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Egypt J Biol Pest Control (2021) 31:113
https://doi.org/10.1186/s41938-021-00460-z

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Biological control of *Ralstonia solanacearum* (Smith), the causal pathogen of bacterial wilt disease by using *Pantoea* spp

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Abstract

Background: Bacterial wilt of tomato (BWP) caused by *Ralstonia solanacearum* (Smith) is a very important disease. Biological control of this disease is a very important tool to protect the plant and environment from pollution of chemical control.

Results: Twenty isolates of genus, *Pantoea* were isolated from healthy tomato root. Out of 20 isolates, 2 strains, PHYTPO1 and PHYTPO2, showed highly antagonistic property to control the growth of *R. solanacearum* in vitro conditions. They were identified as *P. agglomerans* by using 16S rRNA nucleotide sequence analysis. The 2 isolates were selected to study their effect (as cell suspension or culture filtrate) on the bacterial wilt under greenhouse conditions. PHYTPO1 inhibited maximum growth reduction of *R. solanacearum* and formed 2.5 cm^2 of inhibition zone, followed by 1.2 cm^2 in PHYTOPO2 under in vitro conditions. Treating with both isolates of *P. agglomerans* was significantly reduced disease severity of tomato wilt disease. The disease severity was reduced to 74.1 when treated as cell suspension, while when treated as culture filtrate, it reduced the disease severity up to 69.4 than infected control.

Conclusion: The strains of *Pantoea* can be used as an ecofriendly method to control of the most economic pathogen of tomato under greenhouse conditions. Further study is needed to find an appropriate formulation and approving application of these bacteria under field conditions.

Keywords: Tomato wilt, Endophyte, Biological control, *Pantoea agglomerans*

Background

Bacterial wilt of tomato (BWP), caused by *Ralstonia solanacearum* (Smith) (Yabuuchi et al. 1995) is a serious problem in many countries. This bacterium could destroy 100% of the crop production (Wang et al. 2019). BWP disease is difficult to be managed under field conditions due to its ability to survive for a long time in the soil. The long-term use of chemical products such as bactericides and fungicides induced resistance in the pathogen making it tolerant to chemical applications (Maji and Chakrabarty 2014). Generally, this disease could be managed by cultural practices, use of resistant cultivars and application of copper compounds, but all of these methods have a limited success (Bereika et al. 2020). Biological control can be a part of integrated disease management to control the BWP disease (Bereika et al. 2020). Entophytes or phylosphere and rizobacteria are safe for human, and very effective in reducing phytopathogenic bacteria by inducing antibiotic or secondary metabolites (Al-Sman et al. 2019) as well as inducing resistance in plant (Bereika et al. 2020).

Pantoea agglomerans is a very important biocontrol agent that could be used for controlling many pathogens, *e.g.* fungal diseases, tomato early blight caused by (*Alternaria solani*), rot in cotton (*Pythium* sp.), bacterial diseases *e.g.* fire blight in apple and pear caused by (*Erwinia amylovora*) (Laux et al. 2001), cotton blight (Bora et al.

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1993) and plant parasitic nematode, *Meloidogyne javanica* (Bonaterra et al. 2014). It produces single antibiotic or multiple compounds which could inhibit the bacterial growth (Laux et al. 2001). Also, it has the ability to promote the growth of plant by producing some hormones or solubilized of phosphorus as well as production of siderophores (Gutierrez Manêro et al. 2001). *P. agglomerans* mostly has been used to control fire blight disease in Europe or USA (Özaktan and Bora 2004). Recently, Singh et al. (2020) used *P. agglomerans* for studying its effect on the growth of *R. solanacearum* in vitro.

The present study aimed to evaluate the potential of 2 strains of *P. agglomerans,* isolated from Egypt, for inhibiting the growth of *R. solanacearum* in vitro and in vivo as well as enhancing plant growth.

