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Improving the biocontrol method of potato wilt caused by *Ralstonia solanacearum* (Smith) using *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34

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Abstract

This study aimed to evaluate the efficiency of *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34 in the control of potato wilt, caused by *Ralstonia solanacearum* (Smith), under greenhouse and field conditions. In vitro, the endophyte *E. cloacae* PS14 caused the highest reduction of the pathogen growth among 7 screened bacteria. It produced an inhibition zone as 16.9 mm compared to a specific antibiotic (20.0 mm). *E. cloacae* PS14 was selected as an effective antagonistic bacterium to be compared to *T. asperellum* strain T34 for reduction of the disease as well as increasing the crop yield of potato plants. Both *E. cloacae* and *T. asperellum* reduced the disease severity up to 10.7–26.5%, respectively, under greenhouse and up to 26.6–36.6%, respectively, under field conditions. The results approved that both *E. cloacae* and *T. asperellum* increased the yield of the crop by 20.44–40.96%, respectively. Their mode of action was indicated by suppression of the pathogen as well as induction of plant systemic resistance. The induction of systemic resistance was confirmed by increasing the total phenol and salicylic acid contents as well as increasing the activities of peroxidase, lipoxygenase, and polyphenol oxidase in potato plants than the healthy or only infected plants. Production of siderophore, indole-3-acetic acid (0.577–0.884 μ M), hydrogen cyanide (2.34–3.61 μ g/ml), and salicylic acid (0.436–1.488 μ g/ml) was confirmed by *E. cloacae* PS14 and *T. asperellum* T34, respectively, in vitro. The study recommends the new strain *E. cloacae* PS14, as new endophytic effective bacteria, in the control of *R. solanacearum* causing the potato wilt disease.

Keyword: Potato wilt, Endophyte, Biological control, *Enterobacter cloacae*, *Trichoderma asperellum*, Systemic resistance

Background

Bacterial wilt disease caused by *Ralstonia solanacearum* (Smith) is one of the serious plant diseases worldwide (Peeters et al. 2013). In Egypt, as well as in many countries, it has become a severe problem for the production of potato plant (Abo-Elyousr and Bagy 2018).

Traditional control methods such as short rotation, resistant cultivars, and soil fumigation have been suggested. However, such methods are not always enough effective since *R. solanacearum* can remain with the infested plant debris in the soil for a long time. Recently, the research focuses on some alternative methods to control the disease that are characterized by being environmentally safe, long lasting and effectiveness (Abo-Elyousr and Bagy 2018).

Biological control, using beneficial microorganisms, could therefore, be a choice for managing the disease

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(Abd-El-Khair 2020). Many beneficial microbes such as *P. fluorescens* spp., *Acinetobacter* spp., *Bacillus* spp., Bacteriophages, *Enterobacter* spp., *Pseudomonas putida*, *Paenibacillus macerans*, *Streptomyces*, and *Trichoderma* spp. have been documented as effective biocontrol agents against *R. solanacearum* (Ling et al. 2010). *Trichoderma* spp. have been focused on their antagonistic and mycoparasitic ability to reduce the disease incidence caused by phytopathogens (El-Sharkawya et al. 2018). They play an important role in controlling of soil-borne pathogens, stimulating the plant growth, and enhancing the crop productivity (Sallam et al. 2019). Some of *Trichoderma* spp. are manufactured and marketed worldwide as commercial biological products, used as soil fertilizers, plant biostimulant, and biofungicide (Woo et al. 2014). Many reports indicated that *Trichoderma* could induce resistance in plants not only by producing proteins, but also by producing secondary metabolites in the soil amended with decomposed organic fertilizer, which has decreased the *R. solanacearum* population (Konappa et al. 2018).

The endophytic bacteria are those that could internally colonize plants without induction of any apparent negative effects on the plant morphology and physiology (Gaiero et al. 2013). They are increasingly characterized by their possible use in the phytoremediation, promotion of plant growth, reduction of abiotic stress, and antagonism against many pathogens (Mercado-Blanco and Lugtenberg 2014). Interestingly, wilt disease of some crops such as tomato, caused by *R. solanacearum*, has been controlled by endophytic bacteria: *Pantoea ananatis*, *Pseudomonas oleovorans*, and *Enterobacter cloacae* (Upreti and Thomas 2015). It was reported that common genera such as *Sphingobacterium*, *Comamonas*, *Arthrobacter*, *Curtobacterium*, *Paenibacillus*, *Xanthomonas*, *Serratia*, *Pantoea*, *Variovorax*, *Stenotrophomonas*, and *Enterobacter* were frequently isolated from the rhizospheric soil of potato, and many of them were used as potential biocontrol agents (Chamedjeu et al. 2019). For example, Götz et al. (2006) used *Enterobacter cowanii* as biocontrol agents against bacterial wilt of tomato. *E. cloacae* was used successfully for controlling many plant diseases such as *Fusarium wilt* of spinach (Tsuda et al. 2001), bacterial blight of rice (Yang et al. 2000), dry rot of potato (Al-Mughrabi 2010), and damping-off of tomato (Yuliar et al. 2019).

To the best of the authors' knowledge, this is the first time to use *E. cloacae* and *T. asperellum*, as a field trial, against *R. solanacearum*, the causal pathogen of potato wilt. The aim of this study was to investigate the biocontrol efficiency of *E. cloacae* PS14 and *Trichoderma asperellum* T34 to manage the wilt disease of potato under field conditions as well as exploring the biochemical responses in relation to defense enzymes that induce the resistance of plants against pathogen.

Materials and methods

Bacterial pathogen

R. solanacearum isolate PHYRS3, the causal agent of potato wilt disease, was isolated in a previous work by Bereika (2008). In brief, samples of diseased potato plants showing wilt symptoms were washed 2–3 times by tap water, followed by sterile water and sterilized with 2% sodium hypochlorite solution; the disinfected plant samples were homogenized with 5 ml of sterile 0.05-M potassium phosphate buffer in a sterilized mortar and pestle. A loop of the resulted suspension was streaked onto 2,3,5-triphenyl tetrazolium chloride (TZC) agar medium in 9.0-cm Petri plates and then incubated at 27 °C for 48 h and examined daily for bacterial growth. The single colony technique was used to obtain pure cultures of the isolated bacteria by growing on the same media used and kept at 4 °C for further studies.

Microorganisms

Trichoderma asperellum strain T34

T. asperellum strain T34 was obtained from Biocontrol Technologies S.L. Company Spain and reactivated and maintained on potato dextrose agar (PDA).

