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Biological Control of Pepper Soft Rot Disease Caused by *Pectobacterium carotovorum* Using *Rahnella aquatilis*

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PECTOBACTERIUM and *Dickeya* species are the main causative agents for soft rot disease that adversely affect fruits and vegetables leading to considerable economic losses. Biological management with beneficial microorganisms is a promising alternative to hazardous bactericides. Therefore, the antagonistic activity of two different strains of *Rahnella aquatilis* was *in vitro* and *in vivo* evaluated against nine soft rotting bacterial strains. The antagonistic soil bacteria *R. aquatilis* strains 17 and 55 restricted the growth of nine soft rotting bacterial strains on nutrient agar plates, (7 *Pectobaterium carotovorum* strains and 2 *Dickeya chrysanthmi* strains). Transmission electron micrographs of *P. carotovorum* Pep3B cells antagonized with *R. aquatilis* strain 17, showed damaged cells with disrupted plasma membrane releasing the cellular contents. To examine whether *R. aquatilis* 17 could be an effective biological control agent for pepper soft rot disease, two applications were conducted. The pepper seedlings were pretreated, before the pathogen, with *R. aquatilis* 17 through leaves and roots. All seedlings pretreated with the antagonistic strain 17 showed reduced susceptibility towards the *P. carotovorum* Pep3B, increased fresh, dry weights and seedlings' height relative to controls. *R. aquatilis* 17 inoculation has positively influenced the physiological parameters evaluated, such as chlorophyll content, carotenoids, phenolics, flavonoids, protein concentration as well as proline concentration. The obtained results revealed that *R. aquatilis* 17 mitigated the effect of *P. carotovorum* on pepper seedlings and promoted their growth, which means that it has a high probability of being an effective biological control agent and a plant-promoting bacterium.

Keywords : Antagonistic bacteria, Biological control, *Pectobacterium carotovorum,* Pepper seedlings, *Rahnella aquatilis,* Soft rot.

Introduction

Pepper (*Capsicum annuum* L.) belongs to the *Solanaceae* family. It contains a variety of nutrients such as dietary fiber, protein, vitamins, and minerals (Rubio et al., 2002; Igwemmar et al., 2013; Kim et al., 2014). It contains bioactive compounds with medicinal value such as carotenoids which include capsaicin, capsanthin, capsorubin, beta-carotene, cryptoxanthin, lutein, fanthophyl, and xanthophyll (Cervantes-Hernández et al., 2019; Fabela-Morón et al., 2020; Rezaul Karim et al., 2021). Unfortunately, pepper is often attacked by different microbial diseases, bacterial soft rot disease is one of the most destructive diseases affecting pepper in the world (Zhao et al., 2013). *Pectobacterium* spp. are implicated in this disease (El-Hendawy et al., 2002; Hua et al., 2020; Li et al., 2023). They invade plant cell, and produce pectic enzymes which macerate plant cell walls (El-Hendawy et al., 2002) causing symptoms of soft rot, starting as water-soaked area on pepper fruits, then the tissues become mushy and finally completely macerated (Gillis et al., 2017; Djami-Tchatchou et al., 2019). The pathogen can invade different vegetable crops both pre- and post-harvest, causing economic losses. To control bacterial soft rot disease, applications of chemicals such as copper-based compounds and antibiotics are

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mainly used. However, long-term application of these chemicals is not favored due to their harmful side effect, non-persistence, high expense, and development of resistant species among the bacterial populations which increase the difficulty of disease prevention and control (Vanneste, 2000; Huang et al., 2021; Ouf et al., 2023). Therefore, biocontrol of vegetables diseases' using antagonistic microorganisms has been considered as an effective method (Hallmann et al., 2009). *R. aquatilis* is considered to have great potential as an antagonist to many bacterial pathogens (Laux et al., 2002; El-Hendawy et al., 2003; Chen et al., 2007, 2009; Li et al., 2020a). Also, plant growth promotion by *R. aquatilis* has been reported (Yuan et al., 2020; Li et al., 2021; Kong et al., 2022; Landa-Acuña et al., 2023). *Rahnella aquatilis* strains 17 and 55 has been previously reported as potential biological control agents of bacterial spots of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* (El-Hendawy et al., 2005).

This study was carried out to screen *R. aquatilis* strains 17 and 55 for their ability to inhibit the growth of nine soft rotting *Pectobacterium* and *Dickeya* spp. isolated from rotted pepper fruits, cabbage leaves and carrot root. Also, evaluating the role of the most potent strain in protecting pepper seedlings from soft rot disease caused by *P. carotovorum* was achieved.