Methods

Isolation of bacterial bioagent

Different soil and tomato plant samples were collected from different location of Assiut Governorate. Dilution technique methods was used to isolate *Pantoea* spp. from samples onto semi selective medium containing KH_2PO_4 (0.8 g), D- trehalose (2.0 g), Yeast extract (10.0 mg), NaCl (15.0 g), 2,6- diminopurine (0.2 g), $MgSO_4$.7 H_2O (0.2 g), K_2HPO_4 (0.8 g) and agar, Bacto (20.0 g) and adjusted pH 4.9 with 1 NH_4Cl_2 and then added cycloheximide (30 µg/ml) (Manulis et al. 1991), after that the plates were incubated for 2 days at 28 °C. Single colony was selected and transferred to NSA medium slants for further study. *R. solanacearum* PHYRS7 (MT510004), was isolated previously (Seleim et al. 2011).

Effect of *Pantoea* sp. against *R. solanacearum* PHYRS7 in vitro conditions

Methods of Bereika et al. (2020) was used to test the potential of the isolates to antagonize against *R. solanacearum* PHYRS7 in vitro. Bacterial isolates were cultured in NA medium for 2 days at 27 °C. A 100 µl (10^8 CFU/ml) of 48 h-old culture of PHYRS7 have inocula load medium to make a lawn for each isolate separately with 3 replications (each contained 5 plates). After that, 3 wells of 5.0 mm in diameter, using sterile cork borer, were formed on the Petri plate. A 50 µl (10^8 CFU/ml) culture of each isolate of *Pantoea* sp. was inoculated into each well separately and incubated in Petri plates at 27 °C for 2 days as well as a 50 µl of culture filtrates of each isolates was inoculated in well, Culture filtrates of each isolate of *Pantoea* spp. were obtained after growth for 7 days at 28 °C in NA broth. Zone of inhibition, formed by strains of *Pantoea* sp. was recorded.

Identification of the bacterial bioagent using 16S rRNA sequence analysis

Isolation of DNA was performed according to Saghai-Maroof et al. (1984) with some modifications. One ml of overnight liquid culture was placed in a 1.5 ml disposable centrifuge tube. The cell was collected through centrifugation at 7500 rpm for 10 min. The supernatant was discarded, and the pellet was re-suspended in 0.2 ml of Phosphate buffer. 10 µl of lysozyme were added and incubated at 37 °C for 60 min. 0.4 ml of CTAB extraction buffer was added, followed by 40 µl of β-merca btoethanol and gently mix. The tube was placed in 60°C water bath for 60 min. After cooling an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed. This mixture was centrifuged for 5–10 min at full speed, and the aqueous supernatant was transferred to a new tube. An equal volume of cold ethanol 100% was added then cooled at -4°C for 30 min. Then, it centrifuged for 5 min. at 1300 rpm to pellet the DNA. Washing was done by ethanol 70%, followed by centrifugation for 5 min. Finally, the pellets were kept for drying for 1 h at room temperature and then, dissolved in warm dist. H_2O. Amplification of 16S rRNA gene of size 709 bp was done by using the primer set (UNI_OL5:5′GTGTAGCGGT GAA ATGCG3′) and UNI_OR (5′ ACGGGCGGTGTGTACAA-3′) (Wattiau et al. 2001). To perform several parallel reactions, a reaction containing water, 2 × master mix, primers and appropriate DNA template dilution was prepared in a single tube with the quantities (8 µl dH_2O, 10 µl 2X Master mix, 1 µl 20 µM Forward and Reverse primers, 1.0 µl Templet DNA). PCR conditions were carried out in a Lab Cycler SensoQuest, with the following parameters: template denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at (65 °C for 1406f and 23S Primers and 57 °C for MM5F and MM5R) for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min and soaked at 4 °C. The obtained PCR products of each reaction (20 µl) and a 100 bp DNA Ladder were electrophoresed onto submerged agarose gel of 1% concentration containing ethidium bromide in 1X concentration TBE buffer (89 mM Tris–borate; 2.5 mM EDTA). The submarine gel was supplied with a direct current of 100 V for approximately one hour, using a power supply. The gel was then visualized and photographed, using gel documentation system. Sequence of amplified fragment was blasted in NCBI nucleotide BLAST. The phylogenetic tree was prepared by the help of Bio-informatics tool MEGA 5 software.