Endophytic bacteria

The endophytic bacteria were isolated from healthy potato plants, collected from Assiut Governorate, Egypt, during winter 2016. Segments of potato stems (2 cm) were surface-sterilized, using 2% sodium hypochlorite for 3 min, then with 70% ethanol for 30 s. The segments were rinsed by sterile distilled water 3 times and homogenized in 10 ml acetate buffer (pH 5.2). The homogenized plant tissue was taken by a loop and streaked on the surface of Petri dishes (9 cm) containing nutrient agar medium (NA). The plates were incubated at 28 °C for 48 h. Seven bacterial strains were recovered and their pure cultures were kept on NA slants and preserved at 4 °C for further use.

Evaluation of antagonistic potentially of the endophytic bacteria against *R. solanacearum*

The antagonistic and pathogenic bacteria were grown individually in 250-ml Erlenmeyer conical flasks containing 100 ml of nutrient sucrose broth and incubated at 28 °C for 48 h, and 150 rpm. After incubation, the bacterial growth was centrifuged at 10000×g in sterile microfuge tubes. The supernatants were excluded, and the bacterial cells were collected. Using spectrophotometer (at 600-nm), the bacterial cell density was adjusted to 10⁸ cell/ml. The antagonistic activities of seven isolates of the endophytic bacteria were evaluated using the dual culture against *R. solanacearum* PHYRS3 based on the method described by Abo-Elyousr et al. (2012) with modification. Briefly, a suspension of *R. solanacearum*

PHYRS3 ($100 \mu\text{l}$ at 10^8 cell/ml) was spread over agar surface. After drying, $100 \mu\text{l}$ of each antagonistic isolate (10^8 cell/ml) were individually pipetted into 5-mm punches in the same agar inoculated with the pathogen. The antibiotic streptomycin (1.0 mg/ml) was used as positive control. After 2 days from incubation at 28°C , the antibacterial effect of the strains was monitored by measuring the diameter of the inhibition zone (mm). The experiment was repeated twice with four replicates for each treatment.

Identification of the potent antagonistic bacterial strain

Based on the above test, the potent antagonistic bacterial strain was selected for identification via 16S rRNA sequencing according the following protocol.

DNA isolation

The genomic DNA was extracted from the endophytic bacterial strain using the genomic DNA Prep kit (SolGent, Daejeon, Korea) following to the manufacturer's instructions. The extracted DNA was used as a template for PCR to amplify the 16S rRNA gene. Universal bacterial primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') were used to amplify the nearly complete 16S rRNA gene (Abd-Alla et al. 2012).

PCR amplification

The PCR amplification was performed in a 25- μl reaction volume containing 0.4 μM of each primer, 0.75 U of EF-Taq DNA polymerase (SolGent, Daejeon, Korea), 0.2 mM of each d NTP, 10–50 ng of the template DNA, and 1 \times EF-Taq reaction buffer. The thermo cycling conditions included an initial denaturation step at 95°C for 15 min, followed by 30 cycles at 95°C for 20 s, 50°C for 40 s, and 72°C for 1.5 min with a final extension step at 72°C for 5 min. The PCR product was separated by 1.5% agarose gel electrophoresis containing ethidium bromide with buffer of 0.5 \times Tris-acetate-EDTA (TAE) and visualized using a UV illuminator (Abd-Alla et al. 2012).

DNA sequencing

The PCR product was purified according to the manufacturer's instructions using a SolGent PCR purification kit (SolGent, Daejeon, Korea) (Sanger et al. 1977). The partial 16S rRNA gene sequence was compared to full sequences available in the GenBank database, using a BLAST search (NCBI) for identification of the bacterial strain. Sequences obtained with those retrieved from GenBank database and the sequenced data were deposited in the GenBank under a specific accession number.

Effect of *E. cloacae* PS14 and *T. asperellum* T34 on diseases severity under greenhouse conditions

The greenhouse trials were conducted at the Experimental Greenhouse of Department Plant Pathology, Assiut, Egypt, during the 2017 growing season. Healthy potato tubers (*Solanum tuberosum* L.) cv. Berema were surface-sterilized by soaking in 2% sodium hypochlorite for 3 min, washed thoroughly with sterilized distilled water and planted directly in sterilized 25-cm diameter pots. Pots and soil were sterilized by 5% formalin and left for 15 days before planting. Pots filled with 5-kg sterilized sandy-clay soil (3:1 w/w) were kept in the greenhouse under natural temperatures and photoperiods during the growing season. Plants were fertilized every 15 days with (20 g/pots) of 46% urea and irrigated with water when necessary. Forty-five days after planting, potato cultivar was inoculated with *R. solanacearum* PHYRS3 (the pathogen) suspended in water at 10^8 cell/ml, by using an alcohol knife which was inserted 4–5 cm into the soil to cut the roots along two sides and 20 ml of *R. solanacearum* PHYRS3 suspension was added to each plant around the basis of each plant (Bereika 2008). Infected control plants were inoculated with the pathogen only and treated with 20 ml sterile distilled water, while healthy control did not receive any treatment (without infection and without treatment). After inoculation, the potato cultivar was kept in a moist chamber at 25°C for 2 days before being transferred to the greenhouse at 25 – 30°C . Two days after inoculation, 20 ml from each agent suspension (*E. cloacae* PS14 or *T. asperellum* T34) was added surrounding the bases of plants. Four replicates were used for each treatment. After 6 weeks, the development of bacterial wilt symptoms was observed. The disease severity was monitored and the percentage of disease severity (DS%) was estimated using the formula suggested by Kempe and Sequeira (1983).

Disease assessment

Disease severity was recorded, using the scale of Kempe and Sequeira (1983) as follows:

0 = no symptoms, 1 = 1–25% of leaves wilted, 2 = 26–50% of leaves wilted, 3 = 51–75% of leaves wilted, 4 = more than 75% and less than 100% of leaves wilted, and 5 = all leaves wilted and died. Disease severity percentage was calculated by the following equation:

$$\text{DS\%} = [\sum d/d (\text{max} \times n)] \times 100$$

where “*d*” is the disease rating on each plant, “*d* max” is the maximum disease rating possible, and “*n*” is the total number of plants examined in each replicate.

