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Induction of systemic resistance in faba bean (*Vicia faba* L.) to bean yellow mosaic potyvirus (BYMV) *via* seed bacterization with plant growth promoting rhizobacteria

Induzierte systemische Resistenz gegenüber dem Bohnengelbmosaikvirus in der Dicken Bohne (*Vicia faba* L.) durch Saatgutbehandlung mit wachstumsfördernden Rhizobakterien

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Summary

In a greenhouse experiment, two plant growth promoting rhizobacteria „PGPR" strains (*Pseudomonas fluorescens* FB11 and a *Rhizobium leguminosarum* bv. *viceae* FBG05) isolated from roots of faba bean plants were tested singly and in combination as seed inoculants for induction of systemic resistance in faba bean against bean yellow mosaic potyvirus (BYMV). The results demonstrated that BYMV challenged plants emerged from *Pseudomonas* inoculated seeds not only showed a pronounced and significant reduction in percent disease incidence (PDI) but also a significant reduction in virus concentration (ELISA) in the challenged plants, compared to the nonbacterized, challenged plants. *Rhizobium* singly also showed a significant reduction in both PDI and ELISA value, but the reduction was less pronounced than that resulting from *Pseudomonas* inoculation. Combined inoculation with *Pseudomonas* and *Rhizobium* showed no additional significant reduction in PDI or ELISA value compared with *Pseudomonas* singly. Appreciable and significant increase in both salicylic acid level and peroxidase activity was observed in leaves of all PGPR inoculated plants compared to other treatments. Since the PGPR inoculants (*Pseudomonas* and *Rhizobium*) and the pathogen (BYMV) remained spatially separated, it can be concluded that the tested *Pseudomonas* or *Rhizobium* strains induced systemic resistance in faba bean against BYMV.

Key words: bean yellow mosaic potyvirus (BYMV), faba bean (*Vicia faba* L.), induced systemic resistance, *Pseudomonas fluorescens*, *Rhizobium leguminosarum* bv. *viceae*

Zusammenfassung

Zwei das Pflanzenwachstum fördernde Rhizobakterienisolate (PGPR) aus Wurzeln der Dicken Bohne, *Pseudomonas fluorescens* FB11 und *Rhizobium leguminosarum* bv. *viceae* FBG05, wurden einzeln und kombiniert als Saatgutbehandlungsmittel hinsichtlich einer induzierten Resistenz der Dicken Bohne gegenüber dem Bohnengelbmosaikvirus (BYMV) im Gewächshaus überprüft. Dem BYMV ausgesetzte Bohnenpflanzen zeigten nach einer *Pseudomonas*-Behandlung im Vergleich zur unbehandelten Kontrolle nicht nur eine deutlich verminderte Befallshäufigkeit im Bestand (BHB), sondern darüber hinaus eine signifikant verminderte Viruskonzentration im ELISA-Test. Eine *Rhizobium*-Behandlung des Saatguts bewirkte ebenfalls signifikant verminderte BHB- und ELISA-Werte, die allerdings unter denen *Pseudomonas*-behandelter Pflanzen lagen. Eine kombinierte Saatgutbehandlung mit *Pseudomonas* und *Rhizobium* übertraf die Wirkung der *Pseudomonas*-Einzelbehandlung nicht. Die Blätter aller PGPR-behandelten Pflanzen zeigten gegenüber der unbehandelten Kontrolle erhöhte Salicylsäurekonzentrationen und Peroxidaseaktivitäten. Die räumliche Trennung der als Saatgutbehandlungsmittel verwendeten *Pseudomonas*- und *Rhizobium*-Isolate vom Pathogen (BYMV) deutet darauf hin, dass sie in den Bohnenpflanzen eine induzierte Resistenzreaktion gegenüber dem Virus auslösten.

