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Effect of *Bacillus subtilis* on potato virus Y (PVY) disease resistance and growth promotion in potato plants

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Abstract Potato virus Y (PVY) has become the most important viral pathogen of potato. The *Bacillus subtilis* EMCCN 1211 (*B. subtilis*) isolate was investigated in the current study as a biocontrol agent for the management of the PVY and Induced Systemic Resistance (ISR) in potato plants under greenhouse conditions. Foliar and soil applications of a *B. subtilis* suspension at a concentration of 10^8 CFU/mL was applied at 48 h and 10 days respectively, before and after inoculation with PVY. Treatment of *B. subtilis* before virus inoculation resulted in a significant reduction in symptoms and entirely negative enzyme-linked immunosorbent assay (ELISA) results compared to untreated infected potato plants. In contrast, the RT-PCR showed PVY amplification (825 bp) in all bacterially treated plants. The soil application using *B. subtilis* before the PVY inoculation efficiently induced plant resistance and reduced the PVY accumulation level (32.79%) at 10 days post-inoculation (dpi) and continue with percentage increase of virus inhibition up to 72.26% at 35 dpi. The *B. subtilis* stimulated the plant growth that the potato plants fresh and dry weights increased by 61.40 and 56.6% at $p \leq 0.05$ respectively. The transcriptional changes of pathogenesis -related gene (*PR-1*) was tested at 10 dpi. The results showed that the lower PVY accumulation was associated with the lower suppression of *PR-1* defense related gene expression at 10 dpi and showed 0.9659 fold change value comparing with the mock-inoculated control. This data revealed that the *soil application of B. subtilis* efficiently suppressed, reduced the PVY accumulation level and symptoms severity; therefore it can be used as an antiviral biocontrol agent.

Keywords Potato virus Y (PVY) · *Bacillus subtilis* · Virus inhibition and pathogenesis -related gene (*PR-1*) · Promote growth

Introduction

The potato (*Solanum tuberosum* L.) is an essential crop grown globally and is a basic source of nutrition for many people in developing countries (Bond, 2014). Diseases are one of the biotic factors that significantly affect the productivity of the potato crops. Potato production is threatened by a number of different bacterial, viral, fungal, and nematode diseases. The most aggressive and harmful infections that attack the potato are viral illnesses. Potatoes are reported to be commonly susceptible to more

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than 50 viruses and viroids. The major viruses are potato leaf roll virus (PLRV), potato virus Y (PVY), potato virus X (PVX), potato virus M (PVM), potato virus S (PVS), potato virus A (PVA), alfalfa mosaic virus (AMV) and tobacco rattle virus (Jones, 2014). Numerous studies have revealed the existence of PVY, AMV, TRV and PLRV viruses in Egyptian potatoes (El-Kammar et al., 2016, Abdel Aleem et al., 2018; Elwan et al., 2021; Mazyad et al., 2021). PVY is considered the most important and economically harmful virus to grown potatoes (Valkonen, 2007; Scholthof, et al., 2011). It is the type species of the genus Potyvirus of the Potyviridae family and possesses a positive-sense single-stranded RNA genome of approximately 9.7 kb that encodes a large polyprotein (Wylie et al., 2017). The virus occurs as a complex form of several strains and depending on the potato cultivar, the infecting PVY strain(s), the period of infection incidence, and environmental conditions, it can cause a variety of symptoms in potato leaves and tubers. These include leaf mosaic, mottle, crinkling, vein necrosis, necrotic spots, stem and petiole necrosis, leaf drop, and plant stunting. Some PVY strains also cause potato tuber necrotic ringspot disease (Nasr-Eldin et al., 2019). Apart from tuber transmission, PVY also has a number of vector aphid species that transmit the virus non-persistently (Jones, 2014; Lacomme et al., 2017). PVY affects both yield and tuber quality, causing yield losses of up to 86% (Alyokhin et al., 2022). The virus has caused significant economic damage in the USA, Switzerland, the European Union, and Egypt. In the USA, yield losses have been estimated as 0.13 tonnes/ha, while in Switzerland economic damage has been estimated at around 2000 and 200 CHF/ha. The European Union experienced annual losses of 187 million euros in seeds and production. In Egypt, PVY infection has been isolated, causing tuber necrotic ringspot symptoms in vulnerable cultivars, affecting potato quality and yield (Abdalla et al., 2018; Dupuis et al., 2023). Additionally, the virus is responsible for severe disease in other common crops such as tobacco, tomatoes and pepper (Kerlan, & Moury, 2008). Plant disease control is essential for ensuring high-quality, plentiful food, feed and fiber to maintain a healthy and rapidly growing world population.

Various types of plant disease management strategies can be applied to control or eradicate plant diseases. Most of the world's farmers often rely on chemical-based pesticides and fertilizers for bountiful agricultural production (Shafi et al., 2017). However, chemical antiviral compounds are toxic to plants, animals, and humans, and selection for virus resistance and the genetic transformation of plants is expensive and time-consuming with limited applications (Lee & Ryu, 2016, Rabiey et al., 2019).

Environmentally friendly protection of plants from viruses is now associated with the use of plant growth promoting microorganisms (PGPM) and their metabolites as new antiviral agents and biological triggers (Maksimov et al., 2019; Zhang et al., 2022). Previous studies have repeatedly reported that many plant pathogenic diseases can be controlled by natural antagonistic microorganisms (Cook et al., 2002). The relationship between antagonistic microbes and plant pathogens may be complex. The pathogen is directly affected by antagonists acting through hyperparasitism and antibiosis. These interactions, which frequently combine with other forms of action, are very regulated cascades of metabolic events (Köhl et al., 2019).

Many of the bacterial antagonists come from the genus *Bacillus*, though there are a few other species that are more significant but less useful (Verschuere et al., 2000). *Bacillus species* have the unique capacity to replicate quickly, are resistant to tough environmental circumstances and possess broad biological control capabilities (Shafi et al., 2017). Additionally, the volatile substances that *Bacillus subtilis* (*B. subtilis*) produces are crucial for supporting plant growth and activating plant defense mechanisms by causing induced systemic resistance (ISR) in plants (Compant, et al., 2005).

The term ISR refers to induced systemic resistance promoted by plant growth-promoting rhizobacteria (PGPR) regardless of the signaling pathway involved in the process, while the term systemic acquired resistance (SAR) is used to describe induced systemic resistance that is salicylic acid-dependent and caused by a localized infection (Vleesschauwer & Höfte 2009). Many reports mentioned that PGPM have a direct and indirect effect on the viruses either by secretion of enzymes or by inducing ISR (Kumar et al., 2016; Lee & Ryu, 2016; Walaa et al., 2016; Beris et al., 2018; Abdelkhalek et al., 2020; Miljakovic et al., 2020). The development of both SAR and ISR activated by PGPM leads to multiple cellular responses in plants, including the synthesis of pathogenesis-related proteins (*PR*-proteins) (Pieterse et al., 2014; Robert-Seilaniantz et al., 2011). The objective of the present study is to evaluate

the activity of *B. subtilis* as biological control agent against PVY in potato plants, estimate the PVY accumulation and the relative expression of the pathogenesis related gene-1 (*PR-1*) as an SA marker gene which implements SAR induction and participates in the salicylic acid (SA) biosynthesis pathway, which is associated with the immune response of plants and promotes plant growth.