Application of *E. cloacae* PS14 and *T. asperellum* T34 under field conditions

The experiments were carried out at the farm of Faculty of Agriculture, Assiut University, Egypt. Four replicates

were used for each treatment and the treatments were distributed in a completely randomized block design. The experimental plot area was 25-m² containing 5 rows, each row was 4.5-m in length and the distance between each row was 0.5 m. Potato seed tubers (cv. Berema) were sown on the middle of the ridge at 0.4-m apart. After one month from planting, each treatment was added singly to the plant's soil by drenching around the plants at 20-ml from each microorganism before 48 h of inoculation with *R. solanacearum* PHYRS3 (10⁸ cell/ml) as described in greenhouse experiment. The control plants were treated by 20-ml distilled water after cutting (Winstead and Kelman 1952) and the disease severity was recorded after 6 weeks after inoculation according to Kurabachew and Wydra (2013). At harvest time (110 days after planting), potato plants of 6 plants from each replicate were pulled for measuring a total tuber yield (kg) per replicate and the yield was expressed as ton/hectare.

Biochemical analyses

The effects of *E. cloacae* PS14 and *T. asperellum* T34 on biochemical changes of the inoculated potato plants by *R. solanacearum* PHYRS3 were investigated. Leaves samples were taken at zero time and at 2, 4, 6, and 8 days after inoculation for determination of total phenol and salicylic acid contents and enzyme activities.

Determination of total phenol and salicylic acid contents

One gram of potato plant's leaves was crushed in liquid nitrogen and homogenized in 10-ml of 80% methanol. The homogenate was centrifuged at 10,000 g for 30 min at 4 °C. The pellets were wasted after adding ascorbic acid (0.1 g/5 ml). The homogeneous product was evaporated at 65 °C in a rotary evaporator and the process was repeated 3 times each for 5 min. The residues were dissolved in 5 ml of 80% methanol. For each treatment, 4 replicates were used (Rapp and Ziegler 1973). The method of Abo-Elyousr et al. (2008) was used to determine phenol content as milligrams gallic acid/gram plant material. Salicylic acid content was estimated, using a modified method by Dat et al. (1998) as micrograms salicylic acid/gram plant material.

Enzymes activity

For determination of activities of peroxidase (PO), polyphenol oxidase (PPO), and lipoxxygenase (LO), 1 g of fresh potato plants' leaves was treated with liquid nitrogen and homogenized with 10-ml of 0.1 M Na-acetate buffer (pH 5.2). The mixture was centrifuged at 10,000 g for 30 min at 4 °C and the enzyme activity was determined in the supernatants. Four replicates were used for each treatment. Total protein was estimated based on the method described by Bradford (1976) using Bradford

reagent spectrophotometrically at 595-nm using Bovine serum albumin as standard.

Peroxidase (PO) activity The enzyme activity was determined spectrophotometrically by the method of Putter (1974), using guaiacol as a substrate. The reaction mixture was composed of 0.2 ml supernatant, 1 ml of 0.1 M Na-acetate-buffer (pH 5.2), 0.2 ml of 1% H₂O₂ and 0.2 ml of 1% guaiacol. The mixture was incubated for 5 min at 25 °C and then measured at 436 nm. The blank was used with extraction buffer. The PO activity was calculated according to the change in absorbance and expressed as enzyme per 1 mg protein.

Polyphenol oxidase (PPO) activity The enzymatic activity was determined, using the method described by Batra and Kuhn (1975). The reaction mixture was 0.5 ml of the supernatant, 2 ml of 50 mM Sorensen phosphate buffer (g/l: KH₂PO₄, 6.8; Na₂HPO₄·2H₂O, 8.99; EDTA, 0.372 and distilled water up to 1000 ml, pH was adjusted to 6.5) and 0.5 ml of the substrate Bren catechol (Sigma Aldrich). The mixture was incubated for 2 h in water bath at 37 °C and measured at 410 nm. Activity of PPO = OD at 410 nm/mg protein.

Lipoxxygenase (LOX) activity

The enzymatic activity was evaluated according to the protocol of Axelred et al. (1981), the increasing in lipoxxygenase activity was measured according to the increase in absorbance at 234-nm resulting from the conjugated double-bond system in the hydroperoxide produced from the substrate, linoleic acid (10-mM sodium linoleate; pH 9). The mixture contained 10 µl of mixture, 20 µl of the substrate, and 1 ml of 50 mM sodium phosphate buffer (pH 6). Absorbance readings were made spectrophotometrically for 3 min at room temperature. A mixture containing buffer and substrate was used as a blank. The activity was measured from the extinction coefficient of 25 mM⁻¹ cm⁻¹.

Possible mode of action exerted by *E. cloacae* PS14 and *T. asperellum* T34

Salicylic acid production

Salicylic acid (SA) produced by *E. cloacae* PS14 and *T. asperellum* T34 were determined, following the method described by Meyer and Abdallah (1978). The microorganisms were grown in the standard succinate medium at 28 °C for 48-h components from K₂HPO₄ (11.5 g/l), (NH₄)₂ SO₄ (28.7 g/l), and distilled water up to 1000 ml. The pH was adjusted to 7.0 with 20% NaOH prior to autoclaving. Cells were collected by centrifugation at 8000×g for 5 min and re-suspended in 1 ml of 0.1 M phosphate buffer. A 4-ml of cell-free culture supernatant was acidified with 1-N HCl to pH 2.0 and SA was

extracted in CHCl_3 . Four milliliters of water and 5 μl of 2-M FeCl_2 were added to the pooled CHCl_3 phases. The absorbance of the purple iron-SA complex, developed in the aqueous phase, was read at 527 nm. A standard curve was prepared with SA dissolved in succinate medium and quantity of SA produced was calculated.

Indole acetic acid production

E. cloacae PS14 and *T. asperellum* T34 were inoculated by TSB with tryptophan as a precursor (100 $\mu\text{g}/\text{ml}$) on a shaker for 30 min. The culture's supernatants were collected after centrifugation for 10 min at 2000 rpm and 1 ml of cell-free culture filtrate was mixed with 2 ml of Salkowski reagent (1 ml of 0.5 M FeCl in 50 ml of 35% perchloric acid) and incubated at 28 °C for 30 min. Quantification was done calorimetrically at 530 nm comparing to indole acetic acid production (IAA) standard curve (Nandhini et al. 2012).

Quantitative detection of hydrogen cyanide and siderophore

The antagonistic microorganisms were grown in trypticase soy broth (TSB, Hi media, India). Filter paper was cut into uniform strips of 10-cm-long and 0.5-cm-wide saturated with alkaline picrate solution and placed inside the test tubes in hanging positions. After incubation for 48 h at 28 °C, the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the quantity of hydrocyanic acid evolved. The color was eluted by placing the filter paper in a clean glass test tube containing 10 ml of distilled water and absorbance was measured at 625 nm (Sadasivam and Manickam 1992).