Stichwörter: Bohnengelbmosaikvirus (BYMV), Dicke Bohne (*Vicia faba* L.), induzierte systemische Resistenz, *Pseudomonas fluorescens*, *Rhizobium leguminosarum* bv. *viceae*

1 Introduction

Faba bean (*Vicia faba* L.) was an important legume food crop in ancient civilization and is still a major crop in many countries. The family Fabaceae is known to be naturally infected by some of 114 viruses (Brunt et al. 1990). Bean yellow mosaic potyvirus (BYMV) is one of the most economically significant plant viruses affecting production of field-grown legumes and some non-legumes. This virus is probably distributed worldwide and causes mosaics and necrosis in legumes depending on host genotype and virus strain (Derks et al. 1980). In Egypt, the faba bean cultivated area declined up to 34% in 1993 due to an epidemic viral disease disaster in Central Egypt. Results of laboratory tests conducted on 1414 faba bean samples collected from different locations in Egypt indicated that natural field infection with BYMV reached about 21% (Mahmoud et al. 1998). BYMV is difficult to control because of its extremely broad natural host range in excess of 60 plant species and the ability to be transmitted by many aphid species in the non-persistent manner and *via* seed in some legume species (Frison et al. 1990).

Viruses basically differ from other crop pathogens and pests because they cannot be eradicated chemically. Management of plant viral diseases can be accomplished through the induction of plant's natural defenses, e.g., systemic acquired resistance (SAR) (Ryals et al. 1994). SAR against viral infection has been documented using biological and chemical inducing agents (Ross 1961; Mann 1969; Raskin 1992; Kessman et al. 1994). In most cases, the biological agents consisted of plant pathogenic bacteria, fungi, or viruses. An alternative method to induce plant defense is through the use of nonpathogenic rhizobacteria which have the ability to induce a state of systematic resistance in plants that provides protection against a broad spectrum of phytopathogenic microorganisms. This approach has been referred to as induced systemic resistance (ISR) (Van Loon et al. 1998). Maurhofer et al. (1994) evaluated the root colonizing bacterium *Pseudomonas fluorescens* as an inducing agent against the lesion-inducing tobacco necrosis virus (TNV) in tobacco. They observed a reduction in TNV induced lesion number in *P. fluorescens*-treated plants. Zehnder et al. (1999) identified PGPR strains that protected tomato against systemic infection by CMV un-

der greenhouse and field conditions. Pseudomonad determinants that are claimed to produce ISRs include siderophores, the *O*-antigen of lipopolysaccharides and salicylic acid. The latter compound has even been indicated to cause an ISR when present in nanogram amounts (DeMeyer et al. 1999).

In this study, we therefore investigated the response of faba bean plants subjected to inoculation with two PGPR strains (*Pseudomonas fluorescens* FB11 and a *Rhizobium leguminosarum* bv. *viceae* FBG05) singly or in combination to BYMV infection. Also, the performance of *Rhizobium* in case of BYMV challenged plants was investigated.

2 Materials and methods

2.1 Screening of fluorescent pseudomonad isolates for salicylic acid production

Fourteen rhizobacterial fluorescent pseudomonad strains isolated from roots of faba bean plants were screened for salicylic acid (SA) production. The isolates were grown in standard succinate medium (Meyer and Abdallah 1978) and the quantity of SA in culture filtrate was determined as described by Leeman et al. (1996) and expressed as μg ml^{-1}. The isolate FB11 exhibited the highest SA level of 212 μg ml^{-1} (data not shown). The fluorescent pseudomonad isolate FB11 was presumably identified as *Pseudomonas fluorescens* strain based on growth at 4°C and 42°C, lecithinase, utilization of sorbitol, inositol and trehalose. API 20NE system containing 21 biochemical standardized tests (bio Mérieux, Marcy-L'Etaile, France) was used to confirm the identification.

2.2 Isolation of Rhizobium strains

Rhizobium strains which nodulate roots of faba bean plants (cv. Giza1) were isolated and purified according to the methods recommended by Vincent (1970). The isolates were tested for symbiotic effectiveness with faba bean plants cv. Giza 1 using the methods described by Somasegaran and Hoben (1994). The *Rhizobium* isolate FBG05 was the most effective (data not shown).

2.3 Test for antagonism

In vitro antagonism between *Rhizobium* and *Pseudomonas* isolates used in combined inoculation of faba bean plants was tested in agar media according to the defense antagonism procedure described by Gross and Vidaver (1978).

