The Effectiveness of The Bacteria *Rhizobium Leguminosarum* Against Bean Yellow Mosaic (BYMV) Potyvirus Infecting Faba Bean (*Vicia faba L.*) Plants.

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Abstract

A virus causing different symptoms of mosaic, mottling, leaf rolling and distortion in (*Vicia faba*) was found in various fields in Menofia Governorate, Egypt. The virus isolate was detected and identified to bean yellow mosaic virus (BYMV) using symptomatology, indicator plants, serological tests (DAS-ELISA and Dot blot immunoassay (DBIA)) and Light microscope. Also, the study conduct to evaluate the efficiency of two strain of *Rhizobium leguminosarum* (*R. leguminosarum* strain MNF-EM-R4 and *R. leguminosarum* bv.*viciae* strain MNF-EM-R9) for inducing systemic acquired resistance in faba bean to bean yellow mosaic virus (BYMV). Our findings showed that grown faba bean plants from treated seeds with rhizobia and inoculated with BYMV presented a imperative reduction in disease occurrence percentage and ELISA reads. While, these treatments caused an increase in Peroxidase enzyme activity compared with control. SDS-PAGE analysis showed that treatment with *R. leguminosarum* strain MNF-EM-R4 showed one band, this band did not appear in negative control, positive control and treatment with *R. leguminosarum* bv.*viciae* strain MNF-EM-R9 at molecular weight (40 kDa). Treatments with *R. leguminosarum* strain MNF-EM-R4 and treatment with *R. leguminosarum* bv.*viciae* strain MNF-EM-R9 showed one band did not appear in negative, positive control, at molecular weight (85 kDa).

Key words: *Vicia faba*, BYMV, ELISA, SAR, *Rhizobium leguminosarum* and SDS-Page

Introduction

Faba bean plant is one of food legume crop in the most countries of the world. Because of it has a lot of protein content, it can be eaten as green or dried seeds as part of a healthy diet (Robinson *et al.*, 2019). In Egypt, this crop is recognized to be naturally infected by various viruses. From them, Bean Yellow Mosaic (BYMV) belongs to *Potyvirus* genus in *Potyviridae* family of plant viruses (*Zeid*, 2016). Symptoms induced by BYMV on Faba bean plants were, mosaic, deformation of the new leaves, leading to stunting of plants, reduction of nodulation and considerable yield losses (*Efaisha*, 2005; *Zeid*, 2016; Moury and Desbiez, 2020 and Hosny, *et al.*, 2021).

Control of plant viral diseases can be accomplished over inducing plant defense mechanisms, e.g., systemic acquired resistance (SAR) (*Rylls*, *et al.*, 1994). SAR to viral infection can be recognized by using non-pathogenic rhizobacteria (*Van Loon* *et al.*, 1998). Useful rhizobacteria can stimulate plant growth directly over releasing secondary metabolites that have the ability to facilitate the approval of certain nutrients from the root environment ( *Plant Growth Promoting Microorganisms, PGPM*) and indirectly over SAR in plants against pathogens, including viruses (*Whitps*, 2004 and *Elbadry*, *et al.*, 2006). Between of PGPM, *Rhizobium* spp. Were reported to activate plant defense compounds (*Phytoalexins*, *phenols*, pathogenesis-related protein genes, flavonoids and others) related with disease management of alfalfa and beans (*Dakora* *et al.*, 1993 and *Dakora*, 2003).

One of the enzymes involved in the plant defensive response to pathogen infection is peroxidase, which is generally the first to show changes in activity. Peroxidase activity is stimulated the most in hypersensitive hosts, while improved activity has also been documented in hosts that allow systemic infection (*Mojca* *et al.*, 2001).