Effect of *P. agglomerans* as cell suspension or culture filtrate on seed germination and seedling vigor in vitro

The in vitro effect of seed pre-treatment with *Pantoea* was examined on tomato seed germination. Seeds (1 and 2) were soaked in each *Pantoea* sp. suspension (1×10^8 cfu/ml) for 15 min. (3 and 4) were dipped in an culture filtrate of each isolate, (5) seed soaked in PHYTR7 as negative control and (6) untreated seeds soaked in sterile distilled water served as control. Each treatment contained 3 replicates/50 seeds each. The treated seeds were placed on wet filter paper and incubated at 28 °C for one week. Germination, mean shoot length (MSR) and mean root length (MRL) were recorded. Seedling vigor index (VI) was determined, using the equation as follows (Abdul Baki and Anderson, 1973):

$$\mathrm{VI} = (\mathrm{MSL} + \mathrm{MRL}) \cdot \text{germination\%}.$$

Effect of *P. agglomerans* as cell suspension or culture filtrate on diseases severity under greenhouse conditions

Greenhouse experiments were performed at BLINDED FOR PEER REVIEW. Seeds were sown in 20 cm diameter plastic pots containing a soil mixture consisting of soil: sand (1:1 w/w) and fertilizer 1% N.P.K. (12: 4: 6). Plants were irrigated when necessary. Twenty-eight day-old tomato seedlings cv. 'Tala F1' were inoculated by *R. solanacearum* PHYRS7 (108 cell/ml) by cutting the roots from 2 sides, with an alcohol knife inserted 4–5 cm into the soil and inserting 20 ml of *R. solanacearum* PHYRS7 suspension around the base of each plant (Bereika et al. 2020). Only the pathogen was inoculated into infected control plants, which were then treated with 20 ml sterile distilled water. After inoculation, the tomato plants were kept in a moist chamber at 25 °C for 2 days before being transferred to the greenhouse at 25–30 °C. A 20 ml of each bioagent suspension *Pantoea* spp., as well as the culture filtrate of each isolate surrounding the plant bases, were added 2 days after inoculation. Four replicates were used per treatment (3 pots each). The development of bacterial wilt symptoms was observed after 21 days. The severity of the disease was measured, and the percentage of disease severity (DS percent) was calculated, using the formula of Bereika et al. (2020).

$$\text{Disease index (\%)} = \left[\sum (ni \times v_i) \div (V \times N)\right] \times 100$$

where *ni* represents the number of plants for each disease rating, *vi* represents the disease rating, *V* represents the highest disease rating (5), and *N* represents the total number of plants observed. The following scale was used to determine disease severity: 1 = no symptoms, 2 = one leaf wilted, 3 = two to three leaves wilted, 4 = four or more leaves wilted and 5 = whole plant wilted.

At the end of experiment, 5 plants were taken to determine the fresh and dry weights of roots and shoots.

Effect of *P. agglomerans* as cell suspension or culture filtrate on population of *R. solanacearum* PHYRS7 in tomato plant

For determination of bacterial multiplication in tomato plant treated with bacterial cell or culture filtrate, one gram of the stem tissues (3 cm above the soil) of each treatment after 6 weeks from inoculation was cut and washed with tap water, surface sterilized with 3% sodium hypochloride and washed several time with sterile water. A 10 ml of 0.1 M potassium phosphate buffer was used to homogenize the samples in a sterile mortar and pestle (pH 7.0). Stem homogenates were serially diluted in 0.1 M potassium phosphate buffer from 10–1 to 10–9. 200 µl of each dilution was transferred to a selective medium (TTC) and distributed with a glass rod. The number of bacterial colonies was counted after 48 h. of incubation at 27 °C.

Statistical analysis

SPSS 22.0 was used to conduct all statistical analyses. For antagonistic capability, the data were analyzed using one-way analysis of variance (ANOVA), while for the rest of the experiments, two-way analysis was used. According to Gomez and Gomez (1984), the least significant difference (LSD) test was used at P 0.05 to distinguish significant variations among the means of the treatments.

