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Potential of *Pseudomonas putida* F1 to manage *Bean common mosaic virus* of bean

Mohsen Mohamed Elsharkawy[1]* and Mohamed Mahmoud El-Sawy[2]
1- Department of Agricultural Botany, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt.
2- Plant Virology and Phytoplasma Research Dept., Plant Pathology Institutes, Agriculture Research Center, Giza, Egypt.
*mohsen.abdelrahman@agr.kfs.edu.eg

ABSTRACT

The ability of the isolated rhizobacterial strain F1 of *Pseudomonas putida* to promote bean growth and resistance against *Bean common mosaic virus* (BCMV) was evaluated. Moreover, defense enzymes and the temporal expression profile of defense genes were assessed using quantitative real-time polymerase chain reaction (RT-qPCR) in bean plants. Bean plants treated with *P. putida* exhibited increased shoot and root dry weight relative to the control plants under greenhouse conditions. Similarly, the yield and yield components were increased in rhizobacterial treated bean plants under field conditions. Plants inoculated with *P. putida* showed the best inhibition effect of BCMV under greenhouse and field conditions. BCMV titer was significantly reduced in *P. putida* inoculated plants. Treatment with *P. putida* recorded the highest values of peroxidase and polyphenol oxidase enzymes after BCMV inoculation. The transcriptional profiles of *PR1*, *PR2*, *PR3* and *LOX* genes were highly increased in *P. putida* treated plants. The obtained results in this study elucidate the potential of *P. putida* in controlling BCMV infection in bean plants as well as the involved mechanisms in disease resistance.

Key words: Bean common mosaic virus; Pseudomonas putida; rhizobacteria; induced resistance; defense genes; RT-qPCR

INTRODUCTION

The common bean is considered one of the essential vegetables grown and consumed all over the world (Broughton *et al*., 2003). Among bean pathogens, *Bean common mosaic virus* (BCMV) have been identified as the most dangerous and widespread pathogens (Morales and Castaño 1987; Elsharkawy and El-Sawy 2015). The virus is transmitted through seeds and several species of aphids. Currently, no chemical pesticides are available for management of BCMV infection. Moreover, the intensive use of inorganic fertilizers could lead to harmful environmental effects and increasing costs. Biological management strategies have been reported to reduce the incidence and severity of plant virus diseases (Elsharkawy *et al*., 2012, 2013). The effects of PGPR on plant growth and resistance against different pathogens have been studied earlier (Mahour, 2005; Salem and Abd El- Shafea 2018). Plant growth promoting rhizobacteria (PGPR) is important to increase plant growth and induce resistance against different pathogens (Fernández *et al*., 2007; Shiri-Janagard *et al*., 2012). The colonization of plant roots with PGPR increases the ability to solubilize minerals and enhances plant growth (Jorquera *et al.*, 2008; Uribe *et al*., 2012). Tomato growth was increased in plants inoculated with *Paenibacillus polymyxa* and *Bacillus megaterium* (Ei-Yazeid and Abou-Aly, 2011). Dry matter was increased in cowpea plants treated with *Bradyrhizobium* sp. and *Paenibacillus polymyxa* (Saini and Khana, 2012). Additionally, *Rhizobium* nodulation was increased after application of rhizobacteria in bean plants (Remans *et al*., 2007).

This study was therefore designed to evaluate the effectiveness of *Pseudomonas putida* strain F1, a well-characterized plant growth-promoting rhizobacteria (PGPR) against BCMV in bean cultivar Nebraska.

Materials and methods

Isolation and identification of rhizobacterial isolate

The rhizobacterial *Pseudomonas putida* strain F1 was identified based on morphological and physiological characteristics (Bergey's Manuals Systematic of Bacteriology 2005) and 16S rDNA gene was carried out by Sigma, Cairo, Egypt (Shamseldin *et al*., 2009).