The aggregation of pathogenesis-related (PR) proteins, which are normally thought to be indicators of the defensive response, was linked to an increase in resistance (*Ward* *et al.*, 1991). Several proteins are induced in treated plants which are commonly mentioned to as "pathogenesis-related proteins (PRs). These PRs are classified as plant-coded proteins that are induced in diseased or associated states (*Van Loon* *et al.*, 1994) induced locally and systemically in the infected leaves and are linked to the improvement of systemic acquired resistance (SAR) against increased infection by bacteria, fungi, and viruses.

The induction of PR proteins in several plant tissues is one of the main biochemical and molecular actions when plant are subjected to infections with pathogens for example viroid, viruses, fungi and bacteria (*Van Loon*, 1997). When plants are attacked by different pathogens, one of the major biochemical and molecular responses is the induction of PR proteins in several plant tissues (*Van Loon*, 1997).
2. Material and methods:

2.1. Source of Bean yellow mosaic virus isolate:

BYMV was isolated from Vicia faba L. cv.(Balady) plants that were naturally infected and gathered from garden of faculty of agriculture, Menoufia University. Leaf samples showing mosaic, malformation and leaf curling symptoms, were considered to be caused by virus infection, suspected in following investigates.

2.2. Biological identification (Host range and symptomatology):

The leaf samples were homogenised with phosphate buffer (1:5 w/v, 0.1 M, 0.1ML, pH 7.2) in a sterile mortar (Mahdy et al., 2007). The sap was then filtered twice using a double sheet of cheesecloth. The sap used to mechanical inoculation into healthy indicator plants as: Vicia faba L cv. Balady, Phaseolus vulgaris Lcv karnk, Pisum sativum Lcv Mister B, Chenopodium album, Ch. Quinoa, Datura stramonium L. and Nicotiana glutinosa L.

2.3. Serological identification of BYMV isolate:

Serological assays were done using direct ELISA, positive reaction obtained with the specific antiserum as described by (Clark and Adams, 1977) and Dot blot immunobinding assay was used as described by (Lin et al., 1990).

2.4. Light microscopy:

In the LM laboratory FARP, Faculty of Agriculture Research Park-Cairo University, anatomical changes caused by virus infection were explored using faba bean leaves.

2.5. Seeds sterilization and bacterialization:

Surface sterilization of faba bean seeds was done in stages with 70% (v/v) ethyl alcohol (30 sec.) and 7% (w/v) calcium hypochlorite solution (30 min.) before being rinsed three times. The seeds were kept in a 100mL beaker with 50mL of sterile distilled water overnight (SDW). The water was removed after soaking, and the seeds were cleaned 3-4 times with SDW. The sterilized seeds were submerged in a Rhizobia suspension for two strain of R. leguminosarum (R. leguminosarum strain MNF-EM-R4 and R. leguminosarum bv. viciae strain MNF-EM-R9), these isolates identified by( Enas, et al., 2020) for one hour then leaved seeds at room temperature to dry in a sterilized petri dish. Five seeds were sown in a sterile mixture of soil and sand (2:1) in plastic pots. Treatments were arranged as follows: (T1) Healthy plants without BYMV infection and bacterial inoculation (negative control), (T2) Infected with BYMV without rhizobia inoculation (positive control), (T3) Inoculated by Rhizobium leguminosarum strain MNF-EM-R4 and infected with BYMV and (T4) Inoculated by Rhizobium leguminosarum bv. viciae strain MNF-EM-R9 and infected with BYMV virus. The plants of T2, T3 and T4 were mechanically inoculated with BYMV after two weeks of germination and grew in a Greenhouse for six weeks at 25 ± 2 °C. Three replicates in each.

2.6. Disease incidence (DI %) :

45 days after planting, All plants with BYMV symptoms were documented. The following calculation was used to estimate the DI: DI = (Number of symptomatic plants/Total number of plants) x 100 (Reddy et al., 1983).

2.7. Determination of Peroxidase enzyme activity:

Peroxidase activity was determined using the method defined by (Fehman and Dimond, 1967). Aliquots supernatants were analyzed for measuring the peroxidase activity by SPEKOL spectrophotometer at 470 nm. Three replicates were used in each treatment This study was conducted in Central Lab, Faculty of Agriculture, Menoufia University.