Results

Antagonistic potential of the isolates

Out of 20 isolates, 2 strains showed antagonistic potential to control the growth of *R. solanacearum* in vitro conditions. Remaining isolates showed moderate antagonistic activity against *R. solanacearum* (unpublished data). From the obtained results, the most effective 2 isolates were selected to study their culture filtrates and cell suspensions on growth of *R. solanacearum.* As results presented in Fig. 1, isolate PYTOPO2 caused the highest reduction of the pathogen growth when treated as cell suspension, followed by cell suspension of PHYTOPO1 and culture filtrate of both isolates. PHYTPO1 inhibited maximum growth of *R. solanacearum* and formed 2.5 cm^2 of inhibition zone, followed by 1.2 cm^2 in PHYTOPO2 in vitro conditions.

Identification of the bioagent isolates

Bioagent isolates were molecularly characterized by means of 16S rRNA sequencing. BLAST search on the NCBI data libraries (16S ribosomal RNA sequences

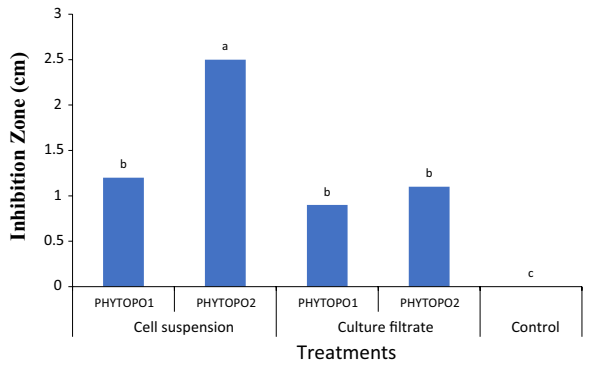

Fig. 1 Effect of two isolates of *Pantoea agglomerans* as cell suspension or culture filtrate on inhibition zone percentage of *Ralstonia solanacearum*. According to the LSD test at $p \leq 0.05$, values in the same column followed by a similar letter are not significantly different

(Bacteria and Archaea) and non-redundant nucleotide collection) for similarities of the 16S rRNA sequences showed it as the most similar to *Pantoea agglomerans* strain PHYTOPO1 and 2 (GenBank accession No. MT605810, MT605811) with 100% identity query coverage (Fig. 2).

PGPR evaluation

Efficiency of *Pantoea agglomerans* in controlling wilt disease of tomato under greenhouse conditions

The results illustrated in the Fig. 3 showed that under greenhouse conditions, treatment with both isolates of *P. agglomerans* PHYTPO 1 and 2 as cell suspension or culture filtrate, significantly reduced disease severity of wilt disease than the infected control (pathogen only). The disease severity was reduced by 74.1 and 70.6% when bioagents were treated as cell suspension, while when in case of the plant treated with culture filtrate, the disease severity was reduced by 69.4 and 68.2%, respectively, than the infected control. Disease severity in infected control was very high (85%), while in treated one, it ranged from 24 to 27%.

Effect of *P. agglomerans* as cell suspension or culture filtrate on seed germination and seedling vigour index

Both isolates and their culture filtrate treatments increased the percentage of germination. Treatment with PHYTOPO2 as cell suspension was the most effective one (79%), it increased the percentage of seed germination up to 75.5% relative to infected control with pathogen, followed by PHYTOPO1 (Table 1). While in case of culture filtrate, it increased the germination with 55.5%. In general, cell suspension increased seed germination higher than culture filtrate. The vigor index was also increased than infected control at all treatments of cell suspension and culture filtrate of both isolates, it ranged between 227.2 and 355.5 and 112.5% in infected one.

Effect of *P. agglomerans* as cell suspension or culture filtrate on *R. solanacearum* population in tomato plants

The results presented in Table 2 indicated that the highest number of cell (CFU) *R. solanacearum* was observed in the infected control. Plant treated with PHYTOPO 1 and 2 as cell suspension or culture filtrate caused reduction of *R. solanacearum* relative to the control DW. Treatment with PHYTOPO 1 as cell suspension was the best treatment, 1.3×10^9 cfu/g, followed by PHYTOPO2, 1.9×10^9 cfu/g while the lowest treatment was observed in culture filtrate of PHYTOPO1, it was 2.9×10^9 cfu/g.