The quantitative estimation of siderophore produced by bioagents was done by CAS-shuttle assay (Schwyn and Neilands 1987). The strains were grown in a succinate medium and incubated at 28 °C for 24 to 30 h and shaking at 120 rpm. After incubation, the fermented broth was taken and centrifuged at 10,000 $\times g$ for 10 min at 4 °C and the cell-free supernatant was mixed by 0.5 ml CAS solution. The color obtained was determined at 630 nm after 20 min from incubation with reference containing 0.5 ml CAS solution with 0.5 ml uninoculated succinate medium. The percentage of siderophore units was determined as the proportion of CAS color shifted using the formula % Siderophore units = $(A_r - A_s/A_r) \times 100$, where A_r = Absorbance of reference at 630 nm (CAS reagent) and A_s = Absorbance of sample at 63 nm

Statistical analysis

Data were initially examined for their normal distribution of errors using Shapiro-Wilk's W test and for homogeneity of variances using Levene's test. All statistical analyses were performed with SPSS 22.0 software.

The data were analyzed for significance of variation using one-way analysis of variance (ANOVA) for antagonistic capability and for the rest of experiments two-way analysis were used. The least significant difference (LSD) test was used at $P < 0.05$ to identify the significant differences among the means of the treatments according to Gomez and Gomez (1984).

Results and discussion

Antagonistic activity and identification of the endophyte *E. cloacae* PS14

The antagonistic activity of 7 bacterial isolates against *R. solanacearum* PHYRS3 was evaluated, using dual culture method (Table 1). The results indicated that the highest inhibitory effect against the PHYRS3 was attributed to the bacterial strain no. 7, which caused production of inhibition zone as 16.9 mm compared with 20.0 mm because of specific antibiotic streptomycin. The potent antagonistic bacterium was identified by 16S rRNA gene and their sequences were compared to data available in the GenBank using BLAST search. The results indicated that the bacterial strain belongs to the genus *Enterobacter* and it could be identified as *E. cloacae* (Table 2) with a similar, percentage as 86.22%. It was given a specific strain number as *E. cloacae* PS14 and it was allocated in the GenBank under accession number Mn385618. The findings could be supported by those of Upreti and Thomas (2015) who isolated *E. cloacae*, *Pantoea ananatis*, and *Pseudomonas oleovorans* as endophytic bacteria from different tomato cultivars. However, a relatively low similarity percentage of the strain identity with those allocated in the GenBank could support our hypothesis that it is a new strain of *E. cloacae* that has specific characteristics such as its antagonistic potency and biological control efficiency against the target pathogen. The use of plant-associated microorganisms in biological control of plant diseases was mentioned as an efficient and eco-friendly method (Xue et al. 2009). The suppressive effect

Table 1 Effect of seven endophytic bacterial isolates on growth of *Ralstonia solanacearum* in vitro

Treatments	Inhibition zone (mm)
Isolate no. 1	3.0 \pm 1.0 d
Isolate no. 2	1.0 \pm 1.0 f
Isolate no. 3	5.0 \pm 0.1 c
Isolate no. 4	2.0 \pm 1.0 e
Isolate no. 5	3.0 \pm 1.0 d
Isolate no. 6	2.0 \pm 1.0 e
Isolate no. 7	16.9 \pm 1.0 b
Streptomycin (1.0 mg/ml)	20.0 \pm 1.0 a
Sterilized water	0.0 \pm 0.0

Values followed by the same letter are not significantly different as determined by the LSD test ($P \leq 0.05$)

Table 2 Identification of endophytic bacterium *Enterobacter cloacae* PS14 by 16S rRNA gene sequences based on the matching similarity in the GenBank

Query length (bp)	Molecule type	Identities (%)	Query coverage	Max score	Total score	E value	Taxonomic name	GenBank accession no.
1971	Nucleic acid	86.22 (870/1009)	94%	1055	12877	0.0	<i>Enterobacter cloacae</i>	Mn385618

of the antagonistic bacterium against the pathogen could be mainly due to its antibiosis activity, where it has the ability to synthesize certain molecules to inhibit the pathogen development that was indicated by an inhibition zone around their growth (Chamedjeu et al. 2019).

Efficiency of *E. cloacae* PS14 and *T. asperellum* T34 in controlling the wilt of potato under greenhouse and field conditions

The results illustrated in Figs. 1 and 2 showed that the antagonists, *E. cloacae* PS14, and *T. asperellum* T34 significantly decreased the disease severity of wilt disease than the control (infected plants with the pathogen only) under greenhouse conditions. The disease severity was reduced by 10.73 and 26.50% due to the 2 respective bioagents, compared to 93.25% due to the pathogenic bacterium. Under field conditions, the bioagents successfully protected the potato plants against the destructive effect of the pathogen (Table 3). The disease severity was reduced by 39.00–49.06% from the infected control as the result of application of microorganisms, during the 2 seasons of the experiment. The used of both agents caused increasing the productivity of the crop by 20.44–40.96% relative to the control during the 2 seasons. Obtained results are somewhat in agreement with those of Upreti and Thomas (2015), who used *P. oleovorans*, *Pantoea ananatis*, and *E. cloacae* to manage the disease caused by *R. solanacearum* on tomato plants. These

authors mentioned that the presence of endophytic bacteria, that have antagonistic potentiality against pathogenic organisms, could reveal a possible role of endophytes associated with the root in natural defense against the pathogens. Also, Podolich et al. (2015) reported that endophytic bacteria may play an important role in crop protection by stimulating their active form in response to environmental stress or pathogen attack as well as improving the growth and plant health.

Effect of *E. cloacae* PS14 and *T. asperellum* T34 on production of biochemical precursors causing resistance's induction in potato plant

Phenol compounds

Salicylic acid Results showed a significant increase in salicylic acid content in the inoculated potato plants with *T. asperellum* T34 and *E. cloacae* PS14 than either only infected control or healthy plants (Fig. 3). The SA accumulation increased, 2 days after inoculation of plants, with the bioagents until the 8th day and SA content in plants, treated with *T. asperellum*, was higher than those treated with *E. cloacae*. The increase in SA content in the treated plants confirms the hypothesis that both rhizobacteria (PGPR) and *Trichoderma* spp. could stimulate the plant resistance, in addition to their suppressive effect on the phytopathogens. It was confirmed that these microorganisms were able to stimulate

