



## RESEARCH

# Plant growth promoting fungi stimulate growth and confer protection against Bean yellow mosaic virus in faba bean

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## ABSTRACT

**Background:** Bean yellow mosaic virus (BYMV) is recognized as one of the most wide distributed virus disease affecting faba bean in Egypt.

**Objective:** The aim of this study is to explore the efficiency of the plant growth promoting fungi (PGPF) of *Penicillium simplicissimum* GP17-2, *Fusarium equiseti* GF19-1 and *Trichoderma asperellum* SKT1 as a sustainable method to control the invasion of BYMV in faba bean plants.

**Methods:** Faba bean plants were treated with PGPF isolates at 2 days prior to BYMV inoculation. Disease severity and virus titer were estimated at 1, 2 and 3 weeks after virus inoculation. The transcription profile related to defense genes was evaluated at 1, 2 and 3 weeks after BYMV inoculation by quantitative real-time PCR analysis.

**Results:** Treatments with PGPF resulted in lower virus severity and titer than the control. GP17-2 treatment exhibited the lowest disease severity and virus titer in comparison with other treatments. Quantitative real-time PCR results showed significant increased expressions of pathogenesis related genes *PRI* and *PR2* relative to the control plants.

**Conclusion:** PGPF isolates of GP17-2, GF19-1 and SKT1 elicited induced resistance against BYMV infection and could be used as a promising strategy to control BYMV.

**Keywords:** *Bean yellow mosaic virus*; induced resistance; plant growth promoting fungi; faba bean; qRT-PCR

## BACKGROUND

The incidence of *Bean yellow mosaic virus* could reach to 67% in infected plants (Makkouk et al., 1989). BYMV is a common disease of legumes and many hosts worldwide (Bos, 1970). This virus is known to infect many other legumes (family Fabaceae) including green beans (*Phaseolus vulgaris*), peanuts (*Arachis hypogaea*), soybeans (*Glycine max*) (Ghabrial et al., 1977), faba beans (*Vicia faba*), several species of clover (*Trifolium hybridum*), alfalfa (*Medicago sativa*), vetch (*Vicia sativa*), lupine (*Lupinus luteus*) (Corbett, 1958), black locust (*Robinia pseudoacacia*), fenugreek (*Trigonella foenum-graecum*), and *Crotalaria spectabilis* (Nienhaus and Castello, 1989). It is also known to infect several non-leguminous plants including *Gladiolus* sp. (Nagel et al., 1983) *Fressia* sp. and *Eustoma russellianum* (Johnson et al., 1955). Moreover, BYMV infection reduced yield (kg/ha) and protein content (Babiker et al., 1995).

In many of recent approaches involving viral components, the induced resistance is very specific to a particular strain or group of viruses (Gholizadeh et al., 2004). The present study aims to isolate *Bean yellow mosaic potyvirus* and to design an integrated program to eliminate or reduce faba bean virus infections including natural alternatives as systemic resistance. The

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management of plant viral diseases may be accomplished through inducing plant defense 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 nutrients from the root environment (Plant Growth Promoting Microorganisms, PGPM), and indirectly through inducing systemic resistance in plants against pathogens, including viruses (Whipps, 2004; Elbadry et al., 2006; Vassilev et al., 2006).