2.8. Protein Electrophoresis:

This trail was done to exhibit the reacted antiviral proteins against BYMV infection which initiated because of treating faba bean leaves with R. leguminosarum comparing with those initiated in faba bean leaves of healthy and infected only with BYMV. In this respect, electrophoretic analysis using SDS-PAGE technique as described by (Laemmli, 1970) was used for this purpose. Screening for induced systemic resistance against BYMV infection by making rabid isolation of initiated antiviral proteins and others according to the described protocol by (Bollag et al., 1996) who used SDS-PAGE analysis.

Statistically analysis:

One-way analysis of modification (ANOVA) was used to analyze the data, following by LSD test for mean separation. Statical significance was defined as P value <0.05 (CoStat-statistic software, CoHort software).

Results

3.1. The isolate of Bean yellow mosaic virus:

BYMV was isolated from naturally infected Vicia faba L. cv. (Balady) plants, were collected from
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Garden of faculty of agriculture, Menoufia University. Leaf samples showing mosaic, malformation, leaf curling symptoms and reduction of root nodulation as clear in (Fig. 1).

**Fig (1):** A&B: Green mosaic, Leaf roll and malformation on infected faba bean leaves. C: Infected root of infected faba bean plant, showing reduction of root nodulation. D: Roots of healthy faba bean plant.

### 3.2. Biological identification:

The virus isolate was mechanically inoculated into seven plant species and cultivars from three different families. Different plants' reactions to BYMV were divided into different categories according to their reaction (Table 1 and Fig. 2).

### Table (1): Susceptibility to mechanical inoculation with the isolated BYMV and responsiveness of different plants, under greenhouse conditions.

<table>
<thead>
<tr>
<th>Family</th>
<th>Scientific name</th>
<th>English name</th>
<th>Variety</th>
<th>Symptoms induced</th>
<th>ELISA test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenopodiaceae</td>
<td><em>Chenopodium album</em></td>
<td>Ouares</td>
<td>—</td>
<td>CLL</td>
<td>+</td>
</tr>
<tr>
<td><em>Ch. quinoa.</em></td>
<td>Gooses Foot</td>
<td>—</td>
<td>CLL</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Leguminosae</td>
<td><em>Phaseolus vulgaris</em> L.</td>
<td>Common bean</td>
<td>Karnk</td>
<td>SYM &amp; Bl &amp;</td>
<td>+</td>
</tr>
<tr>
<td><em>Pisum sativum</em> L.</td>
<td>Garden pea</td>
<td>Mister B</td>
<td>GM &amp; LR</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Vicia fabae</em> L.</td>
<td>Broad bean</td>
<td>Balady</td>
<td>VC &amp; M</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Solanaceae</td>
<td><em>Datura stramonium</em> L.</td>
<td>Jimson-weed</td>
<td>NLL</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Nicotiana glutinosa</em> L.</td>
<td>Virginia plant</td>
<td>—</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviation of symptoms:**

- **CLL** = Chlorotic local lesion
- **LD** = Leaf deformation
- **O** = No symptoms
- **VC** = Vein chlorosis
- **SYM** = Systemic yellow mosaic
- **GM** = Green mosaic
- **LR** = Leaf roll
- **BL** = Blisters
- **M** = Mosaic
- **NLL** = Necrotic local lesion
- **SYM** = Systemic yellow mosaic
- **GM** = Green mosaic
- **LR** = Leaf roll
- **BL** = Blisters
- **M** = Mosaic
3.3. Serological identification:
The isolated virus was detected by DAS-ELISA, virus were examined by light microscope after contrast positive reaction obtained with the specific antiserum. The investigations revealed large difference between healthy and infected tissues. Mesophyll layers technique (DBIA) on nitrocellulose membrane which was reduced and compact in infected plants and the size of plastide cells became short and compact. Also spongy cells were unorganized when compared with healthy ones. The phloem layers and the xylem arms number were reduced and disorganized in the infected cells than in healthy ones (Fig.3 A and B).