Materials and Methods

Bacterial strains

The antagonistic *R. aquatilis* strains 17 and 55 are used in this study, these strains were isolated from cultivated soil located at Giza Governorate, Egypt and identified in a previous study (El-Hendawy et al., 2003). Also, nine soft rotting bacterial strains isolated from different vegetables were used, their names and sources are listed in Table 1.

In vitro antibiosis

The antagonistic activities of *R. aquatilis* strains 17 and 55 were tested against the soft rotting bacterial strains using the semi-solid overlay method according to Ishimaru et al. (1988). A loop full of 24h old bacterial culture of strain 17 or 55 grown on tryptic soy agar (TSA) Petri plates was inoculated into 50 ml sterile tryptic soy (TS) broth and incubated at 37°C±2 in a shaking incubator at 150 rpm (Stuart® SI500, Cole-Parmer, Staffordshire, UK). Then, 50µL of either *R. aquatilis* strains suspension containing $2x10^5$ colony forming unit/mL (CFU/mL), was spotted at the center of nutrient agar (NA) plates, which were incubated at 37ºC±2 for 24h. Plates were then overlaid with 7mL warm semi-solid NA consists of agar (7g/L) seeded with 25µL of soft rotting bacterial strain suspension containing $16x10^8$ CFU/mL, prepared from an overnight shaken culture incubated at 28°C±2 and 150rpm. Plates were then incubated at 28°C±2 for 24h. The antibiosis was confirmed by the formation of clear zones around the antagonistic bacterial strains.

Transmission electron microscope (TEM) imaging

Based on the results obtained from the *in vitro* antibiosis, *P. carotovorum* strain Pep3B treated with *R. aquatilis* strain 17 as well as untreated were subjected to examination by TEM to study the effect of the antagonist on the pathogen according to Lemos et al. (1985). *P. carotovorum* Pep3B was collected from the margin of the inhibition zone in 1.5mL Eppendorf tube containing 2% glutaraldehyde in 1× phosphate buffer solution (1× PBS, pH 7.4). Pep3B cells that were grown normally on a surface of (NA) plates were collected and treated similarly to be served as a control sample. Both treated and untreated samples were transferred to be processed and examined by transmission electron microscope JEOL (JEM-1400 TEM, Jeol Ltd., Tokyo, Japan) in the TEM laboratory at Cairo University Research Park-Faculty of Agriculture (CURP), Egypt.

Biological control of the bacterial pathogen

This experiment was conducted to know whether the antibacterial activity observed *in vitro* could be reproduced in pepper seedlings and, therefore, be responsible for the control of bacterial pathogen. Based on the results obtained from the *in vitro* antibiosis, *P. carotovorum* Pep3B and *R. aquatilis* 17 were used in this experiment.

Pepper seedlings

Two weeks old seedlings of pepper seedlings (*Capsicum annuum*) were purchased from a local nursery located in 6th of October city, Giza, Egypt, and immediately transferred into plastic pots, each of 15cm diameter and containing 0.5kg of loam soil (1 sand:1 clay{v:v}). Three seedlings were planted in each pot at equal distances and watered as required to keep soil moist but not wet, all pots were placed on a bench at room temperature. Inoculation of seedlings was carried out one week after transplanting.

TABLE 1. The name, symbol, and sources of the soft rotting bacterial strains used in this study

Bacterial strains	Strain code	Isolated from	References
D. chrysanthemi	Car2B	Carrot root	(El-Hendawy et al., 2002)
	Car1B	Carrot root	
P. carotovorum	Pep2A	Pepper fruit	
	Pep3A	Pepper fruit	
	Pep3B	Pepper fruit	
	Cab21B	Cabbage leaves	(El-Hendawy et al., 2006)
	Cab21B2	Cabbage leaves	
	Cab30C	Cabbage leaves	
	Cab45B	Cabbage leaves	

Inoculum preparation

P. carotovorum Pep3B and *R. aquatilis* 17 inoculums were prepared from overnight shaken cultures incubated at 28°C±2 and 37°C±2, respectively. Bacterial cultures were centrifuged at 10,000rpm for 10min at 4°C. Then, the pellet was washed twice in sterile distilled water, cells were suspended in sterile distilled water (SDW) and adjusted to $2x10^5$ CFU/mL and $16x10^8$ CFU/mL for *R. aquatilis* 17 and *P. carotovorum* Pep3B, respectively. The inoculum size of both the antagonistic and the pathogenic bacteria was based on the antibiosis experiment.