Plant growth-promoting fungi (PGPF) and plant growth-promoting rhizobacteria (PGPR) are classes of soilborne microbes with beneficial effects on plant growth and the induction of defense resistance (Ryu et al., 2004; Hossain et al., 2007). Several PGPF have been reported such as species belonging to the genera *Trichoderma*, *Fusarium* and *Penicillium* (Hyakumachi, 1994). Using the synthetic elicitors and PGPR strains could be environmentally friendly relative to current pesticides. These characteristics make systemic acquired resistance (SAR) and induced systemic resistance (ISR), and other forms of induced resistance an attractive approach for managing crop pests in a sustainable manner within the scope of a conventional agriculture system. PGPF have been reported to produce substances such as plant hormones, to allow plants to utilize decomposing organic matter through mineral solubilization and to suppress plant pathogens in the rhizosphere by antagonistic mechanisms, such as the production of hydrolytic enzymes, aggressive mycoparasitism, competition for saprophytic colonization, and the induction of plant systemic resistance. Studies of PGPF have concentrated on the mechanisms stimulating plant growth and increased biomass. PGPF have been reported to produce substances such as plant hormones (Blanchard and Björkman, 1996), to allow plants to utilize decomposing organic matter through mineral solubilization (Altomare et al., 1999; Harman et al., 2004), and to suppress plant pathogens in the rhizosphere by antagonistic mechanisms, such as the production of hydrolytic enzymes, aggressive mycoparasitism, competition for saprophytic colonization, and the induction of plant systemic resistance (Haran et al., 1996; Koike et al., 2001; Benítez et al., 2004). Colonization of the roots is considered one of the most important characteristics of PGPF, and helps them to interact with plants to enhance growth and protection. Several studies have demonstrated that PGPF induce systemic protection against phytopathogens (Harman et al., 2004; Hossain et al., 2007). Phytohormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play important roles in the induced defense responses (Kazan and Manners, 2009). The salicylate- and jasmonate-induced pathways are characterized by the production of a cascade of PR proteins which include antifungals (chitinases, glucanases and thaumatin), and oxidative enzymes (viz., peroxidases, polyphenol oxidases and lipoxygenases). Low-molecular weight compounds with antimicrobial properties (phytoalexins) can also accumulate. The treatments of plants with plant extracts and synthetic chemicals under *in vitro* culture will be the alternative method which can lead to the induction of resistance and characterized by restriction of virus multiplication and suppression of disease symptoms compared with untreated plants (Al-Ani et al., 2002; Hammerschmidt, 1999; Walters et al., 2005). The main obstacle to the development of effective chemotherapy is the nature of virus multiplication in the host cells (Yarmolinsky et al., 2009), In addition to that, some viruses persist in a latent infection in the host (Horvath, 1983). The antiviral activity of the products including plant extracts and synthetic chemicals is connected to their components which may act directly by interaction with virus particles in the early stage of infection and block the liberation of its nucleic acid that could finally lead to stopping the virus multiplication (Al-Ani et al., 2011; Abdel-Shafi, 2013).

The aim of this study is to understand the molecular and practical significances of induced resistance against BYMV using some parameters such as growth promotion, disease assessment and signaling pathways. The ability of the PGPF *Penicillium simplicissimum* GP17-2, *Trichoderma asperellum* SKT-1 and *Fusarium equiseti* GF 19-1 to induce resistance against BYMV in faba bean plant will be evaluated.

## MATERIALS AND METHODS

### Plant and pathogen

Broad bean cultivar Giza 843 was used as the test plant in this experiment. *Bean yellow mosaic virus* (BYMV) was previously identified by Plant Virology and Phytoplasma Research Department, Plant Pathology institute, Agriculture Research Center, Giza, Egypt (Elkhyat, 2018). Mechanical inoculation was used for inoculation of the test plants as described by Noordam (1973). Leaves from faba bean plants showing mosaic, leaf deformation, stunting and yellowing were homogenized with a mortar and pestle, with 0.01M phosphate buffer (pH 7.4). The extract was passed through two layers of muslin into a sterilized container and the filtrate used as virus inoculum. All inoculation materials were chilled at 4°C prior the inoculation and maintained on ice during the inoculation.

### Induction treatments

The PGPF used in this study were *Fusarium equiseti* GF 19-1, *Penicillium simplicissimum* GP17-2 and *Trichoderma asperellum* SKT-1 which were obtained from the Laboratory of Plant Pathology, Gifu University, Japan. Autoclaved barley grains (100 g in 100 ml distilled water) were inoculated in a 500 ml Erlenmeyer flask with 10–15 disks (5 mm) obtained from the actively growing margin of 7 days old potato dextrose agar (PDA; 2% agar) cultures of *Trichoderma asperellum* SKT-1, *Fusarium equiseti* GF 19-1 and *Penicillium simplicissimum* GP17-2. After 7 days of incubation at 25°C with complete colonization in the dark, the completely colonized barley grains were air-dried at room temperature (23–25°C). The dried BGI was ground to a 1–2 mm particle size using a hand coffee grinder and stored at 4°C until further use. Seeds of faba bean cultivar Giza 843 were sown on pots at the rate of 2 seeds per pots with four repetitions. Autoclaved potting medium in sterile plastic pots (25 cm) was amended with the powdered BGI (0.5% w/w) of GP17-2, GF19-1 and SKT-1 just before seeds were sown. Autoclaved potting medium supplemented with an equal volume of autoclaved barley grain served as a control. Inoculation by BYMV was carried out 2 weeks after planting.