Molecular analysis

DNA of the isolated strain was extracted using GeneJet genomic DNA purification Kit (Thermo K0721) following the manufacturer protocol. The amplification of 16S rDNA gene was carried out using Maxima Hot Start PCR Master Mix (Thermo K1051) and the primers 27F, 5-AGAGTTTGATCCTGGCTCAG-3 and U1492R, 5-GGTTAC CTTGTTACGACTT-3 (Thermo scientific, Germany) as explained by Jiang *et al*. (2006). The purification of PCR products was done using GeneJET™ PCR purification kits (Thermo K0701). Sequencing was carried out by ABI 3730xl DNA sequencer (GATC Company, Germany). Significant alignments were checked in submitted GenBank database (http://www.ncbi.nlm.nih.gov/blast).MEGA6 was used to make the phylogenetic tree from 1000 bootstrap replicates (Tamura *et al*., 2013).

Seed biopriming

Pseudomonas putida strain F1 was grown on nutrient broth (NB) for 34 h at 27ºC on a rotary shaker at 140 rpm then centrifuged at 7000 rpm for 10 min. The inoculum concentration was adjusted to 1 × 10^8 cfu/ml. Bean seeds were washed, air-dried and soaked in culture suspension with carboxymethyl cellulose (0.4%) to help in adherence of the rhizobacteria to the seeds. Seeds of the control group were treated with distilled water amended with carboxymethyl cellulose.

Chemical induction

Bean plants were drenched with BTH (Benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester, Bion®, Syngenta, CH) at concentration of 0.3 mM at 2 days before BCMV challenge inoculation.

Growth conditions in greenhouse conditions

Five seeds from different treatments were cultivated in each pot (25 cm in diameter). BCMV inoculation was carried out at 2 days after BTH treatment. Each treatment consisted of 3 replications and 15 seedlings each. The growth parameters were evaluated at mid podding after planting. The number of infected plants was recorded and the inhibition percentage of *P. putida* strain F1 treatment was calculated at 2 weeks after BCMV inoculation using the following equation: Inhibition % = {(A-B)/A}×100
Where: A = Number of infected plants in control treatment, B = Number of infected plants in treated plants.

Growth conditions in field treatments

Seeds of different treatments were sown in plots (5 x 5 m). The distance between plants was 20 cm and the distance between rows was 30 cm. Plants were inoculated with BCMV at 10 days old. The number of infected plants was recorded and the percentage of inhibition was calculated as described before. The yield and yield components were evaluated for all treatments.

BCMV inoculation

Bean plants at 2 weeks after sowing were mechanically inoculated with BCMV. The infected leaves were ground in sodium phosphate buffer (50 mM, pH 7). Bean leaves were dusted with carborundum powder (600-mesh) followed by inoculation from the extracted sap of the infected leaves as explained by Elsharkawy and Elsawy (2015).

Enzyme linked immunosorbent assay (ELISA)

Leaves were collected from bean plants at 14 days post inoculation (DPI) and ground in carbonate buffer (1: 10, v/v, pH 9.6). The ELISA assay was conducted using the reagent set for BCMV (Agdia, Inc., Elkhart, IN) following the manufacturer protocol. The samples were visually inspected at 405 nm. Absorbance values were subjected for statistical analysis and the experiment were repeated three times with 9 samples per replicate.

Estimation of peroxidase (POX)

Leaves (1 g) were grinded and homogenized with 5 ml of phosphate buffer (0.1 M, pH 6.5) followed by centrifugation at 10.000 rpm for 10 min at 4 °C. POX activity was measured by adding 2.9 ml of substrate buffer (125 µl guaiacol (0.05 M) and 153 µl 30% H_2O_2 in 50 ml phosphate buffer) to 0.1 ml enzyme extract. POX activity was measured using spectrophotometer at 470 nm/min/mg protein (Hammerschmidt *et al*., 1982).

Estimation of polyphenol oxidase activity (PPO)

Leaves (1 g) were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) followed by centrifugation at 10.000 rpm at 4 °C for 10 min. To measure the activity of PPO, 100 µl of the enzyme extract was added to 1.5 ml sodium phosphate buffer (0.1 M, pH 6.5) and then 200 µl catechol (0.01 M) was added. PPO activity was measured using spectrophotometer at 420 nm/min/mg protein (Mayer *et al*., 1965).