Foliar treatment of pepper seedlings

This experiment was carried out as described by El-Hendawy et al. (2005). Three weeks old pepper seedlings were grouped into four sets, each containing 21 identical seedlings. Three leaves per seedling were inoculated with: $2x10^5$ CFU/mL of *R. aquatilis* 17 only (RF), $16x10^8$ CFU/mL of the pathogen *P. carotovorum* Pep3B only (PF), $2x10^5$ CFU/mL of *R. aquatilis* 17 then *P. carotovorum* Pep3B was inoculated 24h later with $16x10^8$ CFU/mL (RPF), and a negative control group (SWF) inoculated with sterile distilled water. The inoculation process was done by injecting a 1mL sterile disposable syringe with a 25 G needle through the leaves' midribs and stems.

Root treatment of pepper seedlings

Root treatment was carried out according to Sivamani & Gnanamanickam (1988), three weeks old pepper seedlings were lifted off the soil without injuring the roots, the soil particles were removed with running tap water and 1cm of root tips were excised by using a clean sterile scissors, the seedlings were grouped into four groups exactly as the foliar treatment. Except that the bacterial application in each group was applied by dipping the roots for 1h in 30mL of the corresponding bacterial suspension/sterile distilled water: $2x10^5$ CFU/mL of *R. aquatilis* 17 only (RR), $16x10^8$ CFU/mL of the pathogen *P. carotovorum* Pep3B only (PR), $2x10^5$ CFU/mL of *R. aquatilis* 17 for 1h then *P. carotovorum* Pep3B for 1h with $16x10^8$ CFU/mL (RPR), and a negative control group (SWR) inoculated with sterile distilled water. Seedlings were replanted in pots with the same amounts of soil, each pot contained three seedlings. All pots were kept at room temperature for over 3 weeks and watered as required. In all treatments, the development of disease symptoms was observed daily and recorded, then the percentage of infection was calculated 48h after treatment.

Effect of R. aquatilis 17 and P. carotovorum Pep3B on the growth and physiological attributes of pepper seedlings

The vegetative growth parameters

The pepper seedlings height and fresh weight for various treatments were determined for 36 days old seedlings after they were removed from the soil. The seedlings were washed with running tap water and drained with tissue paper. After 72h of air drying at room temperature, the dry weight of the seedlings was measured.

Determination of chlorophyll content

Total chlorophyll was extracted according to Metzner et al. (1965) and the chlorophyll content whether chlorophyll a, b or carotenoids were determined using the following equations:

Chlorophyll *a* = 10. 3 E_{664} - 0.918 E_{645}

Chlorophyll *b* = 19. 7 E_{645} - 3.87 E_{664}

Carotenoids = 4. 3 E_{452} (0. 0265 Chl. *a* + 0. 426 Chl. *b*)

where, E is the absorbance at specific wavelength. Then, the fractions were calculated as mg/g fresh weight.

Determination of total phenolics and total flavonoids

Total phenolic contents were determined according to Kujala et al. (2000) using a calibration curve of gallic acid standard solutions and expressed as mg gallic acid equivalent (GA) per gram of dry weight of extract (mg GA/g dry weight of extract). The total flavonoids were determined using an aluminum chloride colorimetric assay according to Chantiratikul et al. (2009). The total flavonoid content was expressed in mg of quercetin (Q) equivalents per gram of dry weight of extract (mg Q/g dry weight of extract) using a standard curve of quercetin.

Determination of proline concentration and catalase enzyme activity

Free proline was determined as described by Bates et al. (1973). Proline concentration was determined from a standard curve and calculated as mg proline/g dry weight.

The activity of the catalase enzyme in all treatments was determined using the method of Goth (1991), and the activity was expressed as µM H_2O_2 destroyed/g fresh weight/ sec.