3.4. Light microscopy:

Fig. (2): Response of some indicator hosts to mechanical inoculation with the BYMV isolate. A: Chlorotic local lesions on *Ch. quinoa* L. B: Chlorotic local lesions on *Ch. album* L. C: Necrotic local lesion on *Datura stramonium* L. D: Systemic yellow mosaic, blisters and leaf deformations on *Phaseolus vulgaris* L.
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**Fig (3). A:** Dot Blot Immunoassay for BYMV precipitation against specific IgG- BYMV polyclonal. (+: Positive samples -: Negative samples) **B:** A: Light micrograph of healthy leaves (Pc: Plaside cells, Sc : Spongy cell and Xv: Xylem vessels) **C:** Light micrograph of infected leaves showing reducing and compacting of cells (Pc, Sc) and number of xylem arms and phloem layers

### 3.5. Disease incidence:

BYMV-inoculated plants grown from rhizobium- treated seeds showed a considerable decreasing (P=0.05) in disease incidence (DI%) when compared to plants emerged from non-rhizobium treated seeds (Table 2). Data present in table (2) indicated that plants emerged from Rhizopium- inoculated seeds (T4) showed considerable decreasing in DI (22.06 %) followed by those grown from rhizobium treated seeds, 17.27% (T3) compared with 91.33% for the challenged control (infected with virus and without Rhizopium (T2)).

### 3.6. DAS-ELISA:

At the same time, the treated of faba bean seeds with rhizopium reduced significantly the concentration of BYMV in plants as proved by a significantly (P=0.05) decrease in the absorbance values of ELISA reactions (Table 2).
Table 2. Valuation of plant growth improvement rhizobacteria – by inducing systemic resistance to BYMV in 45 – days old faba bean plants.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>DI(1)</th>
<th>ELISA(3)</th>
<th>POA(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>0.00</td>
<td>0.08</td>
<td>0.13</td>
</tr>
<tr>
<td>T2</td>
<td>91.33</td>
<td>1.74</td>
<td>0.20</td>
</tr>
<tr>
<td>T3</td>
<td>17.27</td>
<td>0.55</td>
<td>0.51</td>
</tr>
<tr>
<td>T4</td>
<td>22.06</td>
<td>0.60</td>
<td>0.48</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>1.913</td>
<td>0.5191</td>
<td>0.0998</td>
</tr>
</tbody>
</table>


(2) Disease incidence (%).

(3) ELISA absorbance reads, were measured at 405 nm.

(4) Peroxidase activity expressed as absorbance changes at 470 nm.

3.7. Determination of peroxidase enzyme activity:

The obtained results in table (2) showing a significant increase in (POA) in plants that inoculated with rhizobium compared to the negative and positive control. T3 (*Rhizobium leguminosarum* strain MNF-EM-R4)- inoculated plants showed a very high POA.

3.8. Antiviral protein (SDS-Page):

SDS-PAGE analysis was used to analysis the protein profiles using faba bean leaves (negative control, positive control, inoculated by *R. leguminosarum* strain MNF-EM-R4 and infected by BYMV and inoculated by *R. leguminosarum* bv. viciae strain MNF-EM-R9 and infected with BYMV virus. Fig (4) shows a considerable difference in protein profiles of all treatments with molecular mass from 15 kDa to 254 kDa. All treatments contain the main protein band between (21 to 128 kDa). The SDS-PAGE protein gel is analyzed using Clustering analysis using Jaccard's average (UPGAMA) clustering method, version (3.1). The zero-one (Table 3) was done for the gel according to the molecular mass of marker and existence of each band in the same molecular mass in all lanes of the gel. The zero one results showed that:

- Treatment with *R. leguminosarum* strain MNF-EM-R4 showed one band, this band did not appear in negative control, positive control and treatment with *R. leguminosarum* bv. viciae strain MNF-EM-R9 at molecular weight (40 kDa).
- Treatments with *R. leguminosarum* strain MNF-EM-R4 and treatment with *R. leguminosarum* bv. viciae strain MNF-EM-R9 showed one band did not appear in negative control, positive control treatments at molecular weight (85 kDa).