### Disease severity evaluation

Symptoms developments were assessed after BYMV inoculation. Severity of symptoms was evaluated at 7, 14 and 21 days after virus inoculation following the scale (0-10) with 0 = no disease symptoms and 10 = severe stunting and deformed plants (Ryu et al., 2004).

Virus titer in the leaves of faba bean was estimated at 7, 14 and 21 days after BYMV inoculation using Enzyme-Linked Immunosorbent Assay (ELISA) following the technique described by Clark and Adams (1977) and adopted by Elsharkawy et al. (2013). The ELISA experiment was carried out three times with 10 plants per treatment.

### Analysis of defense related genes expression

RNA was extracted from treated and non-treated plants using the kit (Thermo Scientific, Fermentas, #K0731) following the manufacturer protocol. cDNA was synthesized using the kit reverse transcription kits (Thermo Scientific, Fermentas, #EP0451). The Real-time PCR with SYBR Green method was used to measure expression of mRNAs of target genes, with elongation factor 1 alpha (*EF1 $\alpha$* ) as an internal reference. The isolated cDNA were amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo scientific, USA, # K0221) and gene specific primers. The primers used in the amplification are shown in Table (1). The web based tool, Primer 3 ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)) was used to design these primers based on published sequences.

**Table 1:** List of primers used in RT-PCR analysis.

Gene	Forward primer ('5 ----- '3)	Reverse primer ('5 ----- '3)
<i>PR1</i>	CAGTGGTGACATAACAGGAG CAG	CATCCAACCCGAACCGAAT
<i>PR2</i>	CCAATGGGTACAAAGAAACG	AAACCAAGTAACCAATGAAAGG
<i>EF1<math>\alpha</math></i>	GTGAAGCCCGGTATGCTTGT	CTTGAGATCCTTGACTGCAACATT

Therefore, the quantities critical thresholds (Ct) of target gene were normalized with quantities (Ct) of housekeeping gene (*EF1 $\alpha$* ) by used the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). Throughout the whole real time PCR experiment, the housekeeping gene encoding elongation factor -1 alpha (*EF1 $\alpha$* ) was used as an internal reference for normalization and data was expressed as mean  $\pm$  SEM (n = 3 in triplicate in each group). The expression level of the target gene in control plant at various time points was considered the base line.

### Data analysis

All the data were expressed as means  $\pm$ S.E. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS, 18.0 software, 2011 and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant when  $P \leq 0.05$ .

## RESULTS

### Growth characters

Growth characters were affected by barley grain inoculum (BGI) of plant growth promoting fungi (PGPF) with or without BYMV inoculation at 4 and 6 weeks after planting (WAP) as presented in Tables (2 and 3). Healthy plants were significantly taller than those infected with BYMV. Treatments with PGPF resulted in significant increase in plant heights, number of leaves, leaf area, chlorophyll contents and fresh and dry weights compared with non-treated plants. Faba bean plants treated with BGI and challenge inoculated with BYMV

substantially enhanced growth characters than infected control plants. The best effects were found in plants treated with GP 17-2 followed by GF 19-1 then SKT-1 at the 4 and 6 WAP. Plants infected with BYMV were the lowest ones.

**Table 2:** Plant height, number of leaves plant<sup>-1</sup>, leaf area and total chlorophyll in leaves of faba bean as affected by BGI of PGPF with or without BYMV inoculation at 4 and 6 WAP.