2.4 Inocula preparation

P. fluorescens strain FB11 and *Rhizobium leguminosarum* bv. *viceae* FBG05 were grown for 36-48 h in King's medium B (KB) and yeast mannitol broth (YMB), respectively, at 28°C on a rotary shaker at 150 rpm. Late log-phase cells were harvested by centrifugation (10,000 *g*) at 4°C for 10 min and washed twice with sterile NaCl solution (0.85%). Growth was monitored by absorbance measurements of culture sample at 550 nm. Cell densities were related to viable cell numbers, measured as colony forming unites per ml (CFU ml^{-1}) by standard plate counts and the number of both bacteria was adjusted to 10^8 CFU ml^{-1}.

2.5 Seed bacterization

Seed bacterization was carried out by the method of Dileep Kumar and Dube (1992) with some modification as follows: faba bean seeds (*Vicia faba* L., cv Giza 1) were surface sterilized by placing seeds in 2.5% sodium hypochlorite for 5 min followed by rinsing in 1:29 mixture of hydrogen peroxide : distilled water for 30 min and dried under a sterile condition. Surface sterilized seeds were steeped in bacterial suspensions mixed with 10% gum arabic for 1 h and dried overnight at room temperature in sterile Petri dishes. Seeds steeped in gum arabic solution only served as control.

2.6 BYMV inoculation

A BYMV isolate was obtained from Virology Laboratory, Faculty of Agriculture, Ain Shams University Cairo, Egypt. It was maintained on faba bean cultivar Giza 461. The virus was checked on *Chenopodium amaranticolor* L. and reisolated from a single lesion. Inoculum of the virus was prepared by grinding fresh leaves showing severe symptoms in 100 mM phosphate buffer (pH 7.5) using sterilized pestle and mortar. The pulp was squeezed through tow layer of cheesecloth and the filtrate was centrifuged at 5000 rpm for 10 min. The first two leaves of three leaves-old faba bean plants were lightly dusted with carborundum (600 mesh). The supernatant containing virus was used after dilution 10^{-1} with the same buffer as a virus inoculum.

2.7 Disease incidence

After 45 days from planting, the number of plants exhibiting BYMV symptoms was recorded. Percent disease incidence (PDI) was estimated according the equation: PDI = (Number of symptomatic plants/Total number of plants) x 100 (Reddy et al. 1983).

2.8 DAS-ELISA

Serological examination for the presence of BYMV was carried out using double antibody sandwich enzyme linked immunosorbent assay (DAS-ELLISA) according to McLaughlin et al. (1984). The ELISA kits were obtained from Agricultural Genetic Engineering Institute (AGERI), Giza, Egypt. Five infected plants from each treatment were taken and six symptomatic leaves from each plant were used as replicates to carry out ELISA determination. In case of absolute control plants, a randomly selected leaf samples was used. Absorbance values were recorded using a Dynatech MR700 plate reader at 405 nm. Leaf samples were considered positive for the presence of BYMV if the absorbance value exceeded a threshold value equal to the mean of the absorbance value of healthy control samples + (3) (standard deviation of the mean) (Zehnder et al. 1999).

2.9 Extraction and quantification of salicylic acid in plant leaves

Leaves were frozen in liquid nitrogen and pulverized with mortar and pestle. For determination of SA, 200 ng of the standard ortho-anisic acid was added per gram of fresh leaves. Subsequently, extraction and quantification of free SA expressed as μg g^{-1} fresh leaves were carried out as described by Meuwly and Métraux (1993).

2.10 Peroxidase activity

Peroxidase assays were performed on leaf extracts. One gram fresh weight of leaf tissue was ground in a mortar containing liquid nitrogen. The resulting powder was macerated for 30 s in 10 ml of 0.1 M Tris buffer, pH 7.5, and then centrifuged at 20,000 g for 25 min at 4°C. The supernatants were kept in an ice bath and used for determination of peroxidase activity by

a direct spectrophotometric method described by HAMMERSCHMIDT et al. (1982). Peroxidase activity was expressed as absorbance changes min^{-1} g^{-1} fresh leaf tissue at 470 nm.