Table 3. Zero, one of negative control, positive control, BYMV+ R.leguminosarum (MNF-EM-R4) and BYMV+ R.leguminosarum (MNF-EM-R9).

<table>
<thead>
<tr>
<th>Total MW</th>
<th>Negative control</th>
<th>Positive control</th>
<th>BYMV+ R.leguminosarum (MNF-EM-R4)</th>
<th>BYMV+ R.leguminosarum (MNF-EM-R9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>128</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>107</td>
<td>1</td>
<td>1</td>
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<tr>
<td>103</td>
<td>1</td>
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<tr>
<td>85</td>
<td>0</td>
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<tr>
<td>76</td>
<td>1</td>
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<td>21</td>
<td>1</td>
<td>1</td>
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</table>

Discussion:

Under field conditions, BYMV severely affects various crops including faba bean plant (Vicia faba L.), this plant is among the most important economic legume crops grown in several areas of Egypt (Fegla et al., 2003, Deya Eldeen, 2008 and Zeid, 2016). In the present study, seven plant species from three various families, were tested for their sensitivity to infection by virus isolate. Described symptoms in leaves of Ch. album, Ch. Quinoa, Vicia faba L., Phaseolus vulgaris and Pisum sativum are nearly the same as described by (Sameh, 2005 and Deya El-deen et al., 2008) for bean yellow mosaic virus. Inoculations were further established by positive reactions to the antiserum against BYMV in DAS-ELISA test. But no visible symptoms were induced with Nicotiana glutinosa L (Hammed, 2006).

The isolate of BYMV was detected using serological tests (DAS-ELISA and Dot blot technique) which were positively used. Many investigators have used ELISA and Dot blot tests for serological detection of BYMV from different hosts (Kumar et al., 2015, Pradeep et al., 2015, Zeid, 2016 and Hosny et al., 2021). TBIA approach has the advantage of being able to detect BYMV with a small amount of antigen compared to standard ELISA, as well as providing simplicity, speed, sensitivity, and convenience to large numbers of samples.

Light microscropy (L.M) is still essential in the study of histological abnormalities of tissue, and it can be used to check for the presence or absence of inclusion bodies (Matthews, 1991). Semi thin sections of healthy and infected leaves were scanned after staining with fast green by light microscropy. The investigations revealed significant differences between healthy and infected tissues: the infected tissue's mesophyll layer was reduced and compacted, and the size of spongy and palisade cells was reduced, and the number of xylem arms and phloem layers was decrease compared to the healthy; similar findings were reported by others (Deya Eldeen et al., 2008).

Rhizobium species (PGPR) are widely used in agriculture for the advancement of legume crops due to its capability to fix atmospheric nitrogen in symbiosis with legumes. Improving faba bean plants by inoculation with rhizobia has been examined in the present work for suppression BYMV infection. This suppression induced through rhizobia may be direct by inhibition of plant growth or indirect by stimulating plant defense mechanisms (Kacem et al., 2009).

The first parameter used to assess the occurrence of SAR in faba bean plants treated with rhizobia was the reduction in BYMV percentage disease incidence as well as significant ELISA-value reduction. Our results are in harmony with those reported by (Rakib and Mustafa, 2013) who mentioned that BYMV infection caused a decrease in dry weight of shoot and root also, nodule number, whereas, inoculation with rhizobia induced significant increase in these parameters. Plants grown from seeds treated with Pseudomonas and Rhizobium bacteria showed a significant decrease in % disease incidence as well as a significant decrease in ELISA readings (virus concentration) as compared to untreated seeds with bacteria, according to Elbadry et al., (2006).
Systemic resistance promotion by rhizobacteria against Fusarium wilt in carnations seems to be due to bacterial lipopolysaccharides (LPS) (Van Peer and Schippers, 1992). Furthermore, exopolysaccharides (EPS) and lipopolysaccharides (LPS) constitute the majority of Rhizobium cell surface carbohydrates. Polysaccharides play an important role in the symbiotic connection between legumes and Rhizobium throughout the recognition process (Denny, 1995).