Treatment	Plant height (cm)		Number of leaves plant <sup>-1</sup>		Leaf area (cm <sup>2</sup> plant <sup>-1</sup> )		Chlorophyll content (uE)	
	4 WAP	6 WAP	4 WAP	6 WAP	4 WAP	6 WAP	4 WAP	6 WAP
Healthy	18.5 bc	30.6 cd	7.3 cd	10.9 d	121 c	214 b	0.838bc	0.748 cd
GP17-2	21.4 a	33.5 a	8.3 a	13.8 a	135 a	242 a	0.856 a	0.772 a
GF19-1	20.6 a	32.8 ab	8.1 ab	12.4 b	131 ab	238 a	0.852ab	0.763ab
SKT-1	19.6 ab	31.9 bc	7.6 bc	11.8 c	128 b	233 a	0.845ab	0.756bc
Infected	14.3 d	22.9 e	5.0 f	7.8 f	76 d	153 c	0.667 e	0.606 f
GP17-2 +BYMV	18.3 bc	30.5 cd	7.1 cd	10.6 de	120 c	212 b	0.824 c	0.742 de
GF19-1 +BYMV	17.7 bc	29.8 d	6.8 d	10.5 de	118 c	209 b	0.823 c	0.735 de
SKT-1 +BYMV	17.3 c	29.4 d	6.1 e	10.1 e	116 c	208 b	0.808d	0.733 e
F test	**	**	**	**	**	*	*	**

\* and \*\* indicate p < 0.05 and p < 0.01, respectively. Means of each column followed by the same letter are not significantly different at 5 % level, according to Duncan s multiple range test.

**Table 3:** Total fresh weight and dry matter accumulation of faba bean treated with BGI of PGPF and inoculated or non-inoculated with BYMV at 4 and 6 WAP.

Treatment	Total fresh weight (g/plant)		Total dry weight (g/plant)	
	4 WAP	6 WAP	4 WAP	6 WAP
Healthy	34.7 bc	58.9 b	3.3 b	5.7 b
GP17-2	40.6 a	64.0 a	4.2 a	6.8 a
GF19-1	38.8 ab	62.2 ab	4.0 a	6.1 ab
SKT-1	35.9 ab	60.3 ab	3.4 ab	5.9 b
Infected	19.7 f	37.8 e	2.0 d	3.9 d
GP17-2 + BYMV	31.1 cd	58.5 bc	3.0 bc	5.7 b
GF19-1 + BYMV	27.9 de	55.5 c	2.7 c	5.4 b
SKT-1 + BYMV	24.4 e	51.4 d	2.7 c	4.7 c
F test	**	**	**	**

\* and \*\* indicate p < 0.05 and p < 0.01, respectively. Means of each column followed by the same letter are not significantly different at 5 % level, according to Duncan s multiple range test.

#### Effect of BGI treatments on disease severity and virus titer of BYMV

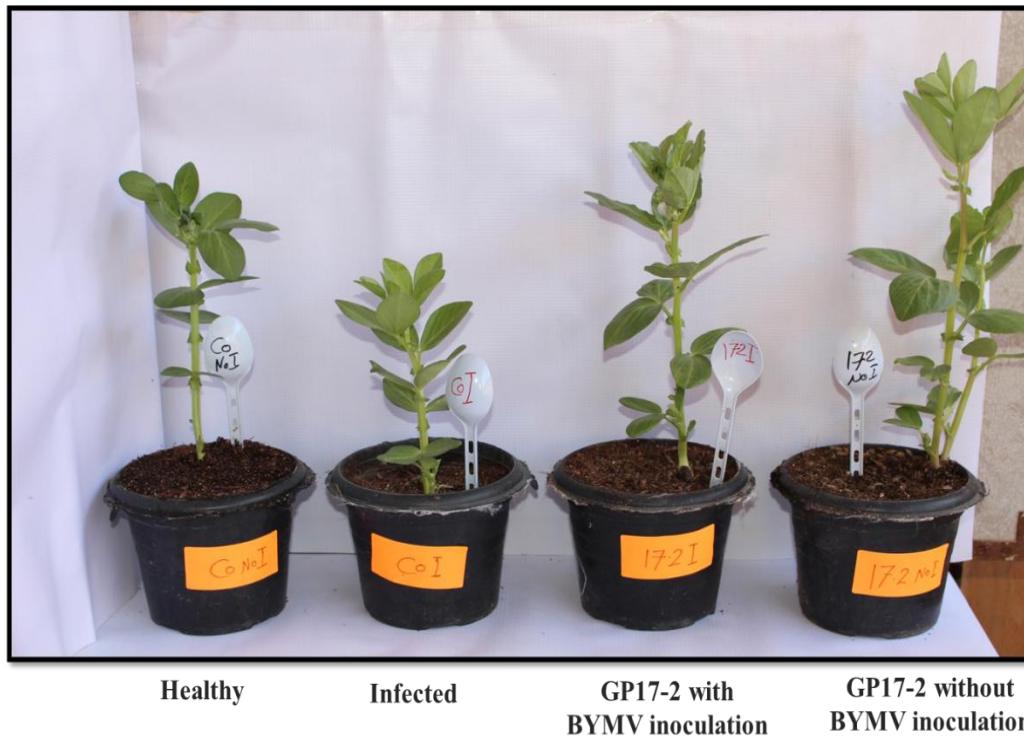
Faba bean plants grown in soils amended with the BGI of GP 17-2, GF 19-1 and SKT-1 exhibited a dramatic reduction in BYMV symptoms and virus titer as compared with the non-treated control plants at 1, 2 and 3 weeks post inoculation by BYMV. The lowest disease severity