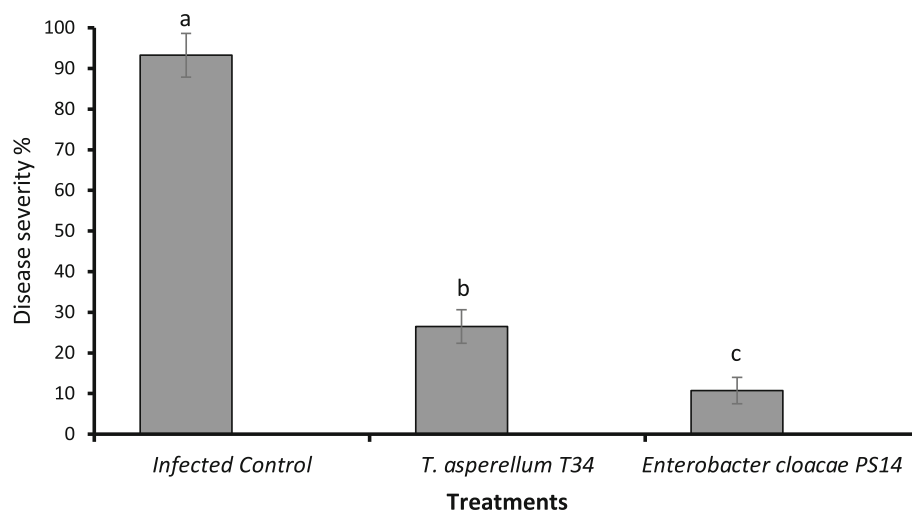


Fig. 1 Effect of *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34 on disease severity% of wilt on potato plants under greenhouse conditions



Fig. 2 Symptoms of wilt disease on potato plants treated with the *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34 compared to healthy and infected plants under greenhouse conditions

plant growth by mediating the plant hormones ethylene (ET) and induction of the resistance in plants through production of salicylic acid (SA) and jasmonic acid (JA) (Hermosa et al. 2012). Hassan and Abo-Elyoussr (2013) suggested that SA accumulation was essential for expression of multiple modes of plant disease resistance. Because SA is a plant defense phenolic compound against pathogens, it should be detected in both infected and distal leaves in response to pathogen attacks. Obtained results confirm that enhanced SA contents are, thus, a prerequisite for expression systemic acquired resistance against *R. solanacearum* in potato plants.

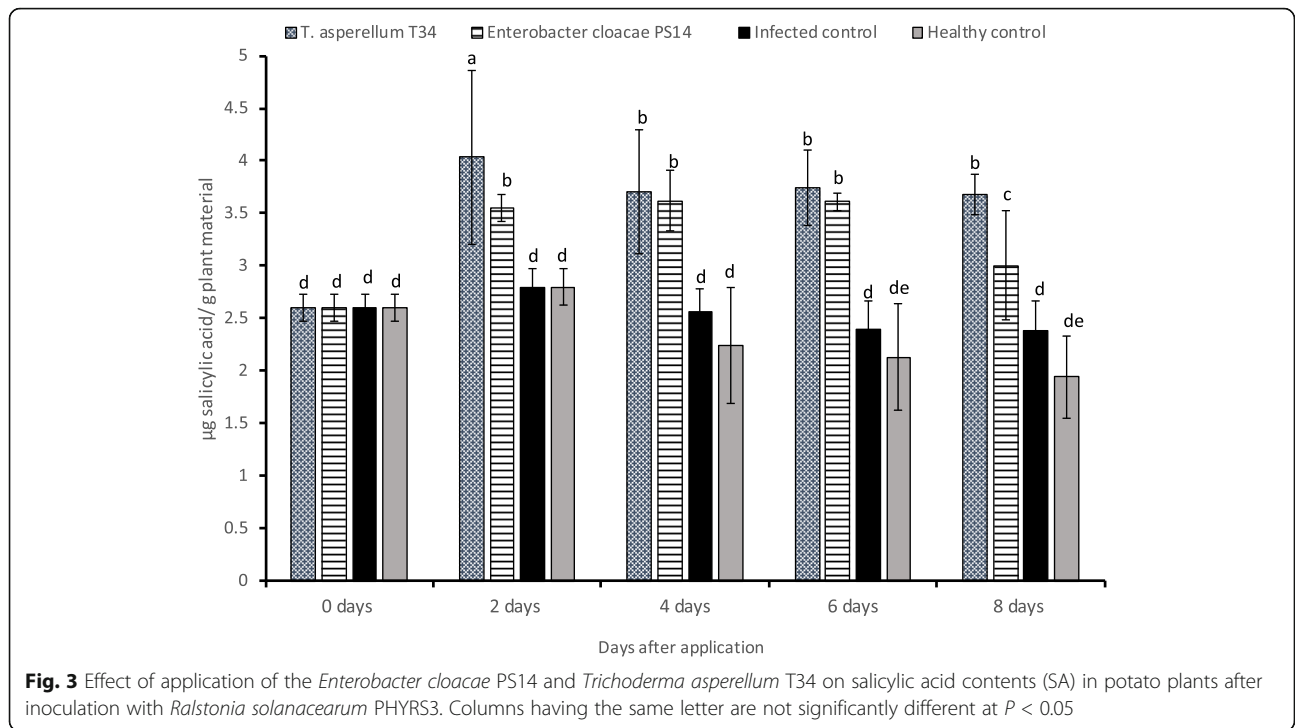
Total phenol content The total phenol contents in the inoculated potato plants with the pathogen and treated

with *T. asperellum* T34 and *E. cloacae* PS14 were significantly higher than that in only infected plants (control) or healthy plants (Fig. 4). The total phenol content in plants, treated with the 2 agents began to increase after the first 2 days from inoculation until the 6th day and then the concentrations began to decrease, however, the increase was not significantly different. These results agree with those of Vinayarani and Prakash (2018) who reported that the accumulation of phenolics was observed in turmeric rhizomes treated with *T. asperellum* after the 2nd from inoculation with the *Pythium aphanidermatum* and the maximum accumulation was observed on the 5th day after inoculation (Vinayarani and Prakash 2018). Khan and Bano (2019) mentioned that treatment of wheat plants with PGPR bacteria increased

Table 3 Effect of the *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34 on wilt diseases severity and tuber yield on potato plants under field conditions during in two trail seasons.