Influence of *P. agglomerans* as cell suspension or culture filtrate on fresh and dry weight of tomato plants under greenhouse conditions

The influence of both isolates on fresh and dry weights of roots and shoots of tomato plants was determined at the end of experiment. Fresh and dry weights of shoots and roots of plants infected with *R. solanacearum* were significantly lower than that of DW treatment. Treatments of both isolates significantly increased fresh weight of tomato shoots (g^{-1} plant), arranged from 135.6 to 138.2 g/plant than *R. solanacearum* 60 g/plants (Table 3). Also, the dry weight of shoots also increased than infected control in all treatments. In case of root weight (fresh and dry), both isolates significantly increased the weight than infected control, when plants treated with cell suspension, it is better than treated with culture filtrate.

Discussion

Numerous studies have shown the role of endophytes as biocontrol agents against various plant pathogens. Endophytes are significant sources of new bioactive compounds and secondary metabolites. In the tissues of various plants, a wide variety of endophytic prokaryotic microorganisms has been reported (Zheng et al. 2018). Twenty bacterial isolates were isolated from different locations at Assiut Governorate Egypt. All isolates were tested against *R. solanacearum,* the causal pathogen of tomato wilt. The best 2 isolates, which inhibited the growth of the pathogen PHYTPR7 were selected to study their effect on the reduction of bacterial wilt of tomato and enhancement the growth of tomato plant under greenhouse condition. Both isolates were identified as *P. agglomerans,* by 16S rRNA, and deposited in the GenBank under accession numbers MT605810 and MT605811 (Li et al. 2008).

