

Bean Yellow Mosaic Virus (BYMV) on Broadbean: Characterization and Resistance Induced by *Rhizobium leguminosarum*

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Bean yellow mosaic virus (BYMV) was isolated from symptomatic plants in Iraq. The virus was characterized by its reaction in indicator hosts, aphid transmission, particle protein size and serological reaction. A study was conducted to evaluate the activity of *Rhizobium leguminosarum* against BYMV in broadbean plants and its role in plant growth stimulation. It was found that broadbean plants grown from rhizobia-treated seeds and sap-inoculated with BYMV showed a significant reduction in disease incidence percentages, 40% compared to 93% in non-treated plants. *R. leguminosarum* induced a significant reduction in virus concentration in plants grown from rhizobia-treated seeds and mechanically inoculated with the virus, as proved by a decrease in absorbance values of ELISA reactions, 0.4 compared with 1.5 in the control. Increases in foliage and root fresh weights and in nodule numbers were observed in plants emerged from rhizobia-treated seeds, 158.25 g, 79.25 g, 100.6 nodules compared to 118.0 g, 72.25 g, 18.5 nodules in control plants. The results obtained indicate that rhizobia may play an important role in BYMV disease management in broadbean.

Key words: Disease management, ELISA, Nodulation, PGPM, *Vicia faba*.

Broadbean (*Vicia faba* L.) is one of the most important grain legumes in the world and Iraq, and represents an important source of protein. Broadbean has the capacity to develop a symbiotic interaction with *Rhizobium leguminosarum* that enhances plant growth through N₂-fixation, improving nutrient acquisition, phytohormone production, and suppression of soil plant pathogens (Sahran and Nehra 2011, Zanatta *et al.* 2007).

Broadbean is known to be naturally infected by many viruses. Among them *Bean yellow mosaic virus* (BYMV, genus *Potyvirus*) is

the most serious problem (Nakazono-Nagaoka *et al.*, 2004). The virus is worldwide distributed and causes mosaic and severe deformation of the new leaves, leading to stunting of plants, reduction of nodulation, and considerable yield losses (Osman and El-Sheikh 1999, Elbadry *et al.* 2006). It was reported that the virus is transmitted from infected to healthy plants by several species of aphids in a non-persistent manner and by seeds of some legumes including broadbean at a low rate, in such a way that the virus cannot be managed chemically (Elbadry *et al.* 2006).

The management of plant viral diseases may be accomplished through inducing plant defence mechanisms by using non-pathogenic rhizobacteria (Van Loon *et al.* 1998). Beneficial rhizosphere microorganisms can stimulate plant growth directly through releasing secondary metabolites that facilitate the uptake of certain

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nutrients from the root environment (Plant Growth Promoting Microorganisms, PGPM), and indirectly through inducing systemic resistance in plants against pathogens, including viruses (Zehnder *et al.*, 2001, Whipps 2004, Elbadry *et al.* 2006, Vassilev *et al.*, 2006). Among PGPM, *Rhizobium* spp. were reported to activate plant defence compounds (phenolics, flavonoids, phytoalexins and others) associated with disease control in alfalfa and beans (Dakora *et al.*, 1993, Dakora 2003, Mishra *et al.*, 2006). *R. leguminosarum* was reported to induce a high activity of peroxidase and phenylalanine ammonia lyase (Mabrouk *et al.* 2007).

This study was conducted to evaluate the efficiency of a seed treatment with a *R. leguminosarum* suspension in the reduction of BYMV effects on broadbean plants.

MATERIALS AND METHODS

Preparation of inoculum

Leaves from broadbean plants showing mosaic, leaf deformation, stunting and yellowing in broadbean fields in the Baghdad area were collected in the springs of 2010 and 2011. The leaves were homogenized with a mortar and pestle, with 0.01M phosphate buffer pH=7.0, containing 0.2% sodium diethyl dithiocarbamate (DIECA), at 1:4 (g/ml). The extract was passed through two layers of muslin and the filtrate used as virus inoculum.