Materials and methods

Bacterial source

A Gram-positive aerobic *B. subtilis* with No. EMCCN 1211 isolated from soil was obtained from the Microbial Inoculants Center (MIC) of the Faculty of Agriculture, Ain Shams University. Bacteria were grown at 37°C for 24 h on nutrient liquid medium (0.1% Beef extract, 0.5% Peptone, 0.2% Yeast extract, 0.5% NaCl) with shaking at 120 rpm. The bacterial culture of the *B. subtilis* suspension was adjusted to 10^8 CFU/mL (10^8 CFU/mL measured by spectrophotometer at OD_{600} nm wavelength).

Virus source

Infected potato plants were collected from different locations in Kafer EL-sheikh Governorate. Leaf samples with symptoms resembling PVY were analyzed serologically by ELISA for different potato viruses (PVY, PVX, PLRV, TRV and AMV) and the results were confirmed by RT-PCR. The positive PVY potato plant was maintained by propagation in an insect-proof greenhouse as a PVY virus source.

Greenhouse experimental design

Bacterial treatment and viral inoculation

Potato tubers were tested directly before planting using ELISA to ensure that they were free of potato viruses. Potato tubers were cultivated in plastic pots filled with sterilized soil. The soil was disinfected at 121 °C for 20 min. Five pots of potato plants with 3–4 plants in each were used for each treatment. Potato plants were inoculated with PVY-infected potato leaf sap diluted 1:1 with distilled water. All plants were kept in greenhouse growing conditions with a photoperiod of 16 h of light and a typical temperature of 25 °C/20 °C (day/night). The treated plants were observed daily for symptom development. All treatments were compared to plants inoculated with PVY (PVY cont.), plants treated with *B. subtilis* only (B cont.), and plants treated with water as a negative control (H cont.). Newly emerged potato leaves were collected and analyzed for the presence of virus by ELISA test and RT-PCR.

B. subtilis foliar spraying (T1)

Potato plants in the fourth leaf growth stage were treated with 10 mL of the bacterial suspension at a concentration of 10^8 CFU/mL. The following treatments, containing 5 replicates, were applied to potato plants: (i) plants treated by foliar spraying with *B. subtilis* 48 h before PVY inoculation (denoted as BV), (ii) plants treated with *B. subtilis* 48 h after infection with PVY (designated as VB). Newly emerged potato leaves were collected at 4, 7, 10 and 16 dpi for further investigation.

B. subtilis soil application (T2)

A bacterial suspension of *B. subtilis* was used in the soil application. The experiment was conducted with five replicates with the following treatments: (i) Plants treated by immersing 10 mL of the bacterial suspension into the soil 10 days prior to PVY inoculation (referred to as BV-soil). (ii) Plants treated with the bacterial cells suspension in soil post 10 days of virus inoculation were (designated as VB-soil). Potato leaves were collected at 10, 20, 27, 35 and 41dpi for virus detection and symptoms development.

Determination of virus accumulation content

Direct enzyme-linked immunosorbent assay (DAS ELISA) using complete kits (LOEWE, Switzerland), was used to determine PVY according to the manual protocol. DAS-ELISA was used to detect viral accumulation and consequently estimate the rate of inhibition of virus replication as previously described (Sorokan et al., 2020). ELISA was performed on both inoculated and non-inoculated newly emerged leaves from five replicate potato plants per treatment. Newly emerged leaves were collected 4, 7, 10 and 16 dpi for the *B. subtilis* foliar spraying treatment (T1) and at 10, 20, 27 and 35 dpi for

the bacterial soil application treatment (T2). The virus concentration was measured at an absorbance value of 405 nm in a microplate reader (CLINDIAG systems Co. LTD). The ELISA was directly applied to the natural infected leaves that were used for the PVY inoculum and to the treated plants. The ELISA results (at A 405 nm) were considered positive for the presence of PVY if the absorption value was greater than twice that of the samples from the healthy controls. The equation for measuring the percent reduction in virus accumulation is as follow: reduction percentage $=\frac{(A-B)}{A} \times 100$, where A is the mean average of virus titer OD values in the positive untreated control plants, and B is the average virus titer OD value in each bacterial treatment.

One step RT-PCR detection

The coat protein gene of PVY was detected using the forward primer PVYCPvBamHI and the complementary primer PVYCPcEcoRI (Table 1) with an expected amplified product of 825 bp (Shalaby et al., 2002). Total RNA was isolated from potato plants treated or not treated with *B. subtilis* using RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Reverse transcription (RT)-PCR reaction was optimized using Verso ™ one-step RT-PCR kit (Thermo scientific, USA). The one-step RT-PCR reaction was performed by combining 12.5 µL 2X One-Step PCR Master Mix, 1µL of each primer pair (200 nM), 1.25 µL of RT-enzyme enhancer, 0.5 µL verso enzyme mix, and 3 ng of RNA template, and the mixture was made up to 25 µL using nuclease-free water. The Reverse transcription reaction started with incubation at 50°C for 30 min, followed by denaturation at 95°C for 15 min. The PCR reaction continued in a thermocycler (Uno) for 35 cycles at 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min and finally at 72°C for 5 min. Five microliter aliquots of RT-PCR products were analyzed on 1% agarose gels in 1X TAE buffer.

Western blotting assay

Total protein was extracted from 0.2 g of potato leaves using 1 mL of 2X loading sample protein buffer (50 mM HCl, pH 8.2, 10% glycerol, 1% β-mercaptoethanol, 0.1% bromophenol blue and 2% SDS) and boiled for 10 min then cooled. The extracted proteins (50 µL) were separated by 12.5% denaturing SDS-polyacrylamide gel electrophoresis for 2 h at 110 V. For western blotting, the separated proteins were transferred to a nitrocellulose membrane using a semi dry electro-blotter (Biometra) with transfer buffer solution at 50 mA. The western blot membrane was then blocked with phosphate buffer saline -Tween-20 (PBST) containing 2.5 g (5% w/v) non-fat dry milk powder without shaking overnight, and the transferred proteins were then hybridized with the primary antibodies produced against PVY CP (LOEWE, Switzerland) (1:200 dilution) and with the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma-Aldrich, Darmstadt, Germany). A color solution (NBT/BCIP) was then used to see the resulting blots.

Evaluation of potato plant growth

In a greenhouse experiment (T2 treatment), plants (41 dpi) were used to track the effect of applying the biological agent (*B. subtilis*) on plant height (cm), fresh shoot and root weight (g), dry shoot and root weight (g) and tuber weight (g) if any. The plants from each treatment were carefully uprooted, washed, and assessed for each growth parameter. The dry weights were determined after drying plant samples in an oven at 50 °C for 72 h.