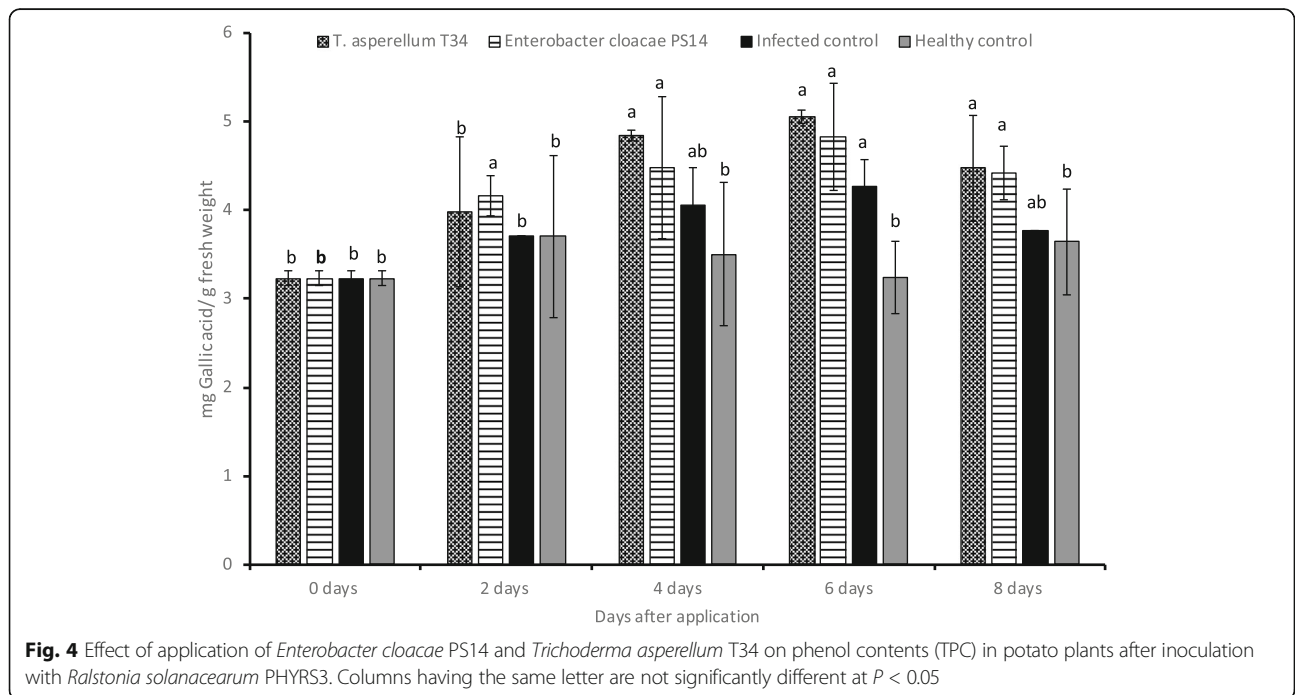
Treatment	Season 2017				Season 2018			
	Disease severity (%)	Reduction of disease severity (%)	Yield (ton/ hectare)	Increasing of yield (%)	Disease severity (%)	Reduction of disease severity	Yield (ton/ hectare)	Increasing of yield (%)
<i>Trichoderma asperellum</i> T34	36.60 ± 5.77 b	39.00	30.45 ± 3.51 ab	33.70	33.7 ± 0.16 b	42.20	31.42 ± 2.03 ab	40.96
<i>Enterobacter cloacae</i> PS14	26.70 ± 2.89 c	48.83	27.46 ± 5.13 bc	20.44	29.70 ± 1.30 c	49.06	29.03 ± 0.91 b	30.24
Infected control	60.00 ± 10.0 a	-	22.80 ± 4.16 cd	-	58.30 ± 0.37 a	-	22.29 ± 0.41 c	-
Healthy control	0.0 ± 0.0	-	32.60 ± 1.46 a	42.98	0.0 ± 0.0c	-	32.80 ± 2.26 a	47.25

Values in the same column followed by the same letter(s) are not significantly different as determined by the LSD test ($P \leq 0.05$)



the phenolic content. Accumulation of phenolic compounds in response to an infection in plants was reported by Hammerbacher et al. (2011) who confirmed that phenolic compounds are produced through the phenylpropanoid pathway and/or rapid translocation and modification of existing compounds. The accumulation

of phenolic compounds, in chili plants, at the site of the infection with anthracnose disease was correlated with the limitation of pathogen development, since these compounds are toxic to pathogens (Jayapala et al. 2019). Resistance may also be increased by changing the pH of plant cell cytoplasm due to an increase in phenolic acid



content that inhibits of development of pathogen (Bereika 2008; Hassan and Abo-Elyours 2013).