was achieved using GP 17-2 at 2 and 3 WPI, followed by SKT-1 and GF 19-1 compared with the control as shown in Table (4) and Figs. |(1, 2 and 3).

**Table 4:** Disease severity and ELISA values of BYMV in faba bean plants treated with BGI of PGPF at 1, 2 and 3 WPI.

Treatment	Disease severity			ELISA		
	1 WPI	2 WPI	3 WPI	1 WPI	2 WPI	3 WPI
Healthy	NT	NT	NT	0.02 d	0.04 d	0.06 e
Infected	NT	2.8 a	7.2 a	0.11 a	0.20 a	0.43 a
GP17-2	NT	0.4 d	1.2 d	0.05 c	0.14 c	0.21 d
GF19-1	NT	0.9 b	1.8 b	0.06 bc	0.15 bc	0.27 c
SKT-1	NT	0.7 c	1.6 bc	0.07 b	0.16 b	0.29 b
F test	NS	**	**	**	**	**

\* and \*\* indicate  $p < 0.05$  and  $p < 0.01$ , respectively. Means of each column followed by the same letter are not significantly different at 5 % level, according to Duncan s multiple range test.

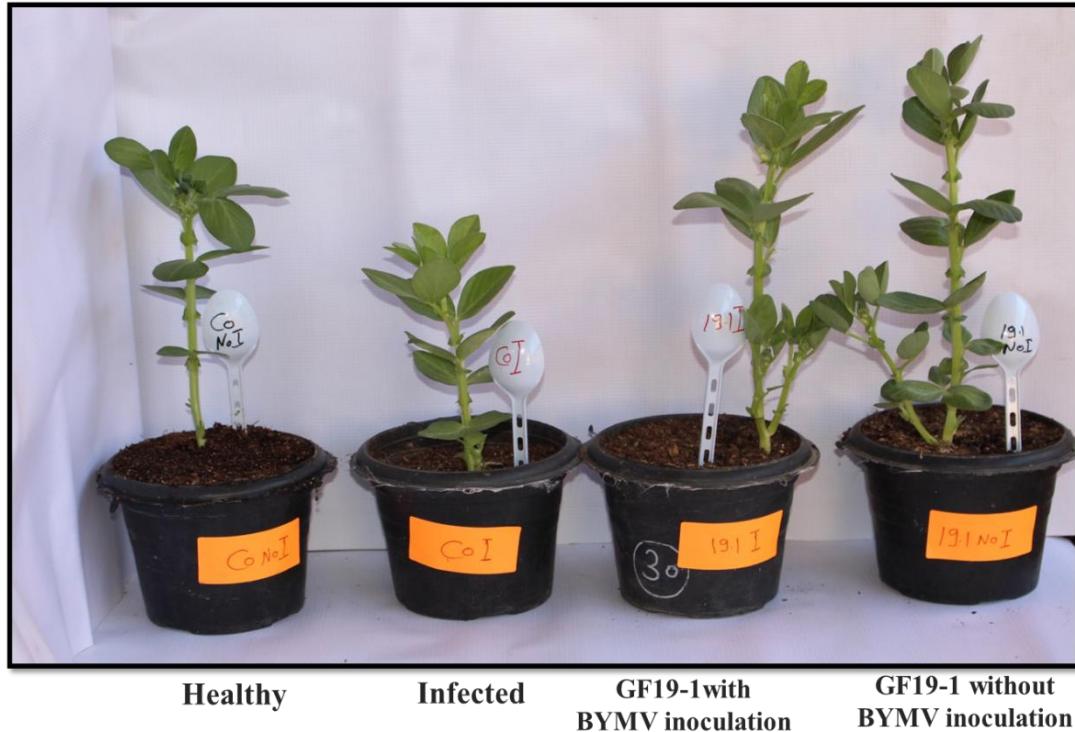


**Figure 1:** Growth promotion effect in faba bean plants treated or non-treated with *P. simplicissimum* GP 17-2 and inoculated or non-inoculated with BYMV at 2 weeks after virus inoculation.