Estimation of phenolics accumulation

Bean leaves (1 g) were homogenized in 10 ml aqueous methanol (80%) at 70 °C (Zieslin and Ben-Zaken, 1993). Total phenols were measured by diluting the extract (1 ml in 5 ml distilled water) then 250 µl of 1 N Folin–Ciocalteau reagent was added and the absorbance was measured at 725 nm as microgram gallic acid per gram tissue.

Real time –quantitative PCR (RT-qPCR)

RNA was extracted from bean leaves using Trizol protocol (Invitrogen Corp., Carlsbad, CA, USA). DNase I treatment was carried out in the presence of a RNase inhibitor (Invitrogen Corp., Carlsbad, CA, USA). Reverse transcription of 1 µg RNA was done using an oligo (dT) 12–18 primer. The analysis was done using the Step One PlusTM System and Power SYBR R Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). SYBR Green PCR Master mix and oligonucleotide (Table 1) were used following the manufacturer protocol. The 2-ΔΔ Ct method was utilized to calculate the relative expression of target and reference genes as described by Livak and Schmittgen (2001).

Statistical analysis

To test the effects of *Pseudomonas putida* treatment on disease inhibition, virus titer and defense-related enzymes and genes, the data were analyzed using analysis of variance (ANOVA). All the results were confirmed by repeating the experiments three times. All statistical analyses were performed at $P \leq 0.05$ by EKUSERU-TOUKEI 2010 (Social Survey Research Information Co., Ltd).

RESULTS AND DISCUSSION

Identification of rhizobacterial isolate

The phylogenetic tree of the *P. putida* strain F1 and the related bacterial species based on the 16S rDNA sequence is provided in Figure (1). It is clear that the rhizobacterial isolate was included in the genus *Pseudomonas* and closely related to the species *P. putida* strain F1. The highest sequence similarities with *P. putida* strain F1 (97%) in Gene bank (Fig. 1).

Plant growth and disease inhibition

Upon seed treatment, the incidence of BCMV infection was significantly reduced in bean plants (Table 2). Seed treatment with *P. putida* led to enhance shoot dry weight and root dry weight (Table 4). As shown in Tables (3 & 4), treatment with *P. putida* significantly increased plant growth along with decreasing BCMV titer in comparison to control plants. Root colonization by rhizobacteria initiated directly after seed germination due to root exudates stimulating disease suppression and plant growth (Haas and Defago, 2005). The potential of PGPR isolates to increase plant growth and reduce disease incidence was reported under greenhouse and field conditions (Kabdwal *et al*., 2019). A similar result was noticed in the current study, wherein disease inhibition was 88%, while in field experiment it was 82% (Table 2). In addition, significant increase in plant growth parameters such as shoot dry weight and root dry weight were also recorded, which was in agreement with Kabdwal *et al.* (2019). Stimulation of bean growth could be through the ability to produce metabolites and enzymes and the increased availability of nutrients (Niranjana and Hariprasad, 2014).

Seed treatment with *P. putida* significantly increased plant growth under field conditions and protected bean plants from BCMV infection. Reduction in the BCMV incidence could be due to the ability of *P. putida* to produce siderphores. The successful outcome of this study is highly correlated with Raj *et al.*, (2004). Plant growth-promoting fungi (PGPF) were reported to induce systemic resistance against *Cucumber mosaic virus* (CMV) through different mechanisms such as defense genes and enzymes (Elsharkawy *et al*., 2012, 2013, 2018). Similarly, in the present study, protection of bean plants against BCMV infection was due to ISR as *P. putida* and BCMV remained spatially separated.

POX activity

High increase in POX and PPO activities were observed at 10 days post-inoculation in treated plants than the control **(Fig. 2)**. But a drastic increase in POX and PPO activity was found in leaves of bean plants treated with *P. putida* compared to all other treatments. Induction of systemic resistance was highly correlated with the synthesis and accumulation of pathogenesis-related proteins such as β-1, 3, glucanase and chitinase (Sendhil, 2003).