Protein quantification and extraction, SDS-PAGE analysis

The protein extraction was performed according to Laemmli (1970) , briefly. Half gram of fresh leaf samples from each treatment was homogenized in 500µL of Laemmli extraction buffer containing [4% SDS, 10% β-Mercaptoethanol, 20% glycerol, 125mM Tris-HCl (pH 6.8), and 5mM EDTA (pH 8)] then centrifuged for 10min at 10,000rpm, 25°C. The liquid phase (crude extract) was transferred to new Eppendorf and kept at -20°C till use. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% SDS-polyacrylamide separation gel in a BioRad apparatus (BioRad- TV 400) vertical electrophoresis apparatus, following the protocol of Laemmli (1970). Pre-stained BLUelf protein marker (Genedirex) was loaded as the molecular size standard. The gel was stained using Coomassie brilliant blue G-250 then de-stained in (methanol: distilled water: acetic acid, 5:4:1) solution till clear background was obtained. The de-stained gel was photo-documented using transilluminator (Vilber Lourmat-Germany). After protein extraction, the total protein concentration in the pepper seedlings of different sets were determined according to the method of Bradford (1976). The total protein content was expressed in µg bovine serum albumin (BSA) equivalent per microliter of protein extract (µg BSA/µL protein extraction) using a standard curve of BSA.

Statistical analysis

The data were subjected to one way analysis of variance (ANOVA) using 'IBM SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, N.Y., USA). The data was represented as an average of the replica. The significance of the data was measured at ($P \leq 0.05$) using Duncan's post hoc test. Skewness and Kurtosis tests were performed to indicate the symmetry of the distribution of the dataset.

Results

In vitro antibiosis

The antagonistic activity of *R. aquatilis* strains 17, and 55 were screened against seven *P. carotovorum* strains and two *D. chrysanthemi* strains using the semi-solid over-lay method. The results showed that both *R. aquatilis* strains 17 and 55 were able to restrict the growth of the

nine soft rotting bacterial strains (Figs. 1, 2). The statistical analysis showed that the data for strain 17 were significant and normally distributed in comparison to strain 55. According to George & Mallery (2010), the skewness or kurtosis value was (<±1.2) for the *R. aquatilis* strain 17 indicating normal distribution of its results. The least coefficient of variation (CV%) for soft rotting causing pathogen isolated from pepper fruit was 16.2% for strain Pep3B that showed an inhibition zone of 30.5±2.5mm when antagonist with *R. aquatilis* 17 (Table 2). Therefore, both strains; *R. aquatilis* 17 and Pep3B were selected for the whole study.

TEM imaging of P. carotovorum Pep3B treated with R. aquatilis 17

Transmission electron microscopy (TEM) analysis of the *P. carotovorum* Pep3B cells treated and untreated with *R. aquatilis* 17 revealed that the untreated cells had intact cell envelops and a typical electron-dense cytoplasm (Fig. 3A). On the other hand, the cells treated with *R. aquatilis* 17 showed damaged cells with convoluted cell envelops (Fig. 3B) and a general disorganization of the cytoplasm that became less electron-dense (Fig. 3C). Disintegration of the plasma membrane was obvious and partial vesiculation of the membrane fragments into small vesicles was observed (Fig. 3D). The lysis of the bacterial cell and leakage of the cytoplasmic contents were also observed (Fig. 3D).

Biological control experiment

Based on the results of *in vitro* antibiosis, *R. aquatilis* 17 and *P. carotovorum* Pep3B, were used in this experiment. Foliar and root treatments of pepper seedlings were carried out with the bacterial inoculum to study the role of *R. aquatilis* 17 in reducing or preventing the deleterious effect of *P. carotovorum* Pep3B on pepper seedlings.

Effect of P. carotovorum on pepper seedlings in presence or absence of R. aquatilis

Differences in the infection percentage of pepper seedlings in different treatments were noticed, for example, 88.9% of seedlings inoculated with *P. carotovorum* Pep3B alone by foliar inoculation (PF) showed disease symptoms. This infection percentage was reduced to 55.6% in seedlings inoculated with both *R. aquatilis* 17 and *P. carotovorum* Pep3B by foliar inoculation (RPF), (Table 3). In contrast, in case of seedlings treated through roots no disease symptoms were detected in all inoculations (Table 3). However, significant reduction in the length of seedlings inoculated with *P. carotovorum* Pep3B either by foliar or root inoculation (PF and PR) was detected in comparison with seedlings inoculated with *R. aquatilis* 17 individually either by foliar or root inoculation (RF and RR) or in combination with *P. carotovorum* Pep3B (RPF and RPR), respectively (Table 3).