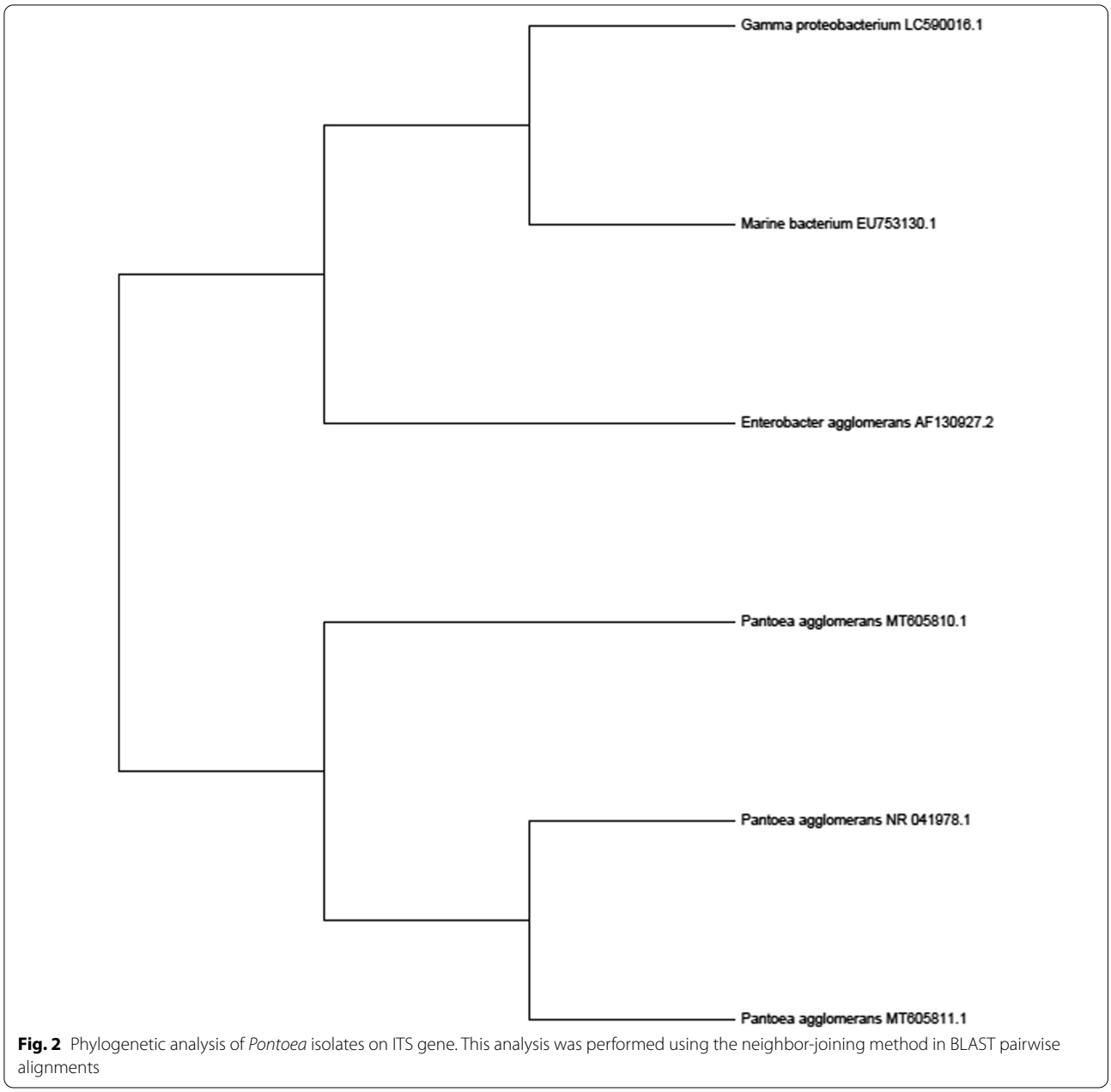

Fig. 2 Phylogenetic analysis of *Pontoea* isolates on ITS gene. This analysis was performed using the neighbor-joining method in BLAST pairwise alignments

In the earlier study of Johnson et al. (1993), reported that *P. agglomerans* was able to suppress diseases caused by different groups of phytopathogenic bacteria *e.g. Xanthomonas albilineans* (blight in sugarcane) *Erwinia amylovora* (fire blight of apple), and *X. oryzae* pv. *oryzae* (Zhang and Birch 1996). Wodzinski et al. (1994) stated that *P. agglomerans* could suppress the diseases by producing various antibiotics, most of these antibiotics most probably were pantocins and herbicolins as mentioned by Wright and Beer (2005).

Many researches showed that a several bacterial bioagent present in phyllosphere or rhizosphere caused enhancing the growth of the plants and suppressing the pathogens, these bioagents called plant growth-promoting bacteria (PGPR) (Miethling et al. 2000). They can enhance the growth of plants through stimulating the growth hormones and solubization of the minerals in the soil and the siderophors production (Vazquez et al. 2000).

The results reported herein showed that the disease severity was reduced by 69.4–47.1% than the control,

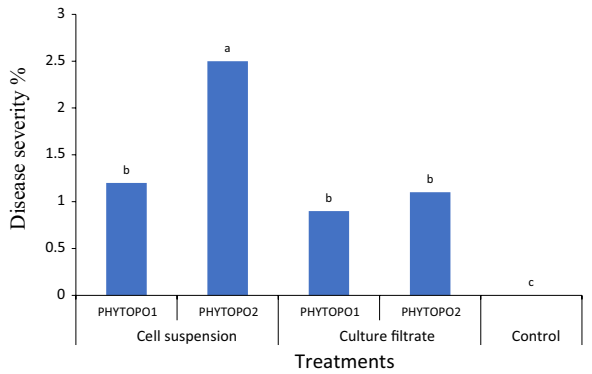

Fig. 3 Disease reduction percentage of treated tomato plants under greenhouse conditions. Columns with the same letters are not significantly different according to Fisher's protected least significant difference at $p \leq 0.05$

when both isolates were applied as cell suspension or culture filtrate, under greenhouse experiment. Using both bioagent decreased the cfu/g in plant as well as increased biomass of the plant. These results are in agreement with those reported by Thomas and Upreti (2014), who mentioned that *Pantoea ananatis* can be used to control bacterial wilt of tomato plants that caused by *R. solanacearum*. They found that this endophytic bioagents had an antagonistic potentiality against phytopathogenic microorganisms, and it could be used for controlling such pathogen. In addition, endophytic bacteria, according to Podolich et al. (2007) can help protecting crops by stimulating their active form in response to environmental stress or pathogen attack, as well as improving plant growth and health.

The study in greenhouse revealed that fresh and dry weights of shoots and roots of bioagents-treated tomato plants were higher than the untreated and infected tomato plants. Several works have reported that plant treated with pathogens could increase the biomass *e.g.* maize (Goswami et al. 2016), tomato (Hernàndez-Suàrez et al. 2011) and potato (Bereika et al. 2020). The plant growth promotion which was observed in the PGPR-treated tomato plants could be indirectly related to the potential of the PGPR strains to reduce the severity of *R. solanacearum* on tomato plants.