**Healthy                  Infected                  SKT-1with BYMV inoculation                  SKT-1 without BYMV inoculation**

**Figure 2:** Growth promotion effect in faba bean plants treated or non-treated with *T. asperellum* SKT-1 and inoculated or non-inoculated with BYMV at 2 weeks after virus inoculation.



**Healthy                  Infected                  GF19-1with BYMV inoculation                  GF19-1 without BYMV inoculation**

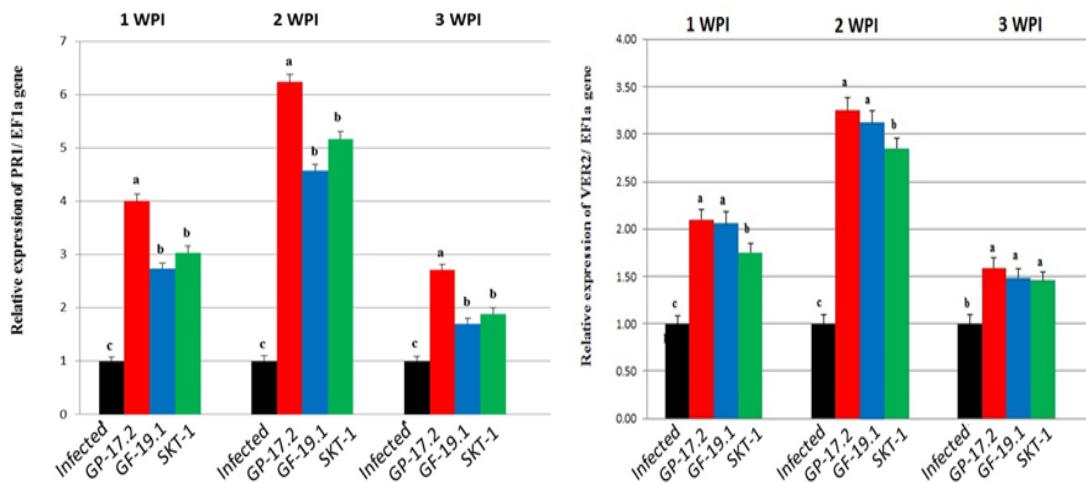
**Figure 3:** Growth promotion effect in faba bean plants treated or non-treated with *F. equiseti* GF 19-1 and inoculated or non-inoculated with BYMV at 2 weeks after virus inoculation

## Effect of PGPF isolates on the expression of defense related genes

Real time PCR was used to detect the transcription levels of two pathogenesis-related genes encoded by the genes *PR1* and *PR2* ( $\beta$ -*I*, 3-glucanase) in faba bean cultivar Giza 843 after treatment with BGI and virus inoculation.

To conduct real time PCR, we first isolated total RNA from the leaves of all groups. The quality and concentration of total RNA were assessed by Nanodrop which showed pure RNA with considerable higher concentrations of RNA (ranged from 950 to 2050 ng/ $\mu$ l).