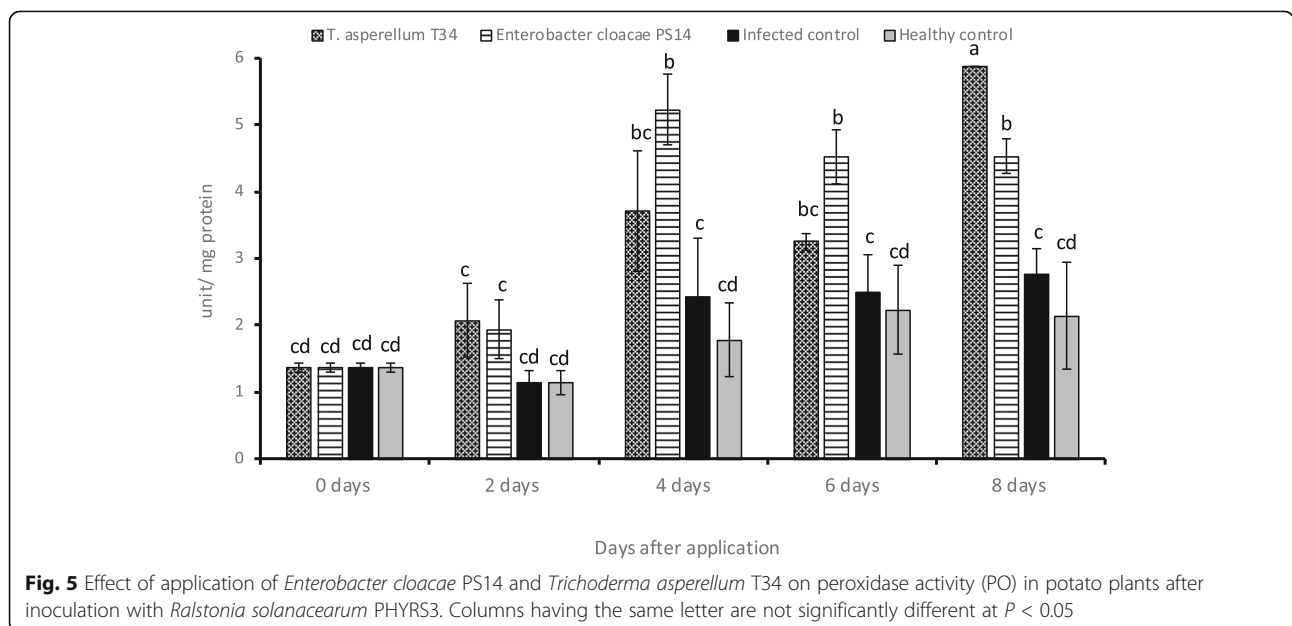
Enzymatic activities

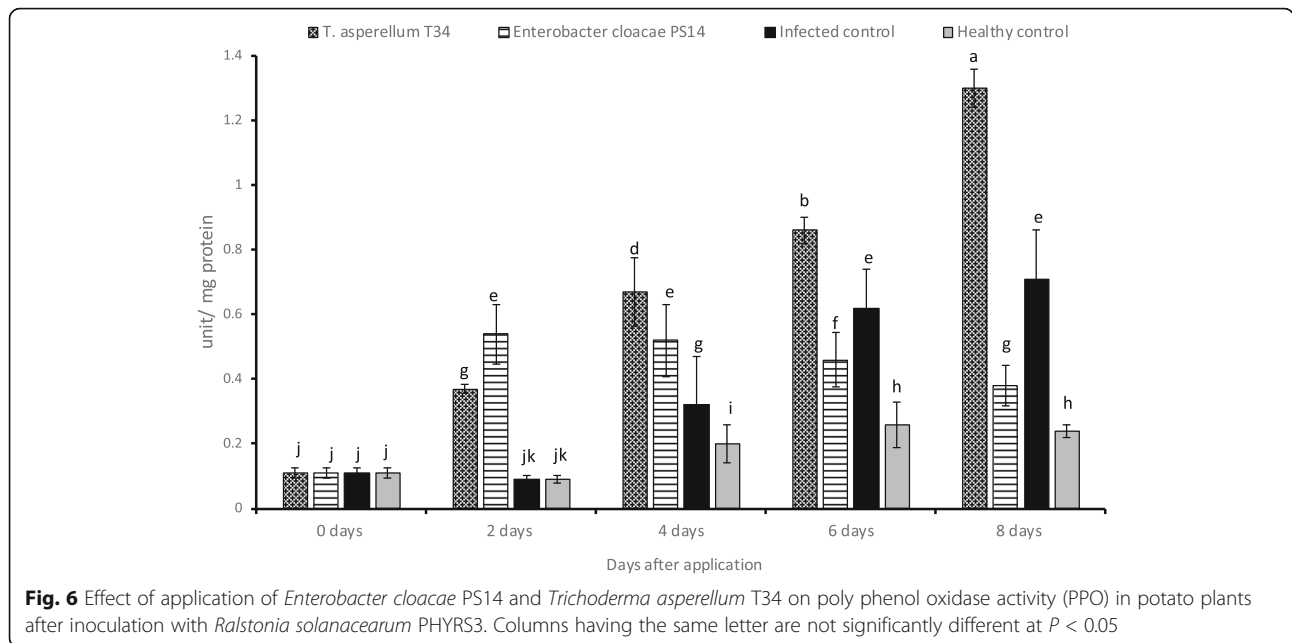
Peroxidase (PO) activity The results displayed that *T. asperellum* T34 significantly induced the maximum levels of PO activity in potato leaves infected with pathogen on the 8th day after inoculation than the either infected or heathy plants (Fig. 5). However, *E. cloacae* PS14 showed the highest level of PO activity in potato leaves on the 4th day after inoculation. This finding is supported by several reports that mentioned the enhanced PO activity in plants, when they were subjected to fungal, bacterial, and viral infections (Safdarpour and Khodakaramain 2018). Peroxidase caused lignin biosynthesis that provides a physical barrier and/or limits the degree of pathogen invasion and spread throughout the plant (Vidhyasekaran 2008). POX increases due to systemic resistance, a rapid synthesis of reactive oxygen derivatives by oxidative burst contributes to cell death and prevents pathogenic activity (Prasannath et al. 2014).

Polyphenol oxidase (PPO) activity Treatment of potato plants with *T. asperellum* T34 and *E. cloacae* PS14, after infection with the pathogen, induced the PPO activity in the potato plants in varied levels (Fig. 6). After 2 days from inoculation, potato plants treated with *T. asperellum* T34 exhibited a significant gradual increase in PPO activity until the 8th day than the only infected or healthy plants. *E. cloacae* PS14 showed a high level of PPO activity from

the 2nd until the 4th day, and then PPO decreased on the 6th and 8th days than the only infected plants. The significance of PPO activity in disease resistance probably be due to its property of oxidizing phenolic compounds to quinines, it is often more toxic to microorganisms than the original phenol (Safdarpour and Khodakaramain 2018). Generally, toxicity of PPO-created quinones to pathogens and plant cells, inducing cell death, alkylation and decreased bioavailability of cell proteins to the pathogen, crosslinking protein or other phenolic quinones, forming a physical barrier to cell wall pathogens and quinone redox cycling leading to H₂O₂ and other reactive oxygen specificities (Konappa et al. 2018).

Lipoxygenase (LOX) activity *T. asperellum* T34 and *E. cloacae* PS14 significantly increased the activity of lipoxygenase (LOX) in potato leaves infected with the pathogen than either only infected or healthy plants (Fig. 7). Increase in LOX level began after 2 days from inoculation of plant with the bioagents till 8 days; however, *T. asperellum* T34 showed higher activity of LOX than *E. cloacae* PS14. The results agree with those of Kurabachew and Wydra (2013). This might be due to the fact that pathogen inoculation does not affect linoleate-consuming LOX. In potato plants, which could be due to an increase in the level of transcription of the two isoforms of LOX: Tomlox D and Tomlox E. LOX products contribute to the defense reaction in plants-pathogen interaction by inhibiting pathogen growth, phytoalexin accumulation, and signal transduction (Mariutto et al. 2011).