Accumulation of phenolic compounds

The accumulation of phenols in treated bean plants and challenge inoculated with BCMV was increased significantly relative to the control (Fig. 3). The highest accumulation of phenolics was observed at 10 days post virus inoculation (470 μg/g tissue) compared with the control (272 μg/g tissue). Phenolic compounds were accumulated in treated plants through phenylpropanoid pathway leading to disease suppression and restricting pathogen infection (Hammerbacher *et al*., 2011). Long chains of phenolics (lignin) is toxic to several pathogens (Basha *et al*., 2006). PGPR induce systemic resistance against several pathogens through increased thickening of cell wall, papillae formation and accumulation of phenolic compounds (Benhamou *et al*., 1998).

RT-qPCR analysis

Dramatic increase in the transcription levels of defense-related genes (*PR1, PR2, PR3* and *LOX*) were reported at 4 days after BCMV inoculation **(Fig. 4)**. Plants treated with *P. putida* showed the best results in this respect. The highest levels of expression were registered for *PR1* (29-folds increase). This increase was correlated well with the increased virus inhibition. After recognition of pathogen attack, defense respond is activated by the synthesis of different proteins. These proteins have several functions especially antibiotic functions, such as PR proteins. Similarly, Elsharkawy (2019) recorded the upregulation of *PR1* and a *β-1,3-glucanase* (*PR2*) during the incompatible interaction between the virus and the plants treated with biotic inducers such as PGPF isolates.

In conclusion, ISR mediated by *Pseudomonas putida* against BCMV was due to the upregulation of defense-related genes and enzymes.

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Table 1. Oligonucleotide primers used for quantitative real-time polymerase chain reaction (RT-qPCR) analysis.

Gene	Forward	Reverse
PR1	AAAGCCAAGAGCGATTCTCTTTTCA	GAACACTCTGATTTGATAACACTTC
PR2	GAAGATGAGCtCAAAGCTGGTAA	CAAGGATTGGCCAAAAGGTA
PR3	ATTGTTGTGCCAATCCCTTT	CACCGCCATACAGTTCAAAA
LOX	AGCACTGTGCCTGTTTTCAGT	AACACACGAGAAGATTCAACCA
Actin	TGCATACGTTGGTGATGAGG	AGCCTTGGGGTTAAGAGGAG

Table 2. Effect of *P. putida* strain F1 treatment on BCMV infection under greenhouse and field conditions.

Treatments	Greenhouse conditions		Field conditions	
	Infection (%)	Inhibition (%)	Infection (%)	Inhibition (%)
P. putida strain F1	11c	88	16c	82
BTH	22b	75	32b	64
Control	89a	-	87a	-

Table 3. Effect of *P. putida* strain F1 treatment on BCMV titer under greenhouse and field conditions.

Treatments	Greenhouse conditions	Field conditions
P. putida strain F1	0.37c	0.34c
BTH	0.64b	0.59b
Control	1.53a	1.34a

Table 4. Effect of *P. putida* strain F1 treatment on growth of bean plants under greenhouse conditions

Treatments	Shoot dry weight	Root dry weight
P. putida strain F1	3.27c	0.44c
BTH	4.93b	0.68b
Control	9.65a	1.31a

Table 5. Effect of *P. putida* strain F1 treatment on growth of bean plants under field conditions

Treatments	Yield (kg $hectare^{-1}$)	No. of pods $plant^{-1}$
P. putida strain F1	690c	8.34b
BTH	810b	8.55b
Control	1136a	9.71a