2.11 Pot experiment

A pot experiment was carried out to evaluate two PGPR, *P. fluorescens* FB11 and a *R. leguminosarum* bv. *viceae* FBG05 isolated from faba bean roots for inducing systemic resistance (ISR) in faba bean plants against BYMV. Randomized complete block design was used with six treatments and two controls each consisted of five replicate pots and four plants per pot. Treatments included *P. fluorescens* FB11 (T1); *R. leguminosarum* bv. *viceae* FBG05 (T2); T1 + T2 (T3); T1 + BYMV (T4); T2 + BYMV (T5); T1 + T2 + BYMV (T6); in addition to a virus challenged control (ChC) and an absolute control (AC) (no bacteria, no virus).

Bacterized as well as nonbacterized healthy, surface sterilized faba bean seeds (*V. faba* L., cv. Giza 1) were sown in 40 cm earthen pots containing clayey soil (pH 7.5). The pots were maintained in greenhouse under natural lighting, day/night temperature of approx. 25/15°C and 55% mean relative humidity. The three leaves-old faba bean plants in T4, T5 and T6 treatments in addition to the challenged control (ChC) were challenge-inoculated with BYMV. At the beginning of flowering stage, after 45 days of planting, numbers of plants developed typical BYMV symptoms were recorded, ELISA test was performed, and plant height was measured. Thereafter, plants were carefully removed from soil to assay acetylene reduction, counting nodules, determining nodules and shoot dry weight and shoots nitrogen content. Data were subjected to one-way analysis of variance followed by least significant difference (LSD) test at $P = 0.05$ by using M-state software.

3 Results

The response of faba bean plants (*V. faba* L. cv. Giza 1) emerged from seeds bacterized with *P. fluorescens* FB11 or/and *R. leguminosarum* bv. *viceae* FBG05 to challenge with BYMV is quantified in Table 1. Challenged plants emerged from *Pseudomonas* -inoculated seeds (T4) showed pronounced and significant reduction in percent disease incidence (PDI: 27.7%), followed by those emerged from *Rhizobium*-inoculated seeds (T5) (43%), compared with 91% for the challenged control plants (T7). Also, inoculation with *Pseudomonas* (T4) or *Rhizobium* (T5) significantly reduced virus concentration in the symptomatic leaves of the challenged plants, as these treatments showed ELISA values of 0.60 and 0.75, respectively, while the challenged control (T7) showed an ELISA value of 1.74. Inoculation of the challenged plants with *Pseudomonas* along with *Rhizobium* (T6) showed no additional significant reduction in PDI in comparison with *Pseudomonas* treatment (T4) and the ELISA values were equal in both treatments.

In challenged treatments, significantly higher salicylic acid (SA) level of 1.05 μg g^{-1} fresh leaves was observed in leaves of *Pseudomonas* bacterized plants (T4) in comparison with 0.17, 0.18, and 0.08 μg g^{-1} fresh leaves in plants treated with *Rhizobium* (T5), challenged control (T7), and absolute control (T8) plants, respectively (Table 1). The salicylic acid level was further increased when seeds were inoculated with *Pseudomonas* along with *Rhizobium* (T6) and showed 1.28 μg g^{-1} fresh leaves.

Regarding the peroxidase enzyme activity (POA), an appreciable and significant increase was observed in plants if all *Pseudomonas*-inoculated treatments compared to the other treatments. POA values in plants of *Pseudomonas* and *Rhizobium + Pseudomonas* treatments were statistically equivalent. *Rhizobium*-inoculated plants showed a very low POA, equal to that in plants of challenged and unchallenged control treatments. It was also observed that inoculation of faba bean seeds with PGPR increased both SA level and POA in emerged plants independently whether the plants were challenged with BYMV or not, but the increase in the challenged plants was more pronounced (Table 1).

