 | 2/20 | 10 | 2/20 | 10 |
| R2 | 15/20 | 75 | 13/20 | 65 | 8/20 | 40 | 6/20 | 30 | 3/20 | 15 | 2/20 | 10 |
| R3 | 15/20 | 75 | 14/20 | 70 | 6/20 | 30 | 6/20 | 30 | 1/20 | 5 | 1/20 | 5 |
| R4 | 15/20 | 75 | 14/20 | 70 | 7/20 | 35 | 7/20 | 35 | 2/20 | 10 | 1/20 | 5 |
| Mean | 15.25/20 | 76.25 | 14/20 | 70 | 6.75/20 | 33.75 | 5.75/20 | 28.75 | 2/20 | 10 | 1.50/20 | 7.50 |

*Number of infected seedlings/total number of inoculated seedlings.

inoculation access period.

The yield of the purified BLRV was about 1 mg/kg broad bean cv. Giza3 leaves. Infectivity on the tested broad bean Giza3 plants with inoculated BLRV using *Acyrthosiphon pisum* and observed epical BLRV symptoms after 25 days from inoculation when saved under green house at 22°C.

As a matter of fact, these methods were used by Ashby and Huttinga (1979); D'Arcy et al. (1990); Schwinghamer et al. (1999); and Van Leur et al. (2002). On the other hand, El-beshehy (1999, 2006) found that the ratio of A 280/260 was 0.78 and the ratio of max/min was 1.23. The yield of the purified PLRV was about 0.7 mg/kg potato

**Table 3.** Effect of Acquisition period on BLRV transmission by *Acyrthosiphon pisum* on *Vicia fabae* cv. Giza3.

| Feeding period | | | Acquisition periods on Giza3 | |
|---|---|---|---|---|
| | | | *T/I | % |
| Inoculation periods on Giza3 for 3 days | Time in min | 5 | 0/10 | 0 |
| | | 10 | 1/10 | 10 |
| | | 15 | 2/10 | 20 |
| | | 30 | 3/10 | 30 |
| | | 45 | 3/10 | 30 |
| | Time in h | 1 | 3/10 | 30 |
| | | 4 | 4/10 | 40 |
| | | 6 | 4/10 | 40 |
| | Time in days | 1 | 8/10 | 80 |
| | | 2 | 10/10 | 100 |
| | | 3 | 9/10 | 90 |
| | | 4 | 5/10 | 50 |
| | | 5 | 6/10 | 60 |
| | | 6 | 8/10 | 80 |
| | | 7 | 8/10 | 80 |

*Infectivity = Number of infected plants (I) / Total number of inoculated plants (T).

**Table 4.** Effect of inoculation period on BLRV transmission by *Acyrthosiphon pisum* on *Vicia fabae* cv. Giza3.

| Feeding period | | | Inoculation periods on Giza3 | |
|---|---|---|---|---|
| | | | *T/I | % |
| Acquisition periods on Giza3 for 2 days | Time in min | 5 | 5/10 | 0 |
| | | 10 | 7/10 | 10 |
| | | 15 | 7/10 | 20 |
| | | 30 | 7/10 | 30 |
| | | 45 | 7/10 | 30 |
| | Time in h | 1 | 7/10 | 30 |
| | | 4 | 6/10 | 40 |
| | | 6 | 5/10 | 40 |
| | Time in days | 1 | 9/10 | 80 |
| | | 2 | 10/10 | 100 |
| | | 3 | 10/10 | 90 |
| | | 4 | 9/10 | 50 |
| | | 5 | 8/10 | 60 |
| | | 6 | 8/10 | 80 |
| | | 7 | 7/10 | 70 |

*Infectivity = Number of infected plants (I) / Total number of inoculated plants (T).

cv. Diamont leaves and 1 mg/kg Ph. Floridana leaves.

Purified sap preparation of BLRV obtained from broad bean cv. Giza3 plants was used for rabbits immunization. Antiserum titer and antigen dilution end-point were determined using microprecipitation test under paraffin oil shown in Table 5. The titer of BLRV imported antiserum was 1/1024 and 1/8 at purified antigen dilution of 1/32 and 1/128, respectively. The dilution end-point of purified antigen was 1/128 when a dilution of 1/8 of imported antiserum was used.

**Table 5.** Determination of imported antiserum titer and antigen (purified BLRV) dilution end point.

| Dilution of purified sap | Dilution of antiserum | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum dilution | | | | | | | | | | |
| | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/2048 |
| 1/4 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | - |
| 1/8 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | +5 | - |
| 1/16 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | +4 | - |
| 1/32 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | - |
| 1/64 | +2 | +2 | +2 | - | - | - | - | - | - | - | - |
| 1/128 | +1 | +1 | +1 | - | - | - | - | - | - | - | - |
| 1/256 | - | - | - | - | - | - | - | - | - | - | - |

- = No visible reaction, +1 = faint reaction, +2 = weak reaction, +3 = moderate reaction, +4 = strong reaction, +5 = very strong reaction.

**Table 6.** Determination of induced antiserum titer and antigen (purified BLRV) dilution end point.

| Dilution of purified sap | Dilution of antiserum | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum dilution | | | | | | | | | | |
| | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/1028 |
| 1/4 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | - | - |
| 1/8 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | - | - |
| 1/16 | +2 | +2 | +2 | - | - | - | - | - | - | - | - |
| 1/32 | +1 | +1 | +1 | - | - | - | - | - | - | - | - |
| 1/64 | - | - | - | - | - | - | - | - | - | - | - |
| 1/128 | - | - | - | - | - | - | - | - | - | - | - |
| 1/256 | - | - | - | - | - | - | - | - | - | - | - |

- = No visible reaction, +1 = faint reaction, +2 = weak reaction, +3 = moderate reaction, +4 = strong reaction, +5 = very strong reaction.