The obtained results revealed a significant ( $P \leq 0.05$ ) increase of the expression level of *PR1* gene in treated faba bean plants at 1, 2 and 3 weeks post virus inoculation (WPI) as compared to the infected control (Fig. 4). Plants treated with GP17-2 showed the highest expression values of *PR1* gene as compared to GF 19-1 and SKT-1. On the other hand, no significant difference in the expression of *PR1* was noticed between GF 19-1 and SKT-1. The highest expression levels of *PR1* gene were found at 2 weeks after virus inoculation. Similarly, the expression levels of *PR2* gene were significantly increased in treated faba bean plants at 1, 2 and 3 weeks post inoculation as compared to the infected control (Fig. 4). Plants treated with GP17-2 and GF 19-1 showed the highest expression values as compared with plants treated with SKT-1. The highest expression levels of *PR2* gene were detected at 2 weeks after virus inoculation.



**Figure 4:** Expression of defense related genes in faba bean plants treated with PGPF isolates and inoculated with BYMV at 1, 2 and 3 weeks after virus inoculation.

## DISCUSSION

Leguminous crops especially faba bean (*Vicia faba* L.) plants are important food legume in Egypt and other countries of the world. Faba bean crop suffered great losses in Egypt due to infection with mosaic diseases. *Bean yellow mosaic virus* (BYMV) is a plant pathogenic virus in the genus *Potyvirus* and the virus family *Potyviridae*. Like other members of the *Potyvirus* genus, BYMV is the most serious problem (Nakazono-Nagaoka et al., 2004). This virus was reported as one of the most common viruses naturally infecting faba bean.

Pathogenic microorganisms that affect plants represent a major chronic threat to food production and ecosystem stability worldwide. Food producers have become increasingly dependent on agrochemicals as a relatively reliable method of crop protection and fertilization (Compan et al., 2005). Viral diseases are fastidious diseases, and there are few chemical solutions available to combat them which are often ineffective, or cannot be used based on the increasing demand for pesticide-free food (Shoresh et al., 2010). Biological control and fertilization are therefore being considered as alternative or supplemental methods for reducing the use of chemicals in agriculture (Whipps, 2001; Postma et al., 2003). Systemic resistance in plants is induced by plant growth promoting rhizobacteria (PGPR) via several different mechanisms. Plant growth promoting fungi (PGPF) are a class of non-pathogenic soil-borne filamentous fungi that have beneficial effects on plants. PGPF may also induce systemic resistance by different mechanisms (Bent, 2006). Several PGPF have been reported as biotic inducers against plant pathogens such as species belonging to the genera *Trichoderma*, *Fusarium* and *Penicillium* (Hyakumachi, 1994). In the current study, BGI of PGPF significantly reduced severity of BYMV in faba bean plants. Similarly, BYMV titer was significantly decreased and ELISA test showed decreased virus accumulation in faba bean plants relative to control plants (infected). The results are in agreement with the protection observed in other plants (Hossain et al., 2007; Elsharkawy et al., 2012a; Elsharkawy et al., 2012b), indicating the general activity of these PGPF.

In the present study, the results demonstrated that the PGPF of GP 17-2, GF 19-1 and SKT-1 treatments at greenhouse resulted in significant increase of plant height, number of leaves, leaf area, total chlorophyll in leaves, fresh and dry weights of faba bean at 4 and 6 weeks after planting (WAP). A progressive increase in plant height, number of leaves, leaf area and total chlorophyll in leaves at 4 and 6 WAP was recorded in plants treated with GP17-2 and GF 19-1 followed by SKT-1 compared to control group. Moreover, there was no significant difference between GP17-2 and GF 19-1 treatments under infection with BYMV when compared with healthy treatment (control). The highest values in fresh and dry weights were found in plants treated by GP17-2, GF 19-1 and SKT-1 without BYMV infection compared with other treatments. Otherwise, no significant difference was found between GP17-2 with BYMV infection and healthy treatment. These findings are supported by the work done by Elsharkawy et al., (2012a); Elsharkawy et al., (2013).

## CONCLUSION

In conclusion, this study showed the ability of PGPF isolates to control BYMV infection in faba bean plants. GP17-2 was the most effective against BYMV. Moreover, pre-treatment with the PGPF showed strong improvements in faba bean growth characters. To the best of our knowledge, this is the first report to control BYMV by PGPF isolates. PGPF could be included in management methods against BYMV.

## AUTHOR DETAILS

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