Quantitative real-time RT-PCR (qRT-PCR) assay

Transcriptional levels of potato pathogenic related gene -1 (*PR-1*) were assessed and estimated through

Table 1 The oligonucleotide primers sequences used for RT-PCR and Qrt RT-PCR

Gene	Primer sequence (5'-3')	Reference
PVYCPv*Bam*HI	TCAAGGATCCGCAAATGACACAATTGATGCAGG	Shalaby et al., 2002
PVYCPc*Eco*RI	AGAGAGAATTCATCACATGTTCTTGACTCC	
18S rRNA-F	TACGCCCCGCCCAAA	Pignatta et al., 2007
18S rRNA-R	CACTGGCAGTCCTTCGTGAGT	
PR1-F	CCAAGACTATCTTGCGGTTC	Abo-Zaid et al., 2020
PR1-R	GAACCTAAGCCACGATACCA	

the qRT-PCR technique, as previously described (Behiry et al., 2018). The 18S rRNA, was used as a housekeeping gene for the normalization of the expression level of *PR-1* gene (Pignatta et al., 2007; Abo-Zaid et al., 2020). Table 1 indicates the primers used in this study for the 18S rRNA and *PR-1* gene. The qRT-PCR was performed using 2×QuantiTect SYBR Green PCR Master Mix (QIAGEN, Germany) to estimate *PR-1* transcription level in the treated potato plants. The reaction was done in a total volume of 25 µL, containing 3 µL RNA (50 ng), 1 µL of 10 µM of each primer (forward and reverse primers; Table 1), 12.5 µL of 2×QuantiTect SYBR Green PCR Master Mix, 0.5 µL Reverse transcriptase (Thermo Fisher, Catalog number: EP0441) and 7 µL of nuclease-free water. The qRT-PCR reaction was performed using Real time PCR machine (Stratagene MX3005P, USA). The reverse transcription reaction started with incubation at 50 °C for 30 min. The PCR amplification was performed by an initial denaturation step at 95 °C for 15 min, followed by 40 cycles each consisting of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s. Amplification curves and C_T values were determined by the Stratagene MX3005P software. After that, the dissociation curves were obtained to eliminate the inclusion of non-specific products. The relative expression level of the target gene was quantified and calculated according to $2^{-\Delta\Delta C}{}_T$ algorithm (Livak & Schmittgen, 2001).

Statistical data analysis

Data were statistically analyzed using Costate Statistics Software (Sokal & Rohlf, 1995) (version 6.4). ANOVAs variance was used to analyze all data. The significant differences in each treatment were detected at $p=0.05$. Each treatment was performed in triplicate.

Results

Virus source

The natural isolate of PVY showed mosaic and necrosis symptoms (Fig. 1A) was collected from infected potato plants from different locations in Kafer El-sheikh Governorate. The PVY infected plant was serologically analyzed by ELISA for different Potato viruses (PVY, PVX, PLRV, TRV and AMV). Plants did not show ELISA reading value with other viruses than PVY isolate which mentioned above by DAS-ELISA. The ELISA reading value for the PVY virus was 1.416 OD, compared to 0.219 OD for negative control plants. The ELISA result was confirmed by RT-PCR for the PVY coat protein amplification with 825 bp amplicon size (Fig. 1B). No PCR amplicon was observed from healthy potato plant used as a negative control.

Influence of *B. subtilis* on PVY symptoms emergence

The antiviral activity of *B. subtilis* isolate against PVY was evaluated with potato plants at 16, 20, 35 and 41 dpi. The virus symptoms were first observed for bacterial foliar spray (T1) at 16 dpi and increased to 20 dpi in 75% of VB treated potato plants (Fig. 2A). On the other hand, 25% of BV treated potato plants showed mild PVY symptoms (mild mosaic) at 20 dpi as shown in Fig. 2B.

The results showed that soil application (T2) of a bacterial cell suspension 10 days before viral inoculation (BV-soil) delayed the appearance of disease symptoms and increased plant growth comparing to non-treated infected plants (Fig. 2C), whereas only 10% of BV-soil treated plants showed a mild mosaic symptom. On the other hand, 40% of plants

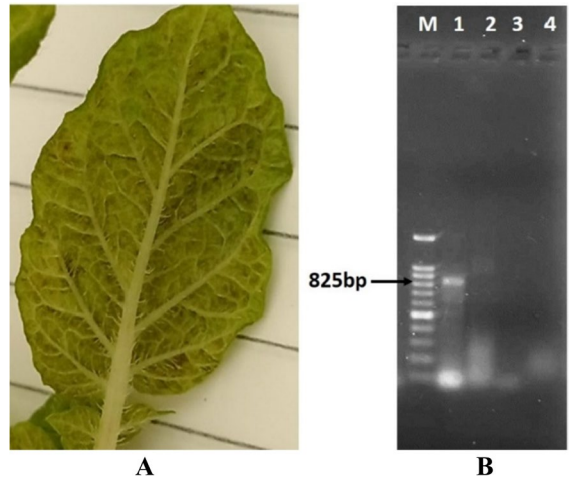

Fig. 1 **A** The natural isolate of PVY showed mosaic and necrosis symptoms **B** 1% agarose gel electrophoresis for the RT-PCR amplicon for: Lanes 1 PVY; Lane 2: AMV; Lane 3: PVX; Lane 4: TRV; Lane M: 100-bp DNA ladder