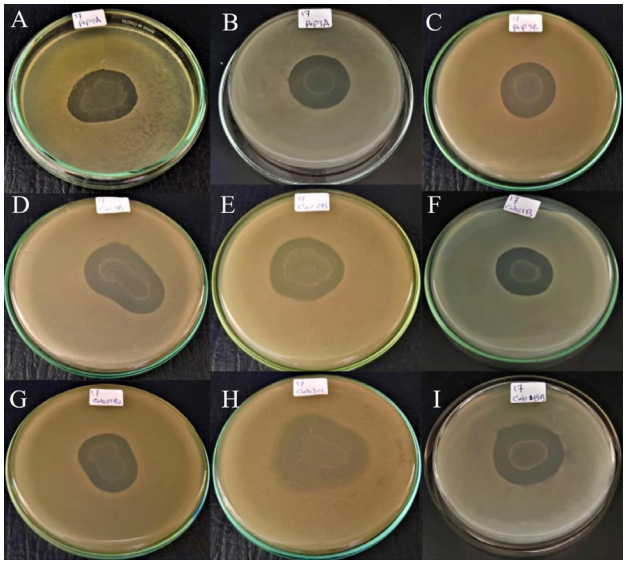

Fig. 1. Antagonistic activity of *R. aquatilis* 17 against nine soft rotting bacterial strains [Photographs from A to I include *R. aquatilis* 17 against *P. carotovorum* Pep2A (A), *P. carotovorum* Pep3A (B), *P. carotovorum* Pep3B (C), *D. chrysanthemi* Car1B (D), *D. chrysanthemi* Car2B (E), *P. carotovorum* Cab21B (F), *P. carotovorum* Cab21B2 (G), *P. carotovorum* Cab30C (H), and *P. carotovorum* Cab45B (I), respectively]

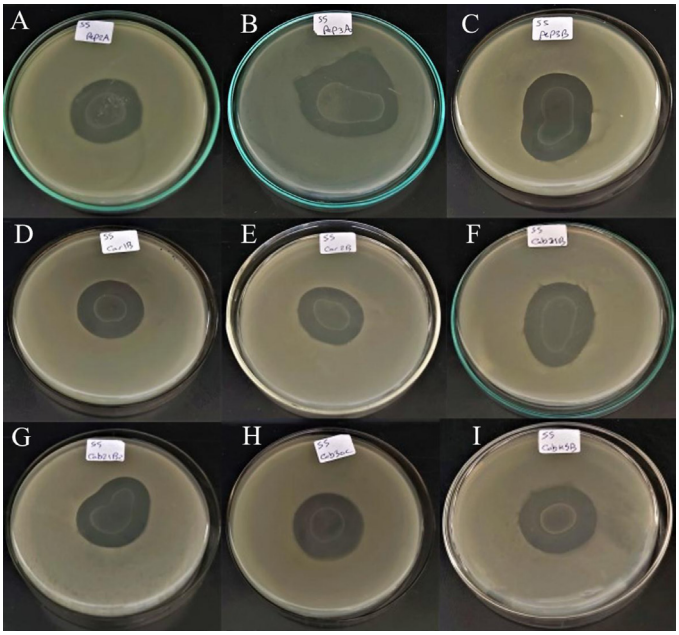

Fig. 2. Antagonistic activity of *R. aquatilis* 55 against nine soft rotting bacterial strains [Photographs from A to I include *R. aquatilis* 55 against *P. carotovorum* Pep2A (A), *P. carotovorum* Pep3A (B), *P. carotovorum* Pep3B (C), *D. chrysanthemi* Car1B (D), *D. chrysanthemi* Car2B (E), *P. carotovorum* Cab21B (F), *P. carotovorum* Cab21B2 (G), *P. carotovorum* Cab30C (H), and *P. carotovorum* Cab45B (I), respectively]

TABLE 2. Antagonistic activity of *R. aquatilis* 17, and 55 against soft rotting bacterial strains

Soft rotting bacterial strains	Inhibition zone diameter (mm)		
	***R. aquatilis* 17**	***R. aquatilis* 55**	**Coefficient of variation (CV%)**
D. chrysanthemi Car2B	33±6	30.5±1.5	18.9%
D. chrysanthemi Car1B	35±8	31±0	32.3%
P. carotovorum Cab30C	47.5±2.5	32±0	7.4%
P. carotovorum Cab45B	36.5±3.5	32±0	11.8%
P. carotovorum Cab21B	33±1	36.5±5.5	17.1%
P. carotovorum Cab21B2	30.5±3.5	34±3	3.8%
P. carotovorum Pep3B	30.5±2.5	36±7	16.2%
P. carotovorum Pep2A	33.5±7	35±1	25.7%
P. carotovorum Pep3A	31±0	39±10	36.3%
	Statistical analysis		
Mean	34.44	34.00	
Std. Deviation	6.91	5.49	
Skewness	0.778	1.643	
Kurtosis	-0.149	2.225	

The activity was quantified by measuring the inhibition zone formed in (mm).