Test plants and inoculations

Seeds of *Chenopodium amaranticolor*, *Ch. murale*, *Ch. quinoa*, *Gomphrena globosa*, and *Tetragonia expansa* were sown in pots (30×20 cm) containing a mixture of soil and peatmoss (3:1). The pots were watered and maintained in a greenhouse (20-25°C). The seedlings were transplanted to other pots (10×10 cm) containing soil mixture. The upper surfaces of test plant leaves, at 4-5 leaf stage, were dusted with carborundum 600 mesh and gently rubbed with virus inoculum. The plants were observed daily for symptom development. The virus was re-isolated from a single lesion on *Chenopodium amaranticolor* and maintained on broadbean plants.

Virus transmission

Aphids from broadbean, alfalfa, and some weed plants were collected and identified at the

Natural History Museum, University of Baghdad. The aphids were previously given access to healthy broadbean plants for several times and then given an acquisition period of 1, 5 or 10 minutes on infected broadbean plants, then transferred onto healthy plants for 30 min in muslin covered cages. The plants were sprayed with an insecticide and maintained under greenhouse conditions until symptom development.

DAS-ELISA

The virus was detected by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). The BYMV-specific ELISA kit was obtained from the Agdia Company (Elkhart, USA). Leaves of infected and healthy broadbean plants were homogenized in phosphate buffered saline (PBS; 10 mM Na₂HPO₄, 0.14 M NaCl, pH=7.0) at 1 g: 10 ml. The extracts were filtered through double layers of muslin and tested by DAS-ELISA as described by Clark and Adams (1977). Absorbance values twice the value of healthy plants at 405 nm were considered positive.

Tissue blot immunoassay (TBIA)

Petioles of infected and non-infected leaves were cut with a razor blade. The cut surfaces were immediately blotted on the surface of a nitrocellulose membrane (0.45 µm pore size) for several seconds and allowed to air dry. The membrane was incubated in blocking buffer (PBS containing 5% skimmed milk powder) for 30 min at room temperature (RT) in a Petri dish. The membrane was washed three times in PBS containing 0.025% Tween-20 (PBST) and incubated with polyclonal antibodies to BYMV (1: 500) (Agdia) for one hour at RT. The membrane was washed three times as before and incubated in alkaline phosphatase conjugated goat anti-rabbit IgG at a dilution of 1:2000 in conjugated buffer (PBS containing 0.2% BSA) for one hour at RT. The membrane was washed three times and incubated in substrate buffer (PBS containing 730 µg/ml of Nitrobluetetrazolium (NBT), and 500µg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), pH=9.5) for 15 min at RT with shaking. The reaction was stopped by washing the membrane with distilled water. Violet to purple color indicated a positive reaction.

Polyacrylamide gel electrophoresis

BYMV particles were extracted and purified according to the procedure described by

Dhar and Singh (1994) for PVY^N. Total proteins from infected and healthy plants were obtained as described by da Rocha *et al.* (1986). The viral and plant proteins in 0.125 M Tris-HCl buffer containing 2% sodium dodecyl sulfate (SDS), 2% β -mercaptoethanol, 15% glycerol and 0.05% bromophenol blue as a tracking dye were incubated at 100°C for 3 min. The proteins then were analyzed by electrophoresis on 10% polyacrylamide vertical slab gel as described by Al-Ani *et al.*, (1979).

The bacterial isolate

A locale isolate of *Rhizobium leguminosarum* was obtained from the Department of Soil and Water Sciences, College of Agriculture, University of Baghdad. The bacteria were grown in yeast mannitol broth (YMB) at 28°C in a shaking incubator. The number of colony forming units per millilitre was determined by standard plate count on yeast mannitol agar (YMA). The original culture was maintained at 4°C.