The data presented in Table (2) show that inoculation with *Rhizobium* and/or *Pseudomonas* significantly improved plant performance in terms of plant height and dry weight, nodulation, nitrogenase activity and plant nitrogen content as compared to unchallenged control plants. However, the coinoculated plants showed the best performance. Challenging with BYMV prevented nodule formation and decreased drastically nitrogenase activity on roots of faba bean plants emerged from non-inoculated seeds. Moreover, *Rhizobium*-inoculated challenged plants showed very poor nodulation and lower nitrogenase activity as compared to plants of the corresponding unchallenged treatment. Coinoculation with *Rhizobium* along with *Pseudomonas* showed non-pronounced improvement in both nodulation and nitrogenase activity (Table 2).

Table 1: Quantification of plant growth promoting rhizobacteria-mediated induced systemic resistance to bean yellow mosaic potyvirus in 45-day old faba bean plants (values are the averages ± standard deviation)

Treatments (1)	PDI (2)	ELISA (3)	SA (4)	POA (5)
T1	0.00	0.14 (± 0.030)	0.50 (± 0.031)	0.27 (± 0.01)
T2	0.00	0.04 (± 0.010)	0.21 (± 0.020)	0.13 (± 0.01)
T3	0.00	0.04 (± 0.030)	0.66 (± 0.026)	0.25 (± 0.01)
T4	27.70 (± 3.22)	0.60 (± 0.059)	1.05 (± 0.042)	0.48 (± 0.01)
T5	43.00 (± 3.61)	0.75 (± 0.053)	0.17 (± 0.015)	0.15 (± 0.01)
T6	25.70 (± 1.53)	0.60 (± 0.025)	1.28 (± 0.053)	0.51 (± 0.01)
ChC	91.33 (± 2.08)	1.74 (± 0.068)	0.18 (± 0.020)	0.13 (± 0.01)
AC	0.00	0.08 (± 0.032)	0.08 (± 0.010)	0.14 (± 0.01)
$LSD_{0.05}$	3.352	0.0719	0.05227	0.02737

(1) *Pseudomonas fluorescens* (T1); *Rhizobium leguminosarum* bv. *viceae* (T2); T1+T2 (T3); T1+BYMV (T4); T2+BYMV (T5); T1+T2+BYMV (T6); Challenged control (ChC); Absolute control (AC).
(2) Percent disease incidence.
(3) ELISA expressed as absorbance values at 405 nm. Leaf sample considered positive for BYMV if the absorbance value exceed 0.18.
(4) Salicylic acid expressed as μg g^{-1} fresh leaves weight.
(5) Peroxidase activity expressed as absorbance changes min^{-1} g^{-1} fresh leaf tissue at 470 nm.

Table 2: Response of faba bean previously subjected to plant growth promoting rhizobacteria inoculation to infection by bean yellow mosaic potyvirus**

Treatments*	Shoot height (cm)**	Shoot dry weight (g)	Nodulation plant^{-1}		N_{ase} activity nmol C_2H_4 g^{-1} h^{-1}	N content mg shoot^{-1}
			Nodule no.	Nodule dry weight (mg)		
T1	62.33 (± 1.53)	7.35 (± 0.36)	78.33 (± 5.03)	220.00 (± 12.77)	179 (± 6.00)	197 (± 16.26)
T2	66.00 (± 1.00)	8.24 (± 0.77)	99.67 (± 6.11)	264.00 (± 12.53)	221 (± 7.00)	300 (± 14.73)
T3	72.00 (± 2.00)	9.37 (± 0.34)	145.33 (± 6.66)	371.30 (± 13.32)	317 (± 7.00)	375 (± 16.17)
T4	63.00 (± 1.00)	7.50 (± 0.20)	6.67 (± 2.08)	017.67 (± 02.52)	15 (± 3.00)	196 (± 03.51)
T5	66.00 (± 2.65)	7.53 (± 0.21)	11.00 (± 2.00)	023.67 (± 05.69)	22 (± 2.00)	204 (± 06.81)
T6	67.00 (± 1.53)	7.64 (± 0.07)	21.00 (± 3.00)	042.33 (± 04.16)	51 (± 3.00)	210 (± 03.61)
ChC	51.33 (± 1.53)	6.43 (± 0.09)	0.00 (± 0.00)	000.00 (± 00.00)	7 (± 1.00)	114 (± 03.61)
AC	53.00 (± 1.00)	7.15 (± 0.15)	36.33 (± 4.04)	067.33 (± 03.79)	101 (± 7.00)	159 (± 04.58)
$LSD_{0.05}$	2.804	0.5996	7.258	14.58	8.783	17.82

* See table (1).
** Means of 20 plants per treatment.