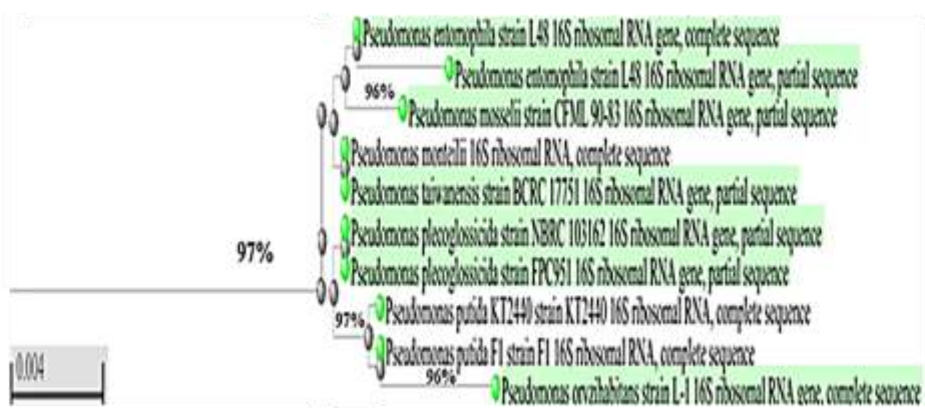

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16SrDNA sequences, showing the position of *Pseudomonas putida* strain F1 among phylogenetic neighbors.

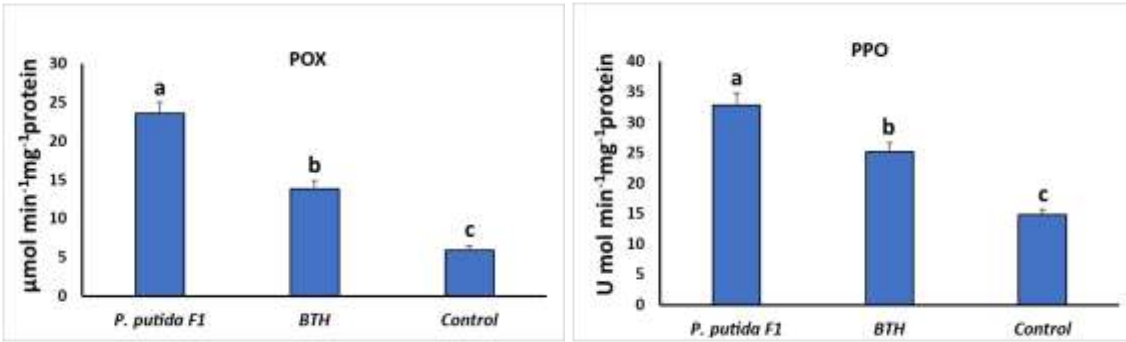

Fig. 2. Effect of *Pseudomonas putida* strain F1 on the production of defense enzymes in detached bean leaf.

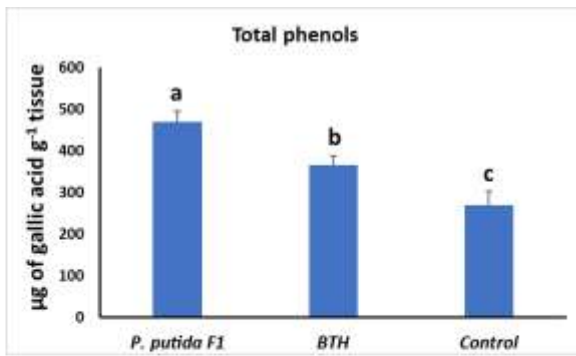

Fig. 3. Effect of *Pseudomonas putida* strain F1 on the production of total phenols in detached bean leaf.