Seed bacterization

Broadbean seeds were surface sterilized by immersing seeds in 2% sodium hypochlorite for 3 minutes, followed by rinsing with sterile distilled water and dried. The sterilized seeds were submerged in a *R. leguminosarum* suspension (10⁸ cells/ml) containing 2% arabic gum for one hour and dried at room temperature in a sterile dish. Seeds submerged in arabic gum without rhizobia served as a control. The seeds were sown in a sterile mixture of soil and peatmoss (3:1) in plastic pots (20×15 cm), at 10 seeds/pot. Treatments were arranged in a complete randomized design with 3 replicates, (T₁) *R. leguminosarum* + BYMV, (T₂) BYMV without rhizobia, (T₃) *R. leguminosarum* without virus, (T₄) no virus, no rhizobia. The plants of T₁ and T₂ were mechanically inoculated with BYMV after two weeks of germination, and grew in a Greenhouse for six weeks at 25 ± 2 °C and humidity 60%. There were ten plants per treatment arranged in a completely randomized design.

Effect of *Rhizobium leguminosarum* on BYMV infection

To assess the effect of *R. leguminosarum* on BYMV infection, the percentage of diseased plants showing BYMV symptoms was recorded at the end of the experiment as described above. Additionally to confirm the presence of the virus in plants and to assess the effect of the treatments on plant growth, ten plants of each of the four

treatments were carefully removed from their pots, washed of soil and air dried. Then, BYMV infection was confirmed by DAS-ELISA as described above, and the number of nodules and fresh weight of shoot and root was also measured.

Statistical analysis

Percent disease incidence, ELISA absorbance readings, shoot and root fresh weight and number of nodules were subjected to analysis of variance. Percentage values were arcsin transformed before analysis. The treatment means were compared using Fisher's least significant difference (LSD) test at $P=0.05$.

RESULTS

Symptomatology and transmission

Symptoms of faint yellowing were developed around the veins on the new leaves of broadbean plants after 7 days of sap inoculation with an extract of infected plants, followed by mosaic and distortion of the new leaves 2 weeks after inoculation (Fig. 1). The virus infected *Chenopodium amaranticolor*, *Ch. quinoa*, *Ch. murale*, and *Tetragonia expansa*, on which it induced chlorotic local lesions on the inoculated leaves 7 days after mechanical inoculation followed by deformation of the upper leaves 12 days after inoculation. The virus only produced necrotic local lesions on the inoculated leaves of *Gomphrena globosa*.

Results of vector transmission indicated that the virus is transmitted by several species of aphids in a non-persistent manner, of them *Aphis fabae* and *Myzus persicae* were found to be the most efficient. Symptoms of mosaic were developed on broadbean plants inoculated by viruliferous aphids given an acquisition feeding period of 1 min on infected plants.

Serological characterization

The anti-BYMV antibodies gave positive reaction with extracts from infected broadbean plants as shown by the development of an obvious yellow color in ELISA microplate wells. The mean absorbance value at 405 nm was 1.5 compared with 0.050 with extracts from non-infected plants.

A positive reaction, as a violet color, was developed on nitrocellulose membranes blotted with cut surfaces from leaf petioles of infected broadbean plants and reacted with anti-BYMV

Table 1. Effect of rhizobia on plant growth and BYMV multiplication

Treatments*	Percent disease incidence	ELISA **	Fresh weight (g)		Nodulation numbers
			Foliage	Root	
T ₁	40.00 b***	0.40 b	158.25 c	79.25 c	100.60 b
T ₂	93.00 a	1.50 a	118.00 d	72.25 c	18.50 c
T ₃	0.00 c	0.05 c	226.25 a	128.75 a	156.75 a
T ₄	0.00 c	0.05 c	207.25 b	114.25 b	20.75 c
L.S.D.	20.97	0.095	11.88	12.49	12.306
P = 0.05					

*(T₁) *R. leguminosarum* + BYMV, (T₂) BYMV without rhizobia, (T₃) *R. leguminosarum* without virus, (T₄) No virus, no rhizobia.** ELISA absorbance values were measured at 405 nm.*** Data were arcsin transformed prior to ANOVA analysis.

polyclonal antibodies (Fig. 2). No reaction was detected with blots of cut surfaces from leaf petioles of non-infected plants treated in the same way.

Proteins analysis

The polypeptide profile of viral particles obtained on the polyacrylamide gel electrophoresis (Fig. 3) revealed a single protein of about 34 kd, representing the BYMV coat protein. A protein migrating at the same speed in the profile of proteins from BYMV-infected plants was also detected; this protein being totally absent in the profile of uninfected plants.