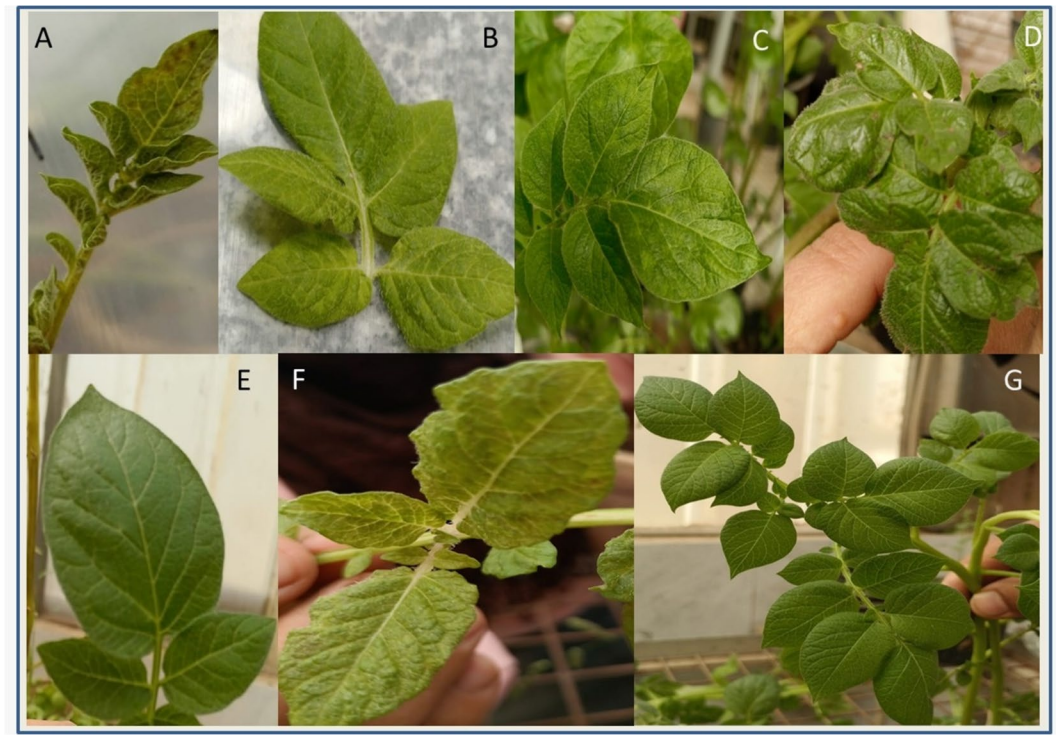

Fig. 2 PVY symptoms on *B. subtilis* treated potato plants infected with PVY. **A** Plants treated with *B. subtilis* spray 48 h after inoculation with PVY (VB) at 20 dpi. **B** Plants treated with *B. subtilis* spray 48 h before inoculation with PVY (BV) at 20 dpi. **C** Plants treated with *soil applied B. subtilis* isolate (BV-soil) at 35 dpi. **D** Plants treated with soil applied *B. subtilis* isolate (VB-soil) at 35 dpi **E** Mock-treated plants (H cont.). **F** Plants inoculated with PVY only (PVY cont.). **G** Plants treated with *soil applied B. subtilis* isolate only (B cont.)

treated with soil applied *B. subtilis* strain (VB-soil) at 35 dpi showed necrotic lesion symptoms on leaves (Fig. 2D). The symptoms of PVY, mosaic pattern and necrosis symptoms on leaves, as shown in Fig. 2F, were obviously detected for the PVY infected potato plants (PVY cont.) at 16 dpi. No PVY symptoms were noticed for either the mock inoculated control (H cont.) as shown in Fig. 2E or *B. subtilis* treated plants (B. cont.) without virus inoculation Fig. 2G.

Influence of *B. subtilis* on systemic PVY accumulation in potato plants

Potato plants were treated with a *B subtilis* bacterial cell suspension at 48 h and 10 days prior or post PVY infection by foliar spray or soil application, respectively. The systemic PVY movement and accumulation of the PVY CP gene at 4, 7, 10, and 16 (dpi) for foliar treatment and at 10, 20, 27 and 35 dpi for soil treatment were analyzed for the presence of the virus using DAS-ELISA and RT-PCR. The ELISA analysis detected the virus accumulation in new emerged leaves at 7 dpi in both BV and VB treatments with 43.27% and 48.54% virus content reduction, respectively Fig. 3. With all replicates at 10 and 16 dpi, the BV treatment resulted in negative ELISA readings for PVY coat protein (0.847 and 0.482) with viral inhibition rates of 41.26% and 66.57% respectively, Fig. 3. On the other hand, ELISA analysis of VB treatment showed a decrease in the PVY inhibition rate (31.28%) in potato leaves at 10 dpi and an increase in the virus titer and accumulation (1.263) at 16 dpi with 13% low percentage of virus inhibition (Table 2 and Fig. 3A). The BV treatment showed totally negative ELISA results (i.e. low PVY accumulation) in the leaves of potato at 10 and 16 dpi. The lowest content of viral particles was achieved in BV plant after 16 day of virus inoculation (Table 2). In the case of *B. subtilis* soil application treatments before and after

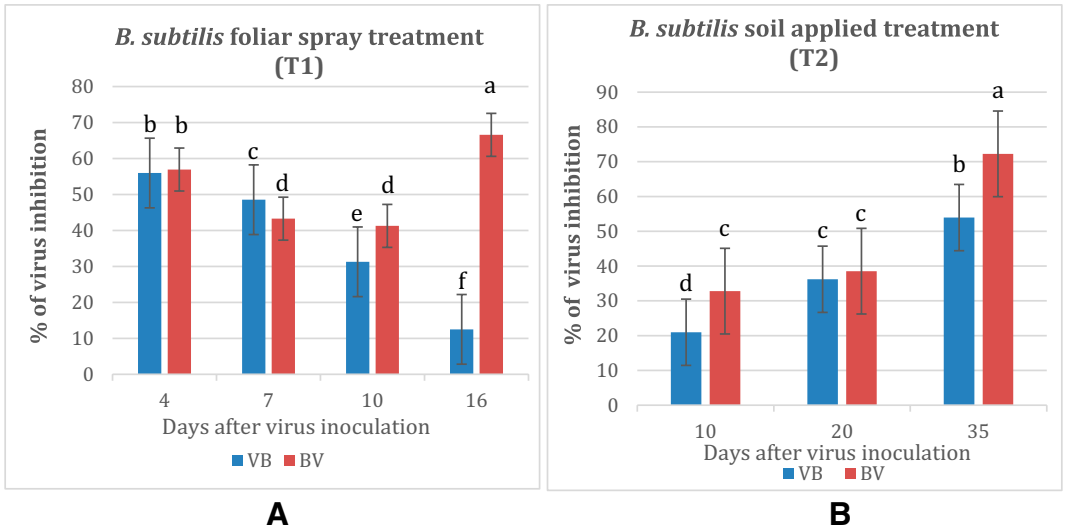

Fig. 3 A histogram showing the percentage of virus content reduction (inhibition) at different days after virus and bacterial treatments where, VB: virus inoculation before bacterial treatment, BV: Virus inoculation after bacterial treatment. Different lowercase letters (a, b, c, d, and e) indicate significant differences between groups at the same time point. Error bars signify standard errors of the mean ($n=5$) at each time point

virus infection (BV-soil and VB-soil), no significant symptoms occurred along with completely negative ELISA data at 10 and 20 dpi. Significant mild symptoms occurred in only 20% of BV-soil treated plants with low ELISA reaction at 35 dpi as illustrated in Table 3. Some of the VB soil treated potato plants displayed local necrotic lesions on leaves despite negative ELISA results at 35 dpi as shown in Fig. 2D. The 10 days pretreatment of potato plants with *B subtilis* soil application before the infection with PVY (BV-soil) suppressed the viral accumulation and showed 32.79%, 38.53% and 72.26% of virus inhibition at 10, 20 and 35 dpi, respectively, as illustrated in Fig. 3B. Meanwhile, the percentage of virus content reduced to 36.2% and 53.94% for VB-soil treatment at 20 and 35 dpi respectively in comparison with the PVY positive control (PVY-cont.), as indicated in Fig. 3B.