Conclusion

The results concluded that the new endophytic strains of *P. agglomerans* were a potent biocontrol agent against *R. solanacearum*—the causal pathogen of wilt of tomato. It caused reduction of the disease severity and increased the crop biomass. Further research is

Table 1 In vitro effect of treatment with the bacterial bioagent as cell suspension or culture filtrate on seed germination and seedling vigour of tomato

Treatments	Method of application	Germination %	MSL (Cm)	MRL (Cm)	VI
PHYTOPO1	Cell suspension	75 c	1.4 a	3.1 a	337.5
PHYTOPO2	Cell suspension	79 b	1.3 b	3.2 a	355.5
PHYTOPO1	Culture filtrate	70 d	1.2 c	2.9 b	287
PHYTOPO2	Culture filtrate	71d	1.1 c	2.1 c	227.2
Healthy Control	Water	85 a	1.5 a	2.9 b	374
Infected control	*R. solanacearum*	45 e	0.9 d	1.6 d	112.5

Values in the same column followed by a similar letter are not significantly different as determined by the LSD test ($P = 0.05$)

Table 2 Effect of treatment with bacterial bioagents as cell suspension or culture filtrate on number of bacteria in plant under greenhouse conditions

Treatments	Method of application	Bacterial numbers (cfu/g stem tissue) after two weeks from inoculation
PHYTOPO1	Cell suspension	$1.9 \times 10^{9\,cd}$
PHYTSTC7	Cell suspension	$1.3 \times 10^{9\,e}$
PHYTOPO1	Culture filtrate	$2.9 \times 10^{9\,b}$
PHYTOPO2	Culture filtrate	$2.2 \times 10^{9\,c}$
Control	Water	0.0^{f}
Infected control	*R. solanacearum*	$4.2 \times 10^{7\,a}$

Values in the same column followed by a similar letter are not significantly different as determined by the LSD test ($P = 0.05$)

Table 3 Effect of some bacterial boiagents on fresh and dry weight of shoots and root under greenhouse conditions

Treatments	Method of application	Root		Shoots	
		FW	DW	FW	DW
PHYTOPO1	Cell suspension	14.8 b	1.3 c	138.0 a	12.1 b
PHYTOPO2	Cell suspension	16.9 a	1.6 a	138.2 a	13.1 a
PHYTOPO1	Culture filtrate	13.5 c	1.3 c	135.6 b	11.6 c
PHYTOPO2	Culture filtrate	13.6 c	1.5 b	136.2 b	13.1 a
Control	Water	13.5 c	1.2 d	130.5 c	10.1 d
Infected control	*R. solanacearum*	6.5 d	0.8 e	60 d	6.4 e

Values in the same column followed by a similar letter are not significantly different as determined by the LSD test (P = 0.05)

needed to find an appropriate formulation and approving application of the bacteria under field conditions.

Abbreviations

BWP: bacterial wilt of potato crop; LSD: least significant difference; IAA: indole acetic acid; MSR: mean shoot length; MRL: mean root length; VI: seedling vigor index; PHYTPO1: strain 1 of isolate *P. agglomerans*.

Acknowledgements

The authors extend their appreciation to Taif University for funding current work by Taif University Researchers Supporting Project number (TURSP - 2020/142), Taif University, Taif, Saudi Arabia..

Authors' contributions

All authors contributed equally in the manuscript, AKAM suggested the idea of the work and contributed to data curation and their validation as well as writing original draft.SH. contributed to the formal analysis of the data., SH and AKAM. contributed to the reviewing and editing the manuscript. All authors read and approved the final manuscript.

Funding

The authors extend their appreciation to Taif University for funding current work by Taif University Researchers Supporting Project Number (TURSP—2020/142), Taif University, Taif, Saudi Arabia.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable. This manuscript is in accordance with the guide for authors available on the journal's website. Also, this work has not been published previously and is approved by all authors and host authorities.

Consent for publication

Not applicable.

Competing interests

No potential conflict of interest was reported by the authors.

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Received: 24 May 2021 Accepted: 14 August 2021
Published online: 19 August 2021

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