The PGPR-suppression SR is frequently linked to the initiation of the defence mechanism, which includes the increased presence of defence enzymes such as peroxidase (Bergstrom et al., 1982). Increased peroxidase activity was recorded in Rhizobium-inoculated plants in our study. This result is in agreement with those of Elbadry et al., (2006) who mentioned that the degrees of PO activity were improved in faba bean leaves inoculated with Pseudomonas and Rhizobium bacteria. PO is one of the oxidative enzyme, play an great role in different physiological in plant specially metabolism and anabolism. It hydrolyzes hydrogenperoxide caused by dehydrogenase enzyme to water (Mengel, 1979).

The proteins of induced faba bean plants were examined using SDS-Page, and our findings revealed the formation of a novel pattern of proteins, as well as several increases in band density among biotic inducer treatments. The produced proteins may help to inhibit virus propagation or multiplications. The proteins of induced faba bean plants were examined using SDS-Page, and our findings revealed the formation of a novel pattern of proteins, as well as several increases in band density among biotic inducer treatments. The produced proteins may help to inhibit virus propagation or multiplications (Mahmoud, 2000). Rakib and Moustafa (2012) mentioned that The polypeptide profile of viral particles obtained on the polyacrylamide gel electrophoresis, revealed a single protein of about 34 kd, representing the BYMV coat protein. A protein migrating at the similar speed in the profile of proteins from BYMV-infected plants was also identified; this protein being completely absent in the profile of uninfected plants. Electrophoretic tests by SDS-Page showed that foliar treatment with two plant extracts and their major components induced resistance against BYMV, therefore resulted in inducing new proteins, which were not established in healthy or infected and untreated plants. It has been recommended that, the induced proteins can help to limit virus infection or virus multiplication (Chen et al., 2006).

**Conclusion**

In conclusion, under greenhouse conditions, *R. leguminosarum* was isolated from faba bean roots and used as a promising inducer for SR in faba bean to BYMV infection. Because it is simple, cheap, and environmentally safe. This result could be a highly important strategy for controlling BYMV infection.

**References**


فعالية بكتريا 

Rhzobium leguminosarum

ضد فيروس موزايك الفاصولياء الأصفر على نباتات الفول البلدي

الخطاب:

تتم الحصول على فيروس بسبب أعراض مختلفة (وزايك - تبقعات - أوراق - عوائل) على نباتات الفول البلدي في مناطق مختلفة في محافظة المنوفية - مصر. تم تعريف هذه العزلات بواسطة الأعراض - العوائل المشخصة - الأختبارات السيرولوجية في Leguminosarum Rhizobium. أجريت الدراسة لقياس كفاءة البكتريا العديدة تحفيز المقاومة الجهازية في نباتات الفول البلدي ضد الفيروسات الفاصولية الأصفر. وقد أظهرت النتائج أن عزلات من هذه البكتريا تكون تأثيراً إيجابياً في نباتات الأعشاب وذلك أثناء التعرض للفيروس (قراءة الإليزا). بينما أدت هذه المعالمة إلى زيادة في نشاط الإنزيم البيروكسيداز مقارنة بالكنترول السليم والمصاب الغير معامل بالبكتريا. أظهر تحليل البروتين باستخدام PAGE أن المعالمة بـ R. leguminosarum strain MNF-EM-R4 بالكترول السليم والمعملولات المصاب الغير معامل بالبكتريا.