PVY RT-PCR detection in *B. subtilis* treated plants

The RT-PCR was carried out on potato leaves collected 20 and 35 dpi to detect the virus in plants with negative ELISA readings as shown in Table 2 and Fig. 4. The PVY PCR amplification from potato plants treated with the bacterial suspension spray (T1) after and before inoculation with PVY (VB and BV) revealed DNA amplicon at the right size (825 bp) at 20 dpi as shown in Fig. 4 lanes 1 to 6. PCR amplification for PVY from potato plants treated by *B. subtilis* soil application (T2) after and before PVY

Table 2 The ELISA absorbance values of potato plants treated with foliar spray by *B. subtilis* cell suspension (T1)

Treatment	4dpi	7dpi	10dpi	16dpi
V B	$0.635^{e} \pm 0.126$	$0.742^{d} \pm 0.044$	$0.991^{b} \pm 0.164$	$1.263^{a} \pm 0.113$
BV	$0.621^{e} \pm 0.048$	$0.818^{c} \pm 0.053$	$0.847^{c} \pm 0.014$	$0.482^{f} \pm 0.360$
H cont	0.407	0.219	0.680	0.366
PVY cont	0.893	1.194	1.401	1.442
L.S.D			0.03	

The LSD test ($n=5$, $p \leq 0.05$). Different lowercase letters (a, b and c) within the same column indicate significant differences between treatments ($p \leq 0.05$)

Table 3 The ELISA absorbance values of potato plants treated with *B. subtilis* soil application (T2)

Treatment	10 days	20 dpi	35dpi
VB-soil	$0.923^{a} \pm 0.014$	$0.745^{b} \pm 0.047$	$0.538^{c} \pm 0.000$
BV-soil	$0.785^{b} \pm 0.115$	$0.718^{b} \pm 0.116$	$0.324^{d} \pm 0.167$
PVY cont	1.340 ± 0.246	1.098 ± 0.211	1.068 ± 0.357
H cont	0.571 ± 0.236	0.522 ± 0.118	0.118 ± 0.304
L.S.D		0.128	

The LSD test ($n=3$, $p \leq 0.05$)

inoculation at 35 dpi showed a low intensity amplified product at the right size (825 bp), as indicated in Fig. 4 lanes 8 to 11. The PVY positive control plant (PVY) showed a sharp band of DNA amplicon at the expected size (Fig. 4 Lane 12). No PCR amplification was observed with the water treated potato plant (H cont.), as shown in Fig. 4 lane 7.

Impact of bacterial treatment on potato plant growth

Soil treatment with *B. subtilis* significantly increased the growth of potato plants inoculated with PVY. Statistical analysis using the Costate program showed significant LSD value of 13.38 at 0.05 P value. No significant difference in total plants lengths (shoot and root) among all bacterial treated potato plants (B cont., BV-soil and VB-soil) compared with PVY cont. (positive control plants without *B. subtilis* treatment) value where they show 55.38, 56.08 and 47.44 cm respectively with percentage increases of 63.3%, 63.78% and 57.19%, respectively (Table 4). The PVY cont. length were 20.31 cm and showed a large significant difference with the bacterial treated plants (B cont., VB-soil and BV-soil), as shown in Table 4. On the other hand, the BV-soil demonstrated the highest total plant (shoot and root) fresh weight with a percentage increase of 58.9%. It showed no significant difference with the healthy untreated plants (71.43 g). No significant difference was observed between the (B cont.) bacterial treated plants without virus inoculation (46.76 g) and the VB-soil total plant fresh weight 50.59 g as indicated in Table 4. Moreover, soil treatment with *B. subtilis* before PVY infection (BV-soil) showed increases in the total plant dry weights of potato plants (5.02 g), with percentage increases of 54.38%, and showed no significant difference with the bacterial treated plants without PVY infection (B cont.) and the healthy control potato plants (H cont.), as indicated in Table 4. Conversely, soil treatment with *B. subtilis* after PVY infection (VB-soil) and PVY cont. plants showed a significant decrease in the total plant dry weights of 3.78 and 2.29 g respectively.

Western blot analysis

After SDS-PAGE analysis, the expression of the virus coat protein gene was detected in all bacterial treated plants in comparison with the PVY control and healthy uninfected potato plants. A protein band with a size of 39KDa was detected with

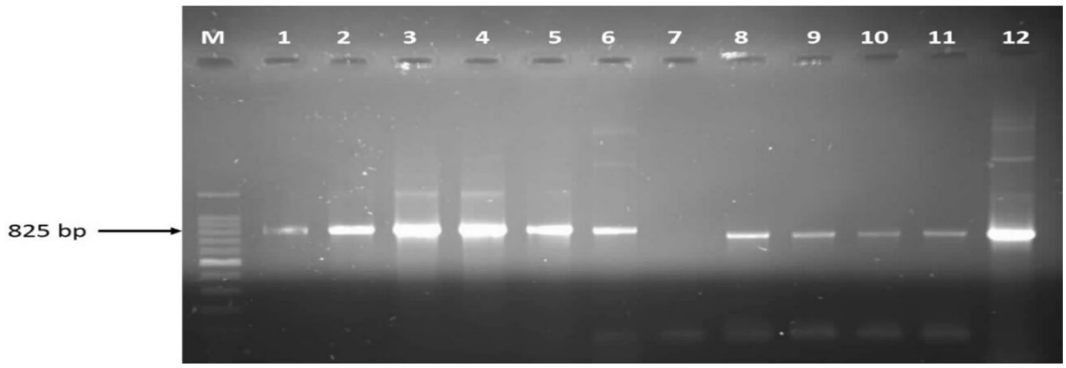

Fig. 4 1% agarose gel electrophoresis for RT-PCR amplicons from *B. subtilis* treated plants at 20 dpi. M: 100 bp DNA ladder; Lanes 1 to 3: potato plants treated with the bacterial suspension spray after inoculation with PVY (VB); Lane 4 to 6: potato plants treated with the bacterial foliar spray before inoculation with PVY (BV); Lanes 8 and 9: potato plants treated by *B. subtilis* soil application after PVY inoculation (VB-soil); Lanes 10 and 11: potato plants treated by *B. subtilis* soil application before PVY inoculation (BV-soil).; Lane 12: PVY positive control plant (PVY cont.); Lane 7: un-inoculated potato plant (H cont.)

Table 4 Impact of bacterial treatment on growth parameters of potato plant under greenhouse conditions

Treatments	Measurements					
	Total plant length (cm)	Increase* %	Total plant Fresh weight (g)	Increase* %	Total plant Dry weight (g)	Increase* %
BV-soil	56.08[b]	63.78%	72.09 [a]	58.9%	5.02 [a]	54.38%
VB-soil	47.44 [b]	57.19%	50.59 [b]	41.43%	3.78 [ab]	39.42%
PVY cont	20.31[c]	-	29.63 [c]	-	2.29 [b]	-
H cont	82.96 [a]	75.5%	71.43 [a]	58.52%	5.36 [a]	57.28%
B cont	55.38 [b]	63.3%	46.76 [b]	36.63%	4.28 [a]	46.5%
L.S.D.0.05	13.38		15.77		1.97	

Different lowercase letters (a, b and c) within the same column indicate significant differences between treatments ($p \leq 0.05$). *: increase % = (treatment –PVY cont.) / treatment × 100

the PVY positive control at 16 dpi (untreated with the *B. subtilis*) as shown in Fig. 5 lane 7. A faint protein band with the expected size was noticed in VB and BV treatment after 4 days of virus inoculation on inoculated leaves (Fig. 5, lane 1 and 4). An adequate protein band was observed with both VB and BV treatment at 7 dpi (lane 2 and 8). The VB treatment at 10 and 15 dpi showed an intense band (Fig. 5 lane 3 and 6), whereas we found that foliar spray treatment with *B subtilis* followed by virus inoculation (BV) reduced PVY coat protein gene expression (Fig. 5) at 10 and 16 dpi (lanes 9 and 10). No virus protein band was detected in healthy uninfected potato plants (Fig. 5 lane 5). The BV-soil and VB-soil treated plants showed a faint protein band at 10, 20 and 27 dpi, whereas a distinct protein band was present in plants treated with soil application of *B subtilis* at 35 dpi (supplementary S1), although 90% of BV-soil and 60% of VB-soil treated plants gave negative readings with ELISA and did not show PVY symptoms.