In vitro production of siderophore, HCN, IAA, and SA by *E. cloacae* PS14 and *T. asperellum* T34 to confirm their possible mode of action

Both microorganism, *E. cloacae* PS14 and *T. asperellum* T34, were able to produce a considerable amount of siderophore, HCN, IAA, and SA (Table 4). The endophytic bacterial *E. cloacae* PS14 produced a higher amount of siderophore, HCN, and SA than *T. asperellum* T34; however, the situation was reversed in the case of IAA. *E. cloacae* PS14 produced 0.884, 3.61, and 1.488 $\mu\text{g/ml}$

of siderophore, HCN, and SA, respectively. *T. asperellum* T34 produced 0.225 $\mu\text{g/ml}$ of IAA. Our findings could be supported by those of Calin et al. (2019) who reported that *T. asperellum* had the ability to produce IAA. That is recognized as the key plant growth-promoting substance regulating plant physiological and developmental functions (Srisuk et al. 2018). *Trichoderma* spp. can produce IAA with varied amounts (Mohiddin et al. 2017). In other work, *E. cloacae* strain OS03 had the ability to produce IAA in varying levels in

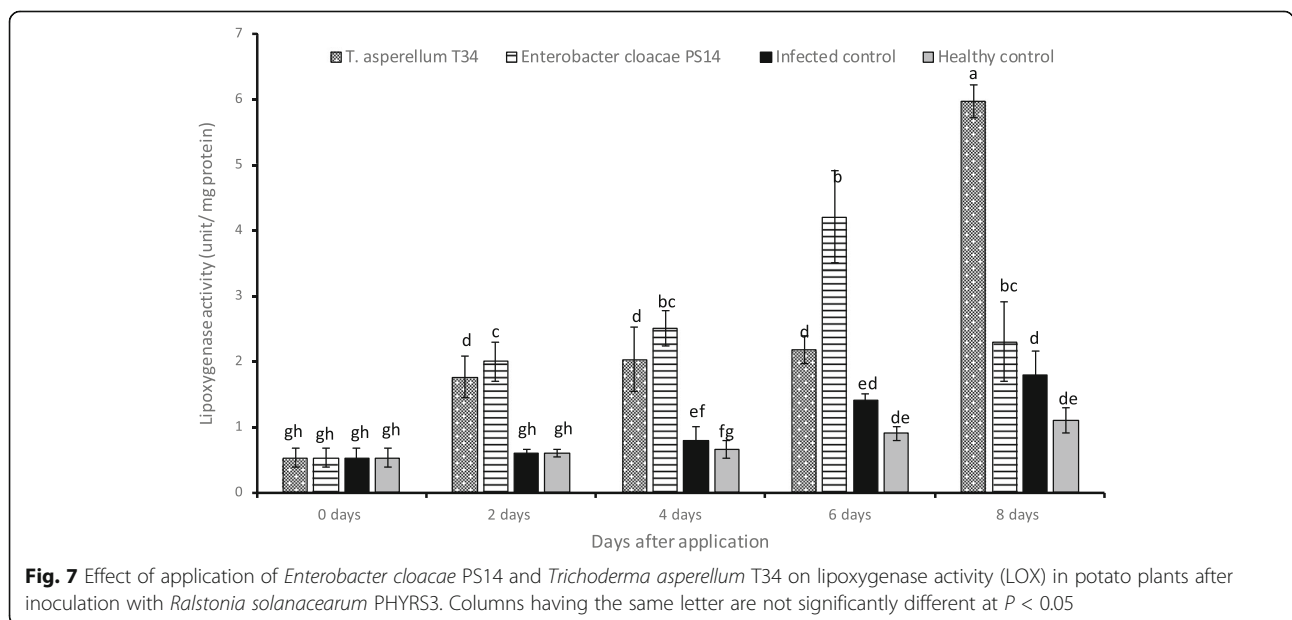


Table 4 Production of siderophore, HCN, IAA, and SA by *Enterobacter cloacae* PS14 and *Trichoderma asperellum* T34 in vitro

Bioagents	Siderophore production (μmol)	HCN production μg/ml	IAA (μg/ml)	SA (μg/ml)
<i>Trichoderma asperellum</i> T34	0.577	2.34	0.225	0.436
<i>Enterobacter cloacae</i> PS14	0.884	3.61	0.133	1.488

the medium containing L-tryptophan than under a cop of physicochemical conditions (Panigraha et al. 2019). Sheng et al. (2008) mentioned that *E. cloacae* could have a significant impact on the growth of plants, in particular by producing IAA (Panigraha et al. 2019). Siderophores are iron chelating compounds that have a serious part in the field such as agriculture, bioremediation, biosensor, and medicine (Venkat et al. 2017). In a similar report to ours, *E. cloacae* strain PGLO9 exhibited activity of siderophore production and showed a positive antagonistic ability to phytopathogens (Verma et al. 2018). *Trichoderma* can produce siderophores to control plant disease and promote growth opportunities to replace chemical pesticides (Calin et al. 2019). Siddiqui et al. (2006) reported that HCN production is an important feature found in different microorganisms, since it indirectly promotes plant growth by controlling certain soil-borne diseases (Siddiqui et al. 2006). Positive correlation has been documented between production of HCN in vitro and plant protection and their defense system. It was thought that HCN direct inhibition of fungi was one of the major mechanisms of action. There are numerous reports on biocontrol activity of HCN production in the rhizosphere in tobacco plants. Also, it can stimulate the root length and root hair germination (Ghosh et al. 2018). Also, specific strain of *E. cloacae* (K2 strain) was reported as a good HCN-producer (Pramanik et al. 2018). *T. asperellum*-cucumber interaction increased SA and peroxidase activity as an induction response in the plants. In addition, after inoculation of *Trichoderma* into the plants, a systemic resistance of plants increased as the increase in SA and JA rates were observed (Hermosa et al. 2012). Also, endophytic bacteria increased the growth of sunflower seedlings and inhibited the growth of pathogenic fungi by developing SA (Klessig et al. 2016).

Conclusion

The results conclude that the new endophytic strain of *E. cloacae* PS14 was a potent biocontrol agent against *R. solanacearum*—the causal pathogen of wilt of potato. The use of *T. asperellum* T34 and *E. cloacae* PS14 successfully suppressed the *R. solanacearum* in vitro, in vivo, and in situ. It caused reduction of the disease severity and increased the crop yield in a level close to *T. asperellum* T34. The main mechanism of action of the new bacterial strain was due to production of antibacterial agent as well as induction of systemic resistance of potato plant. Successful application of the new bacterial

strain on the field scale for two successive seasons encourages its application on a large scale in biological control of plant disease, and further research to find an appropriate formulation and approving application of the new strain for a wide range of plant diseases was recommended.

Abbreviations

SA: Salicylic acid; LOX: Lipoxygenase; PPO: Polyphenol oxidase; PO: Peroxidase; IAA: Indole acetic acid production; HCN: Hydrogen cyanide

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Authors' contributions

All authors contributed equally in the manuscript. B.F.F.M. and N.M. S. suggested the idea of the work and contributed to data curation and their validation as well as writing the original draft. K.E. contributed to the formal analysis of the data. S.A., M.H., and Y.S.M. contributed to the reviewing and editing of the manuscript. All authors reviewed and approved the final version of the manuscript.

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All authors approve to publication.

Competing interests

No potential conflict of interest was reported by the authors.

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