**Table 7.** Determination of imported antiserum titre and antigen ( infectious sap) dilution end point .

| Dilution of purified sap | Dilution of antiserum | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum dilution | | | | | | | | | | |
| | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 | 1/256 | 1/512 | 1/1024 | 1/1028 |
| 1/4 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | - | - |
| 1/8 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | - | - |
| 1/16 | +3 | +3 | +3 | - | - | - | - | - | - | - | - |
| 1/32 | +2 | +2 | +2 | - | - | - | - | - | - | - | - |
| 1/64 | +1 | +1 | +1 | - | - | - | - | - | - | - | - |
| 1/128 | - | - | - | - | - | - | - | - | - | - | - |
| 1/256 | - | - | - | - | - | - | - | - | - | - | - |

- = No visible reaction, +1 = faint reaction, +2 = weak reaction, +3 = moderate reaction, +4 = strong reaction, +5 = very strong reaction.

On the other hand, in Table 6 shown the titer of the prepared BLRV antiserum was 1/512 and 1/8, at purified antigen dilution of 1/8 and 1/32, respectively. The dilution end-point of the purified antigen was 1/32 when dilution 1/8 of prepared antiserum was used.

The titer of BLRV imported antiserum was 1/512 and 1/8 at clarified antigen dilutions 1/8 and 1/64, respectively in Table 7. The dilution end-point of the clarified antigen was 1/64 when a dilution of 1/8 of imported antiserum was also used.

The titer of prepared BLRV antiserum was 1/256 and 1/8 at clarified antigen dilution of 1/8 and 1/16, respectively.

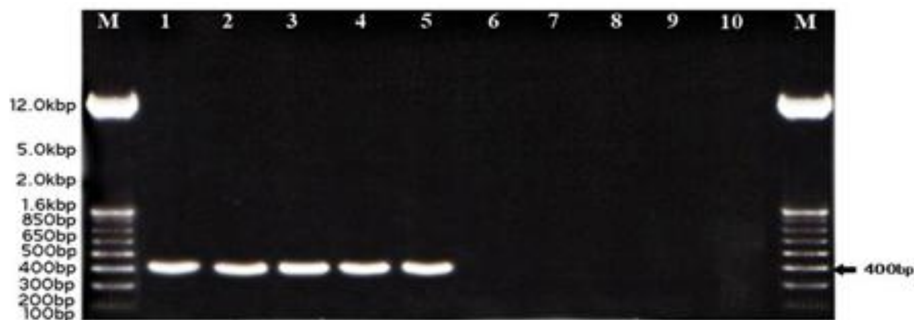


**Figure 2.** RT-PCR for BLRV-RNA extracted from infected tissues. A product of 400 bp was produced from the amplification of BLRV-RNA. Lanes 1, 2, 3, 4 and 5 are BLRV isolated from infected *Vicia fabae* cvs. Giza3, Giza 461, Giza 717, Giza 429 and Giza 714 leaves, respectively. Lanes 6, 7, 8, 9 and 10 are *Vicia fabae* cvs. Giza3, Giza 461, Giza 717, Giza 429 and Giza 714 healthy control leaves, respectively. M: Marker.

The dilution end-point of the clarified antigen was 1/16 when a dilution of 1/8 prepared antiserum was used. On the other hand, the controls of normal serum, healthy plant sap and buffer NaCl 0.85% gave negative reactions in all tests. Similar results had already been observed by D'Arcy et al. (1989); Hewings et al.(1990); Larsen and Webster (1999); Schwinghamer et al. (1999); Van Leur et al. (2002); Kumari et al. (2006).

The original BLRV isolate was obtained from naturally infected broad bean plants collected from El- Tal El-Kaber Farm of Ismailia governorate and the purified sap from BLRV isolate gave positive reaction with imported and prepared BLRV antibody and negative reaction with BYMV and FBNYV antibodies.

In this work, we used a simple, specific and rapid method for the detection of BLRV coat protein gene in plants by RT-PCR. This technique was used as an effective method to confirm the validation of RT-PCR 400 bp product. The results shown in Figure 2 demonstrate that the amplified sequence from total RNA was readily detected in all ELISA-positive samples.

A major PCR product of about 400 bp coat protein gene, *cp*, was present in samples-bearing BLRV. Our results are in harmony with those reported for BLRV by Prill et al. (1990); Cavileer and Berger (1994); Larsen and Webster (1999); D'Arcy et al. (2000); Smith et al. (2000); Terauchi et al. (2001); Terradot et al. (2001); Leslie et al. (2002); Shahraeen et al. (2005); Thekke Veetil et al. (2009) and Chomic et al. (2010). No specific products occurred for healthy samples. Similar results have been obtained by Pantaleo et al. (2001) using the same primers with BLRV isolate from Italy. The procedure is quick, valuable diagnostically and can be used as a preliminary method to detect virus in infected plants.

We found that the serological and molecular procedures used in this study have potential merit for routine testing in diagnostic laboratories. The disease still under investigation using many techniques such as cloning, sequencing and phylogenies of the Egyptian BLRV isolate and other worldwide isolates. It needs more attention as a serious problem that could become uncontrollable and may be fast spreading via insect vectors circulative in persistent manner.

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