QRT-PCR-Gene expression

Under *B. subtilis* (B cont.) inoculation, the *PR-1* gene was up-regulated, and the transcription level was 4.5631 fold higher than that of the H. cont plants. It is well known that relative expression values lower than 1 means a decrease in expression levels (down-regulation). The expression of the *PR-1* gene was down-regulated, with a relative expression level 0.0284 fold lower in PVY infected plants (PVY cont.) compared with the healthy control plants. Both foliar spraying treatments (BV and VB) by the bacterial

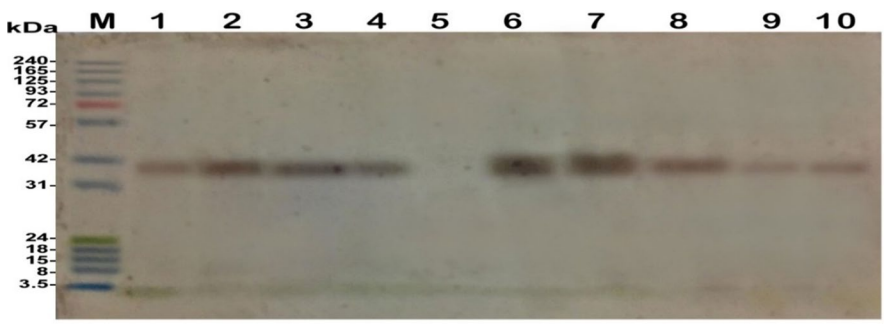

Fig. 5 Western blot analysis with PVY antibody (1:200) against total proteins extracted from bacterial treated potato plants and inoculated with PVY. M: prestained protein molecular weight marker (New England BioLabs, Ipswich, MA).Lane 1; VB treatment after 4 days of virus inoculation (dpi); Lane 2: VB treatment at 7 dpi; Lane 3: The VB treatment at 10 dpi; Lane 4; BV treatment after 4 days of virus inoculation; Lane 5: Health potato plant; Lane 6: The VB treatment at 16 dpi; Lane 7: PVY positive control at 16 dpi (untreated with the *B. subtilis*); Lane 8; BV treatment at 7 dpi; Lane 9: Foliar spray treatment with *B. subtilis* followed by virus inoculation (BV) at days 10 dpi; lane 10: Foliar treatment (BV) at 16 dpi

suspension at 10 days after virus inoculation showed *PR-1* transcriptions with relative expression levels of 0.5141 and 0.3015 fold changes, respectively (Fig. 6). Increased transcript levels of the PR1 gene were found in the *B. subtilis* soil applied treatments infected with PVY (BV-soil and VB-soil) and showed PR1 transcriptions with relative expression levels representing 0.9659 and 0.6878 fold changes, respectively (Fig. 6).

Discussion

Plant viruses are the most hazardous plant pathogens. Viruses infect all known commercial crops, and result in severe crop losses worldwide (Jones, 2021). *B. subtilis* has long been shown to be a PGPR, and broadly elicits significant reductions in the incidence and severity of various diseases on a diverse range of hosts (Raza et al., 2016). Recently, endophytes (*B. subtilis*) have been applied to crops, and have been shown to elicit a defense reaction against Potato viruses X and Y. (Veselova et al., 2022).

In the present study, the antiviral bioactivity of a *B. subtilis* strain against PVY on potato plants, as the most economic important crop, was evaluated. Potato plants were treated with *B. subtilis* bacteria either by foliar sprays (T1) at 48 h or soil application (T2) at 10 days prior (i.e. protective biocontrol) or post (i.e. curative biocontrol) PVY infection. The systemic PVY movement and accumulation at 4, 7, 10, 16 and 20 dpi were assessed using DAS-ELISA and RT-PCR.

Our results showed that the foliar BV treatment decreased PVY accumulation titer in the leaves of potato at 10 and 16 dpi with suppression of disease severity under greenhouse conditions. The lowest amount of PVY was achieved in BV plants 16 days after virus inoculation (66.57% of virus inhibition). These results are in line with Sorokan et al. (2020) who reported that endophytic bacteria have the power to stop viral spread during the initial phases of infection (7–14 dpi). The RT-PCR was conducted on potato leaves collected at 10 and 16 dpi and revealed PVY amplicon (825 bp), confirming the presence of the virus in newly emerged leaves. The decrease in virus accumulation in potato plants can be attributed to the fact that the endophytic bacteria's influence on the overall RNase activity in potato plants was significant for the initial stages of viral dissemination. Extracellular high-molecular weight RNases are produced by a large number of *Bacillus* species (Maksimov et al., 2019). The *Bacillus amyloliquefaciens* (phylosphere bacteria) treated pepper in conjunction with cucumber mosaic virus (CMV) and significantly reduced the relative CMV coat protein RNA content (Lee & Ryu, 2016). Our data is consistent with Veselova et al. (2022) results, in which foliar treatment with endophytic *B. subtilis* (26D and Ttl2) significantly decreased both PVX and PVY accumulation in tomato plants at 14 dpi (Raza et al., 2016). According to Fedorova et al.