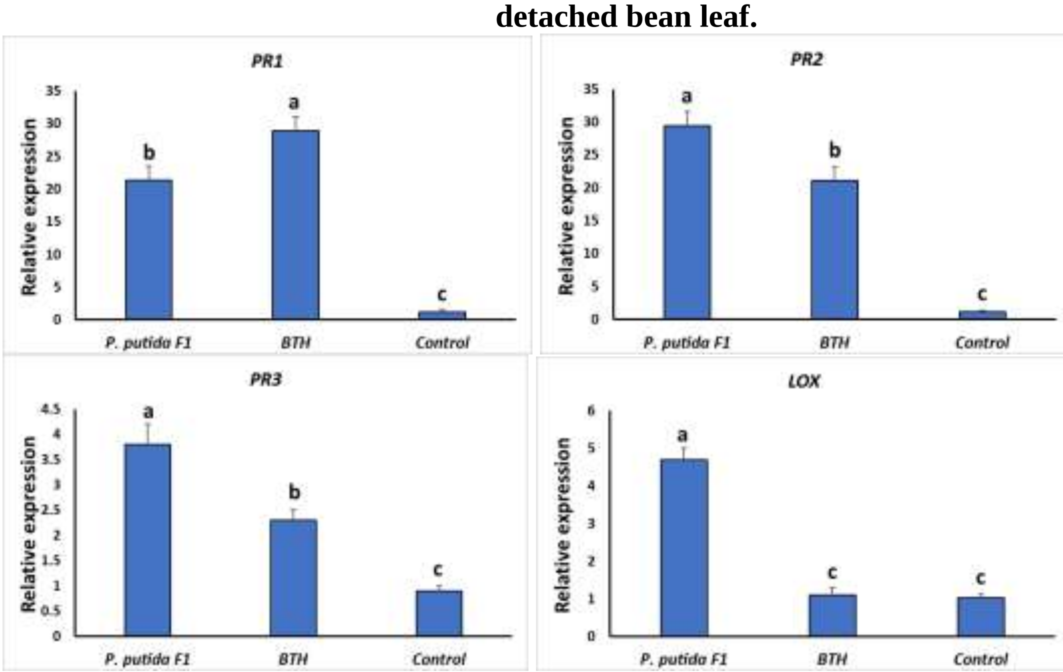

Fig. 4. Expression pattern of pathogenesis-related genes in bean plants in response to *Pseudomonas putida* strain F1 and BCMV inoculation.

Mohsen M. Elsharkawy[1]* and Mohamed M. El-Sawy[2]

قدرة سلالة F1 *Pseudomonas putida* لمقاومة فيروس موزيك الفاصوليا العادى فى الفول

محسن محمد الشرقاوي[1]* ، محمد محمود الصاوي[2]
1- قسم النبات الزراعي ، كلية الزراعة ، جامعة كفر الشيخ ، كفر الشيخ 33516 ، مصر.
2- قسم بحوث الفيروس والفيتوبلازما ، معهد أمراض النبات ، مركز البحوث الزراعية ، الجيزة ، مصر.
*mohsen.abdelrahman@agr.kfs.edu.eg

المستخلص

تم تقييم قدرة السلالة F1 من *Pseudomonas putida* على تعزيز نمو الفول ومقاومته لفيروس موزيك الفاصوليا العادى فى الفول (BCMV). علاوة على ذلك ، تم تقييم إنزيمات المقاومة والتعبير الجينى لجينات المقاومة باستخدام تفاعل البلمرة المتسلسل الكمي (RT-qPCR) في نباتات الفول. أظهرت نباتات الفول المعامله بـ *P. putida* زيادة في الوزن الجاف للنبات والوزن الجاف للجذر بالمقارنه بالكنترول تحت ظروف الصوبه الزراعيه. وبالمثل ، تمت زيادة محصول نباتات الفول المعامل تحت ظروف الحقل. أظهرت النباتات الملقحة بـ *P. putida* أفضل تأثير تثبيط لـ BCMV تحت ظروف الصوبه و الحقل. تم تخفيض تركيز BCMV بشكل كبير في النباتات الملقحة بواسطة *P. putida*. سجلت المعاملة باستخدام *P. putida* أعلى قيم لإنزيمات البيروكسيديز والبوليفينول أوكسيديز بعد تلقيح BCMV. تمت زيادة التعبير الجينى لجينات PR1 و PR2 و PR3 و LOX بدرجة كبيرة في النباتات المعاملة بواسطة *P. putida*. النتائج التي تم الحصول عليها في هذه الدراسة توضح إمكان استخدام *P. putida* في السيطرة على عدوى BCMV في نباتات الفول وكذلك ميكانيكيه عمليه مقاومة المرض.