Effect of *Rhizobium leguminosarum*

BYMV-inoculated plants grown from rhizobium-treated seeds showed a significant reduction ($P=0.05$) in percent disease incidence (PDI), when, compared to plants emerged from non-rhizobium treated seeds (Table 1).

At the same time, the treatment of broadbean seeds with rhizobia reduced significantly the concentration of BYMV in plants as proved by a significant ($P=0.05$) decrease in the absorbance values of ELISA reactions (Table 1).

Results indicated that the treatment with rhizobia significantly ($P=0.05$) improved plant growth in terms of fresh weight and nodule numbers. The foliage fresh weight was higher in both the virus-inoculated and virus uninoculated treatments that received the rhizobia (Table 1). For root fresh weight, however, plants treated or not with the rhizobia showed similar weight when inoculated with the virus. In relation to this, the virus significantly ($P=0.05$) reduced nodule formation on roots of plants from seed treated with rhizobia (Table 1).



Fig. 1. Symptoms of BYMV on the leaves of broadbean. A= Healthy plant; B= Infected plant

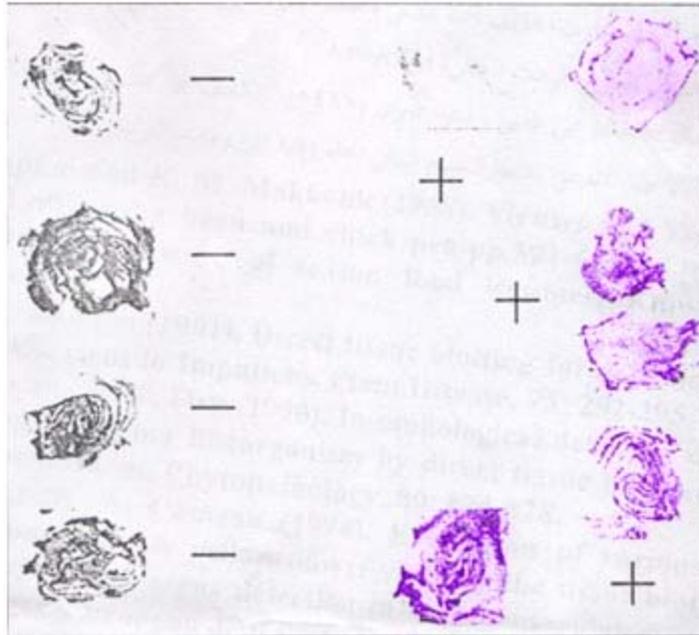


Fig. 2. Tissue blots of cross sections in broadbean leaf petioles of BYMV infected (+) and non-infected (-) leaves. The blots were reacted with polyclonal antibodies to BYMV

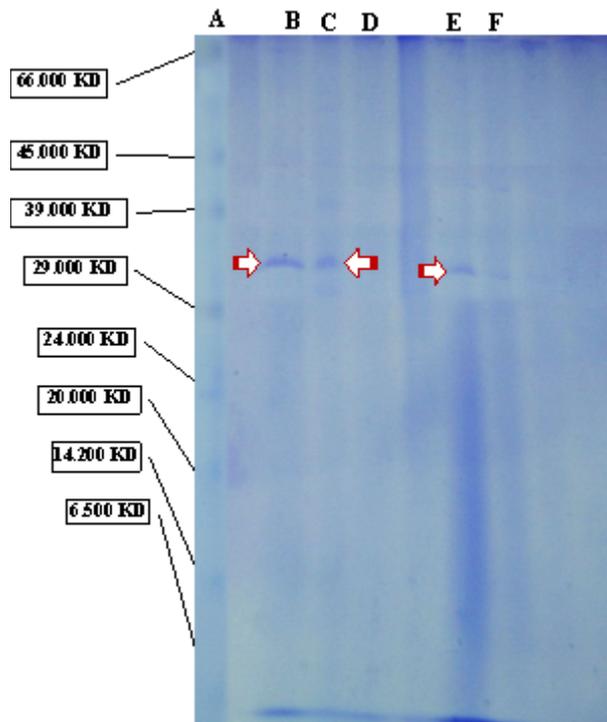


Fig. 3. Polypeptide pattern in SDS-polyacrylamide gel electrophoresis of purified BYMV (Track B), and of total proteins from BYMV-infected (Tracks C & E) and non-infected (Tracks D & F) broadbean plants. Note the presence of a protein of 34 kd (arrows) in tracks B, C & E representing viral coat protein and its absence in tracks D & F