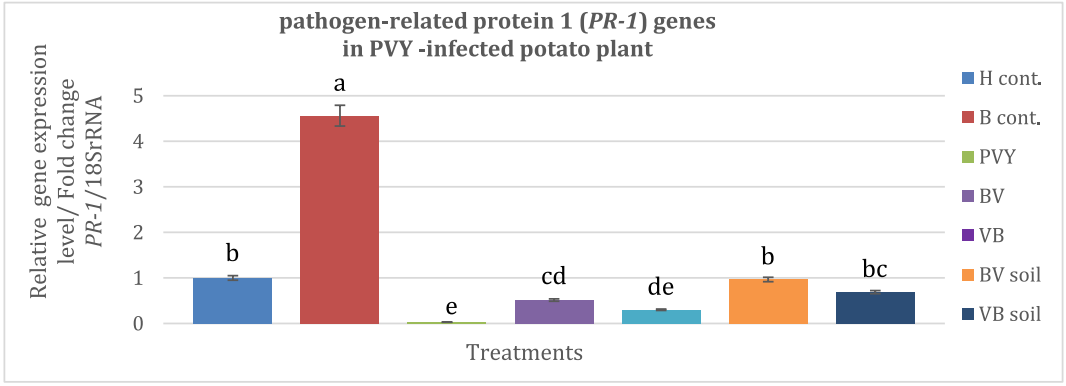

Fig. 6 Relative transcriptional gene expression levels of *PR-1* gene in potato plants treated with foliar and soil application of *B. subtilis* at 10 days after and before PVY inoculation. Where, BV: foliar spray with *B subtilis* before PVY inoculation, VB: foliar spray with the bacteria after virus inoculation, BV-soil: application of *B. subtilis* before PVY inoculation, VB-soil: application of *B. subtilis* after PVY inoculation, H cont. Mock treated plant (healthy control), PVY: plant inoculated with PVY only (PVY cont.). Different lowercase letters (a, b, c, d, and e) indicate significant differences among treatments at the same time point. Error bars signify standard errors of the mean ($n = 3$) at each time point

(2011), tobacco plants treated with *Bacillus pumilus* RNase prevented PVS and PVM infection from developing, almost completely preventing PVX infection, and reduced the amount of red clover mottle virus (RCMV) particle numbers in pea plants.

Soil treatment with *B. subtilis* suspension was performed at 10 days before (BV-soil) and after (VB-soil) inoculation with PVY. The results showed that BV-soil efficiently induced systemic plant resistance (32.79% virus inhibition) 10 dpi, and continues with adequate virus inhibition (72.26%) at 35 days after virus infection. The results showed that the soil application of bacterial cell suspension 10 days before viral inoculation (BV soil) delayed the appearance of disease symptoms and increased plant growth compared to non-treated infected plants. Similar studies reported that treatments with *Bacillus amyloliquefaciens* strain MBI600 as foliar or soil amendments reduced tomato wilt virus infection by up to 80% under two distinct sets of environmental factors. In addition, the use of MBI600 immersion delayed the accumulation of potato virus Y (Beris et al., 2018).

Interestingly, some of the VB-soil treated potato plants displayed necrotic lesions on potato leaves despite having a negative ELISA results at 35 dpi, a result which could be due to a plant hypersensitivity response (HR). The HR is traditionally seen as the plant mechanism that prevents pathogen growth in incompatible plant–pathogen interactions and thus leads to disease resistance (Torrance & Talianksy, 2020). In plants, there is genetic separation between cell death and pathogen growth inhibition that results in disease resistance. The HR cell death may simply occur as a result of increased signaling at the interface of interactions between plants and pathogens, and the resulting increase in toxic intermediates that cause both host and pathogen cell death (Coll et al., 2011). The development of tissue necrosis was once thought to be a frequent and necessary aspect for SAR activation (Vleesschauwer & Höfte, 2009), yet in many instances SAR activation can occur without tissue necrosis. HR occurs as a result of changes to the plant cell wall brought on by an increase in calcium ions, superoxide and nitric oxide radical concentrations, and levels of SA, JA, and H_2O_2. However, Potato Nytbr and Nctbr genes regulate HR reactions against PVY^C and PVY^O strains (Manjunatha et al., 2022).

The western blot analysis for the accumulation and expression of viral coat protein revealed that the T1 treatment (VB) at 10 and 16 dpi showed an intense amount of the viral protein, whereas T1 treatment (BV) reduced PVY coat protein gene expression at 10 and 16 dpi. The western blot analysis for *B. subtilis* T2 treatment revealed an inhibitory effect of *B. subtilis* on PVY protein expression. The BV-soil and VB-soil treatments of potato plant rhizospheres reduced PVY coat protein gene expression and showed a faint protein band at 10, 20 and 27 dpi. On the other hand, it showed an intense protein band with T2 treated plants (BV-soil and VB-soil) at 35 dpi only. These results revealed that treatment first with soil applied *B. subtilis* (BV-soil) was better than the foliar spray treatment with *B. subtilis*, and can a defer the long distance movement and/or the viral protein expression until 35 dpi. This result could be attributed to *B. subtilis* (isolated from soil) rhizobacterial secondary metabolites moving from the root system to the shoots with the aid of water-transporting channel proteins, which play an active role in facilitating the movement of water along with the bacteria and its secondary metabolites across living cells and the phloem cells where the virus is mostly found, consequently affecting the virus directly, either on its replication, expression or its long distance movement. A previous report stated that the endophytes *Bacillus spp*. isolated from plant roots are resistant to infection from the environment. They have a more stable biocontrol effect on infections than those from the surface of a plant (Lopes et al., 2018). According to Van Loon (2000), the ISR, which is induced by the PGPR, provides protection that is much less effective than SAR and that is somewhat genotype-dependent in the ISR generation (Bloemberg & Lugtenberg, 2001). Salicylic acid builds up both locally and, at lower concentrations systemically, in tandem with the emergence of SAR (Van Loon et al., 1998). However, when ISR and SAR, which is induced as a result of virus and localized infection combined, they offer greater protection than each one of them would alone, suggesting that they can function additively to increase pathogen resistance (Van Wees et al., 2000).

PVY infection can cause severe inhibition of plant growth and yield production. In this study, the soil application of *B. subtilis* before PVY infection increased the plant growth parameters, reduced disease symptoms, decreased viral titer and viral protein levels compared to infected potato plants without any bacterial treatment.

In the current study, we determined the growth-promoting effects of *B. subtilis* on potato plants. The bacterial treatment prompted the growth of potato plants inoculated with PVY compared to those plants infected with PVY alone. The fresh and dry weights of the total plant (shoot and root systems) increased in potato plants treated with *B. subtilis* before PVY infection (BV-soil) in comparison to the reduction in the fresh and dry weights of untreated PVY infected potato plants. Our results showed that soil treatment with *B. subtilis* bacteria had a great effect on the increase of the fresh and dry weight. The plant fresh weight of BV-soil (preventive biocontrol) and VB-soil (curative biocontrol) treated plants were higher than the plants treated with the bacteria only (B cont.). This may be due to virus inoculation and bacterial treatment synergy, which stimulates SAR and ISR together to highly accelerate the SA/JA signaling pathway and thus increasing the production of phytohormones two times more than the bacterial treated plants alone (B cont.) which only induced ISR. The growth rate in bacterized plants without PVY infection (B cont.) was restored plant length by an increase of 63.3% at 41dpi compared to control plants. On the other hand, those plants untreated with bacteria but and infected with PVY virus (PVY cont.) could not completely restore their growth rate. The increase in plant growth could be correlated to the ability of the *B. subtilis* strain to induce the plants to produce various plant hormones. PGPR promote plant growth through metabolic adjustments, phytohormone levels such as auxins, exopolysaccharides, root colonization, and nutrient availability by synthesizing siderophores (Spence & Bais, 2015; Nazli et al., 2020), as well as produce rhizobitoxine, which reduce ethylene production and promotes plant growth and expansion under stress, and indirectly improves plant growth by inducing resistance to stresses and controlling pathogens (Gupta et al., 2014; Khan et al., 2019). Similar results were obtained by Veselova et al. (2022) & Zhang et al. (2022). For instance, Veselova et al. (2022) reported that in lettuce plants treated with the rhizosphere strain *B. subtilis* IB-22 (zeatin-riboside producer), the growth and weight of roots increased by 30% in comparison to control plants. Both PGPM and viruses have the ability to control and disrupt hormonal pathways in plants, which in the case of PGPM causes ISR to initiate and viruses to cause symptoms to appear, viral reproduction, and systemic infection (Khan et al., 2019). Additionally, ISR in plants drives the expression of pathogenesis related genes with the aid of phytohormone signaling pathways and defense regulatory proteins to defend plants from pathogen attacks in the future. (Pieterse et al., 2014). SAR and ISR, however triggered by the virus and PGPM result in numerous cellular reactions in plants, including the synthesis of PR-proteins (Vleesschauwer & Höfte, 2009; Pieterse et al., 2014; Robert-Seilaniantz et al., 2011).