However, BYMV challenging showed less detrimental effect on plant growth and plant nitrogen content as compared with its effect on nodulation and nitrogenase activity.

4 Discussion

In the present study, the spatial separation between the *Pseudomonas and Rhizobium* strains in the rhizosphere and the foliar pathogen BYMV in the phyllosphere satisfy the condition of non-specific protection proposed by Steiner and Schönbeck (1995) as a criterion of induced systemic resistance (ISR). The onset of ISR was assayed as a significant reduction in both PDI and virus concentration (ELISA value) (27% and 0.60) relative to challenged control (91% and 1.74).

Because inoculation with *Pseudomonas* caused a significant increase in SA level in plant leaves (Table 1), it is reasonable to assume that the ISR was mediated by salicylic acid. According to Van Loon et al. (1998), rhizobacterially-produced SA can trigger the SAR pathway as well as ISR in some plant species. In radish, induction of SR to *Fusarium* wilt by two *Pseudomonas fluorescens* WCS 374 and WCS 417 was clearly associated with the capacity of these strains to produce SA in culture (Leeman et al. 1996). Also, root colonization of tobacco plants with a SA-producing *Pseudomonas fluorescens* CHAO strain as well as leaf infection with tobacco necrosis virus (TNV) suppressed necrosis caused by TNV and caused up to five-fold increase in SA level in leaves (Mauerhofer et al. 1994). The *P. fluorescens* strain used in the present study produces a high level of SA in culture filtrate (212 μg ml^{-1}). The level of SA in leaves of both unchallenged and challenged *Pseudomonas* inoculated plants was about six times higher than that in both absolute and challenged control plants.

The PGPR-mediated SR is often associated with the onset of defense mechanism including the increased expression of defense enzymes, such as peroxidase (Bergstrom et al. 1982). In the present study, increased peroxidase activity was observed in *Pseudomonas*-inoculated plants (Table 1).

Rhizobium species are widely used in agriculture for legume crops improvement because of their ability to fix atmospheric nitrogen in symbiosis with legumes. The potential for improving faba bean plant performance by coinoculation with *Pseudomonas* and rhizobia has been investigated in the present study. The results (Table 2) show that coinoculation resulted in a significant increase in plant performance as compared with *Rhizobium* inoculation. This result is in agreement with those of Dashti et al. (1998) with soybean and Parmar and Dadarwal (1999) with chickpea.

The results show that inoculation of faba bean seeds with *Rhizobium* induced SR in faba bean plants against BYMV is very interesting, although it was lower than that induced by the *P. fluorescens* strain tested. The bacterial lipopolysaccharides (LPS) appeared to be the trait responsible for systemic resistance induction by pseudomonad rhizobacteria to *Fusarium* wilt in carnation (Van Peer and Schippers 1992) and in radish (Leeman et al. 1995). On the other hand, surface carbohydrates of *Rhizobium* cells consist mainly of exopolysaccharides (EPS) and LPS. The polysaccharides play an important role during the recognition process in the symbiotic interaction between *Rhizobium* and legumes (Denny 1995; Leigh and Coplin 1992). However, further studies are needed to support this finding that inoculation with rhizobia could induce SR in legumes against pathogen infection.

In conclusion, under our experimental conditions, the rhizobacterium *P. fluorescens* FB11 isolated from faba bean roots seems to be a promising inducers for SR in faba bean (*V. faba* L.) to a challenge BYMV infection under greenhouse conditions. This result could be very important practically since it may offer a simple, environmentally safe and economically accepted mean to protect faba bean plants from BYMV infection. However, additional studies are needed to confirm these results under field conditions.

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