Also, a significant reduction was detected in fresh and dry weights of pepper seedlings inoculated with the pathogen only (Pep3B) either through foliar or root inoculation (PF and PR treatment). The reduction reached to 43.47, and 10.6% for fresh weight, 18.18, and 2.36% for dry weight, respectively. However, slight improvement of fresh and dry weights of seedlings inoculated with *R. aquatilis* 17 was observed. Inoculation of *R. aquatilis* 17 individually or combined with *P. carotovorum* Pep3B alleviated the reduction in both fresh and dry weights in both treatments (Table 3).

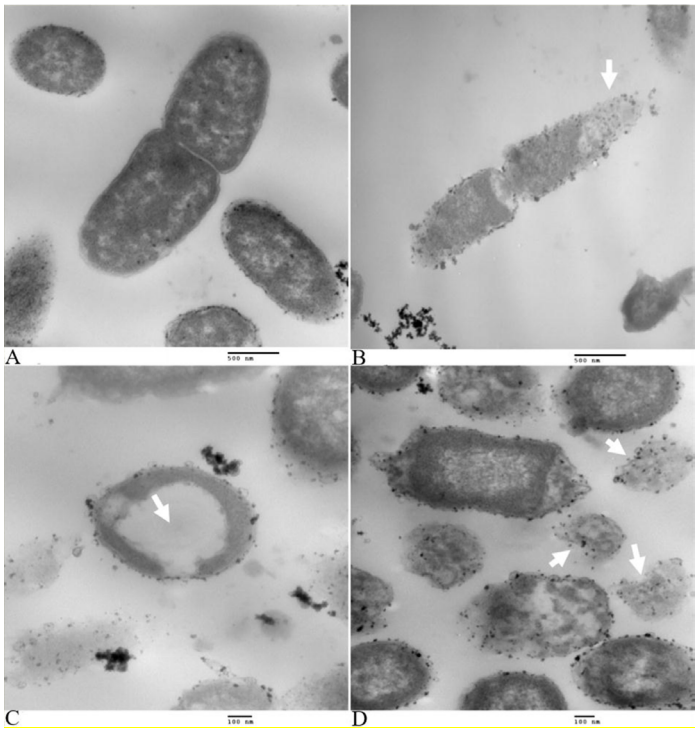

Fig. 3. Transmission electron microscopy images [*P. carotovorum* Pep3B untreated (A) and treated with *R. aquatilis* 17 (B, C, and D). White arrow in (B) represent damaged cells with convoluted cell envelops, white arrow in (C) represent a general disorganization of the cytoplasm that became less electron-dense, white arrows in (D) represent disintegration of the plasma membrane and partial vesiculation of the membrane]

TABLE 3. Effect of *R. aquatilis* 17 on pepper seedlings treated or untreated with *P. carotovorum* Pep3B

Inoculation method		Weight (wt.) expressed as g/seedling				Height expressed as cm/ seedling		% of infection
		Fresh wt.	%*	Dry wt.	%*	Plant height	%*	%
Foliar	RF	2.534±0.77^{ab}	6.74 | 88.8	0.392±0.11^{a}	27.27 | 55.55	25.75±0.35^{c}	-6.36 | 10.75	0
	RPF	1.901±0.52^{bc}	-19.92 | 41.65	0.304±0.08^{ab}	-1.3 | 20.63	26±0.45^{c}	-5.45 | 11.83	55.6
	PF	1.342± 0.42^{c}	-43.47| 0	0.252±0.08^{b}	-18.18 | 0	23.25±0.15^{d}	-15.45 | 0	88.9
	SWF	2.374±0.63^{ab}	0 | 76.9	0.308±0.11^{ab}	0 | 22.22	27.5±0.55^{b}	0 | 18.27	0
Root	RR	2.789±0.81^{a}	7.93 | 20.74	0.357±0.11^{a}	5.3 | 7.85	29.5±0.25^{a}	37.21 | 55.26	ND
	RPR	2.867±0.60^{a}	10.95 | 24.11	0.392±0.07^{a}	15.63 | 18.42	27.5±0.45^{b}	27.9 | 44.74	ND
	PR	2.310±0.54^{ab}	-10.6 | 0	0.331±0.08^{ab}	-2.36 | 0	19±0.2^{f}	-11.63 | 0	ND
	SWR	2.584±0.80^{a}	0 | 11.86	0.339±0.11^{ab}	0 | 2.42	21.5±0.3^{e}	0 | 13.16	ND

*Percent of reduction in treatment (-) or increase (+) over control (SW)/P. The data represent the effect on seedlings fresh, dry weights and heights in comparison to uninoculated plants (SW); ND, not detected. Data represents the means of 21 independent replicas ± SD.