In this work, the *PR-1* gene was chosen because of its link with the primary plant defense responses to viruses (SAR). It participates in salicylic acid (SA) biosynthesis which is caused by a localized infection (Dempsey et al., 2011). Furthermore, the PR proteins contribute to SAR by promoting programmed cell death, which slows the proliferation of pathogens and, eventually, lowers the incidence of infection (Zaynab et al., 2021).

The relative expression of the *PR-1* gene in potato plants treated with the *B. subtilis* and inoculated with PVY at 10 days after virus and/or bacterial treatment was investigated. The relative expression of five defense genes (*PR-1, PR-2, PR-3, PR-5* and *PR-7*) is common varied between induction and suppression, especially in the first 10 dpi (Abdelkhalek et al., 2020).

In this study, PVY infection resulted in dramatically reduced expression of *PR-1* (Salicylic acid (SA) biosynthesis) at the mRNA relative expression level in potato plants compared with the healthy un-inoculated potato plants (Su et al., 2018). Relative expression values lower than 1 means a decrease in expression levels (down-regulation). The down regulation of the *PR-1* gene was associated with the viral coat protein (CP) accumulation. The greater accumulation of the viral coat protein at 10 dpi coincided with greater suppression of the *PR-1* (salicylic marker) gene expression. Infection of the plants with PVY led to a dramatic decrease in the transcript level of the *PR-1* gene with 0.0284 fold change compared with the healthy un-inoculated control. The down-regulation of the *PR-1* gene in infected PVY potato plants (PVY control) reflects the suppressor activity of PVY. Similar findings have found that the viral infection was associated with down-regulation or a decrease in *PR-1* activity (Prins et al., 2008; Veselova et al., 2022; Zhou & Niu, 2009). Conversely, our result is inconsistent with Abdelkhalek et al. (2020), in which it was mentioned that the accumulation of the virus at 10 dpi suppressed the expression of examined pathogenesis-related proteins (*PR-2, PR-3, PR-5* and *PR-7*), except for the *PR-1* (salicylic marker) gene.

From our results, increasing the relative transcriptional level of the *PR-1* gene was present in the potato plants treated with all bacterial treatments. It should be highlighted that the soil application of *B. subtilis* (BV-soil) increased the expression of the *PR-1* gene in infected plants with a 0.9659 fold change at 10 dpi, restoring its level to a similar level to a normal level in the healthy plant. We noticed that as the virus reduction increased as the *PR-1* suppression decreased between all the bacterial treatments (T1 and T2) at 10 days after virus inoculation and/or bacterial application. These results revealed that the *B. subtilis* treatments either downregulated the suppressor which is associated with the expression of *PR-1* gene or suppressed the viral replication and expression.

Additionally, the *PR-1* gene was significantly upregulated with *B. subtilis* inoculation in (B. cont.) potato plants. The *PR-1* gene transcript level showed a 4.5631-fold change in B cont. plants compared to water-treated control plants (H cont.) Remarkably, after virus infection, *PR-1* was oppositely downregulated. It was observed that BV-soil treatment persistently re-induces the expression of the *PR-1* gene. This may be attributed to the salicylic acid induction, the nutritional characteristics, synergy of the SAR and ISR pathways of plant hosts for virus and nonpathogenic rhizobacteria (Zhang et al., 2022). The capability of *B. subtilis* to induce salicylic acid production appears to have a prominent role in viral inactivation (up to 70% inhibition) leading to restriction of viral spread.

Furthermore, the plant root endophytes can enhance plant resistance to pathogens by activating the plant's ISR and SAR defense mechanisms. ISR and SAR have cross effects on the signaling pathway. For example, ISR can enhance plant resistance to pathogens by activating the SAR signaling pathway. In addition, some key genes and proteins in the SAR signaling pathway can also participate in the regulation of ISR (Zhang et al., 2022; Ngou et al., 2022). Similar investigations have demonstrated that PVX and PVY-infected potato plants under the effect of *B. vallismortis* strain EXTN-1 and PVY-infected tomato plants treated with *B. amyloliquefaciens* strain MBI600 both exhibit increased *PR-1* gene expression that increases plant resistance to viruses (Park et al., 2006; Beris et al., 2018). The decrease in PVY accumulation in potato plants treated with SA was associated with the induction of the *PR-1* and *PR-2* genes (Baebler et al., 2014; Yang et al., 2019).

To sum up, the soil application of *B. subtilis* affected the SA signal transduction pathways of potato plant causing ISR and SAR. Our work has demonstrated that the soil application of a *B. subtilis* strain prior to the virus infection (as a preventive biocontrol strategy) promotes potato plant growth, lowers the level of PVY accumulation and induces systemic resistance. Since the gene expression of the virus appeared after 35 days in the case of treatment with bacteria in the soil we recommend treating the soil with *B. subtilis* 10 days after the emergence of seedlings and repeating the treatment after one month of growth. Finally, *B. subtilis* could therefore be used as a biocontrol agent against PVY infection. More research is necessary to analyze possible field application and its commercial uses.

Conclusions

B. subtilis isolate is effective in inducing systemic plant resistance and controlling PVY. PVY symptoms and severity were reduced significantly by *B. subtilis* treatment. Compared to potato plants infected with PVY alone, the *B. subtilis* foliar (T1) and soil (T2) treated plants decreased the PVY accumulation level by 67% and 70%, respectively. Additionally, the application of *B. subtilis* increased the fresh and dry weights of potato plants. The relative expression level of the *PR-1* gene was reduced (downregulation) in potato plants treated with *B. subtilis* and PVY when compared with healthy control plants. Despite this, a significant increase in the relative expression level of *PR-1* gene was observed in potato plants treated with *B. subtilis* (soil application) before virus inoculation compared to plants infected with PVY alone. However, using *B. subtilis* in soil to provide plants with a degree of systemic resistance to the pathogen before infection with the virus can lower the disease's mortality rate.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest The authors declare no conflict of interest.

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