DISCUSSION

The results of symptoms on indicator plants, serological and molecular studies obtained in this study provide evidence that the virus causing mosaic, distortion of the new leaves and yellowing on broadbean plants in Iraq is an isolate of *Bean yellow mosaic virus* (BYMV). Several previous studies reported similar characteristics to those induced by BYMV on broadbean in Iraq (Al-Noaimi *et al.*, 2003, Adhab 2009, 2012). However, this would be the first report in an indexed journal. Treatment of broadbean seeds with *R. leguminosarum* (in the absence of virus) significantly improved plant growth, as shown by an increase of foliage and root fresh weight and nodule numbers. Our results are in accordance with those reported by El-Badry *et al.* (2006) in that, treatment of broadbean plants with rhizobia caused significant reduction in percentage of disease incidence of BYMV, as well as significant reduction in ELISA-values.

Result also agreed with those of Osman & El-Sheikh (1994) in that BYMV caused a decrease in shoot and root dry weight and nodule number, whereas inoculation with rhizobia induced significant increase in these parameters.

The improvement of plant growth by rhizobia is thought to come mainly from the capacity of these bacteria to form nitrogen-fixing nodules as well as of releasing secondary metabolites, including plant growth regulators; as well as from solubilizing phosphate, so facilitating the uptake of nutrients from the root environment (Antoun *et al.*, 1998, Antoun and Prevost 2005, Sahran and Nehra 2011, Zanatta *et al.*, 2007).

Stimulation of plant growth by rhizobia could also be due to the suppression of plant diseases. This suppression induced by rhizobia may be direct, through inhibition of pathogen growth, or indirect, through stimulating plant defence mechanisms. The direct effect of rhizobia is through competition for certain nutrients or antibiosis by extracellular compounds. It was reported that rhizobia produced compounds with direct antimicrobial activities (Kacem *et al.*, 2009), and iron-chelating siderophores that reduce the availability of iron to the pathogen (Arora *et al.*, 2005).

The indirect effects of rhizobia in disease suppression are the activation of plant defence mechanisms when challenged with pathogens through production of several compounds including phenolics, flavonoids, phytoalexins, and proteins. Several previous studies reported that phenolic and flavonoids induction are associated with *Botrytis faba*-infected broadbean plants (Rabie 1998), with disease control in alfalfa (Dakora 2003), and with sheath blight in rice (Mishra *et al.*, 2006) when the plants were pre-inoculated with rhizobia.

Results of ELISA reactions revealed that treatment with rhizobia, prior to inoculation with BYMV, reduced significantly virus concentration in broadbean plants. We also obtained lower incidence of diseased plants, higher foliage fresh weigh, and higher nodule numbers.

Since there is no direct contact between rhizobia in the rhizosphere and the foliar BYMV, the activity of *R.leguminosarum* against BYMV may be due to the stimulation of plants to activate its defence mechanisms referred to as "Induce Systemic Resistance" (ISR). It was reported that inoculation of broadbean seeds with rhizobia induced SR against BYMV, and appeared that bacterial lipopolysaccharides (LPs) are responsible for such induction (Denny 1995, Leigh and Coplin 1992, Elba dry *et al.*, 2006).

The results obtained in this study indicated that *R.leguminosarum* might induce systemic resistance in broadbean plants against BYMV. However, further work should be conducted to demonstrate this effect. Rhizobia, with other PGPM, seem to be a promising management strategy for this disease, since they offer a simple, safe, and economically acceptable way to protect the plants against virus diseases.

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