Physiological evaluation

Chlorophyll content: In general, there was an obvious difference between the chlorophyll content for bacterial application through foliar and root inoculation. In foliar inoculation, a significant reduction in the chl a, chl b and carotenoids observed for all treatments in comparison to the control. However, inoculation of *R. aquatilis* 17 alleviated the reduction in chlorophyll content caused for Pep3B inoculated seedlings, and this was significant for chl a reached up to 0.103mg/g fresh wt for RPF in comparison to 0.095mg/g fresh wt for PF treatment and 0.151mg/g fresh wt for the control.

In root inoculation, significant reduction of chl a, chl b and carotenoids in pepper seedlings inoculated with Pep3B only PR was observed of 0.062, 0.037, and 0.023mg/g fresh wt for chl a, chl b, and carotenoids, respectively in comparison

with the chlorophyll content of other seedlings. In contrast, seedlings inoculated with *R. aquatilis* 17 (RR) showed a highly significant increase in the chlorophyll content with readings 0.284, 0.125, and 0.330mg/g fresh wt for chl a, chl b, and carotenoids, respectively. Inoculation of *R. aquatilis* 17 had significantly alleviating the reduction in chl contents that was observed in Pep3B inoculated seedlings (Table 4).

Total phenolics and total flavonoids: Total phenolics and flavonoids were measured in pepper seedlings from both experimental inoculation groups (foliar and root). In general, the concentration of total phenolics and flavonoids increased substantially in response to inoculation with *R. aquatilis* 17 and/or *P. carotovorum* Pep3B, regardless of whether the inoculation was performed via root or shoot. The highest values for both phenolics and flavonoids were recorded for seedlings inoculated with both bacterial strains; *R. aquatilis* 17 and *P. carotovorum* Pep3B reached up to 12.17, and 21.11 in comparison to 9.35, and 16.51mg/g of the controls, respectively in case of foliar application. While reached to 11.09 and 18.66 in comparison to 9.78 and 15.48mg/g of the controls, respectively in case of root inoculation.

Proline content and catalase enzyme activity: Differences in proline content were detected between seedlings of foliar and root treatments. In foliar inoculated seedlings, an overall significant increase was detected for seedlings inoculated by *R. aquatilis* 17 and/or *P. carotovorum* Pep3B although, was more pronounced for *R. aquatilis* 17 inoculated seedlings reached up to 8.03 in comparison to 3.76mg/g for the control (SWF). In root inoculation, proline content was significantly increased only in the seedlings inoculated with Pep3B reached up to 8.42mg/g in comparison to control of 7.73mg/g and a significant reduction in the other treatments (Table 4).

Similar differences in catalase enzyme activity were detected in foliar inoculation, catalase enzyme activity of pepper seedlings was significantly increased for *P. carotovorum* Pep3B inoculated seedlings whether alone or in combination with *R. aquatilis* 17. In contrast, in root treatment, only a significant increase in catalase activity was observed for *R. aquatilis* 17 alone reached to 0.212 in comparison to 0.176µM/g/sec for the control and non-significant differences were observed for other treatments.

TABLE 4. Evaluation of physiological parameters of pepper seedlings with different treatments

Treatment		Physiological parameters							
		Chl a	Chl b	Carotenoids	Protein concentration µg BSA/µL protein extraction	Phenolics mg GA/g dry wt	Flavonoids mg Q/g dry wt	Proline mg proline/g dry wt	Catalase activity µM H_2O_2 destroyed/g fresh wt/sec.
		mg/g fresh wt							
Foliar	RF	0.084±0.058^{e}	0.057±0.021^{d}	0.059±0.019^{e}	10.79±0.69^{b}	11.01±0.54^{b}	18.69±1.5b^{c}	8.03±0.24^{b}	0.125±0.012^{c}
	RPF	0.103±0.072^{c}	0.063±0.054^{c}	0.080±0.021^{c}	7.41±0.49^{cd}	12.17±0.48^{a}	21.11±0.37^{a}	7.36±0.052^{c}	0.280±0.058^{a}
	PF	0.095±0.053^{d}	0.062±0.092^{c}	0.079±0.053^{c}	6.89±2.26^{d}	10.48±0.28^{bc}	17.42±0.25^{cd}	5.65±0.072^{g}	0.218±0.042^{ab}
	SWF	0.151±0.062^{b}	0.075±0.011^{b}	0.128±0.082^{b}	8.12±1.63^{cd}	9.35±0.29^{d}	16.51±0.29^{de}	3.76±0.52^{f}	0.196±0.086^{bc}
Root	RR	0.284±0.047^{a}	0.125±0.019^{a}	0.330±0.036^{a}	18.17±1.39^{a}	9.13±0.38^{d}	15.78±0.39^{e}	6.47±0.17^{d}	0.212±0.041^{ab}
	RPR	0.106±0.039^{c}	0.060±0.032^{cd}	0.075±0.071^{d}	9.55±0.44b^{c}	11.09±0.66^{b}	18.66±1.07^{bc}	5.8±0.23^{e}	0.166±0.026^{bc}
	PR	0.062±0.055^{g}	0.037±0.056^{f}	0.023±0.014^{g}	11.46±2.16^{b}	11.27±0.76^{b}	19.97±1.18^{b}	8.42±0.72^{a}	0.196±0.015^{bc}
	SWR	0.072±0.049^{f}	0.043±0.079^{e}	0.029±0.032^{f}	11.36±0.39^{b}	9.78±0.075^{cd}	15.48±0.25^{e}	7.73±0.18^{bc}	0.176±0.024^{bc}

The data represents the average of three independent replicas ± SD. Means followed by different letters are significantly different at $P \leq 0.05$ level, Duncan's post hoc test.

Protein concentrations and SDS-PAGE electrophoretic pattern: The SDS-PAGE analysis of the protein extracted from the 21 days old pepper seedlings treated by foliar or root application of *R. aquatilis* 17 and/or *P. carotovorum* Pep3B in comparison to non-treated plants (control) revealed no variation in polypeptide banding pattern. A maximum number of 16 polypeptide bands were observed ranged from 175kDa to 8kDa. The SDS-PAGE profile revealed three major regions (Fig. 4).

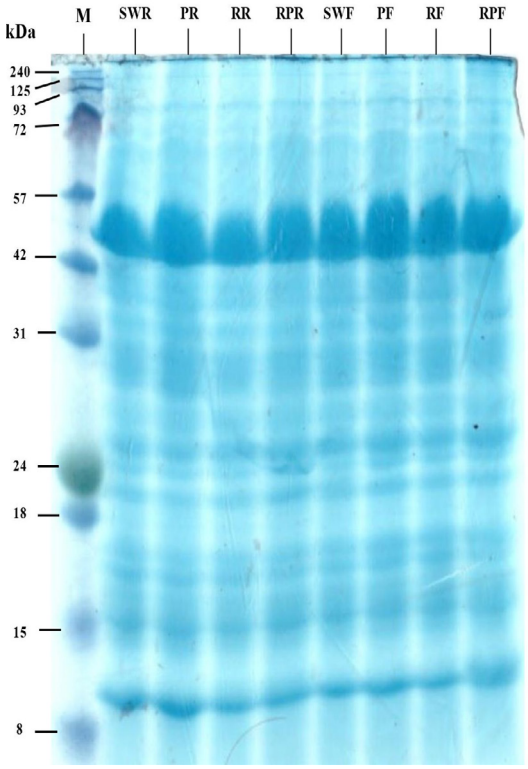

Fig. 4. Protein gel electrophoretic pattern showing banding profile of protein extracted from pepper seedlings [The label M is referred to Bluelf ladder marker, labels SWR, PR, RR, and RPR are referred to seedlings inoculated with, sterile distilled water, *P. carotovorum* Pep3B individually, *R. aquatilis* 17 individually, both *R. aquatilis* 17 and *P. carotovorum* Pep3B, respectively in root treatment, and labels SWF, PF, RF, and RPF are referred to seedlings inoculated with, sterile distilled water, *P. carotovorum* Pep3B individual, *R. aquatilis* 17 individual, both *R. aquatilis* 17 and *P. carotovorum* Pep3B, respectively in foliar treatment]

Different protein concentrations of pepper seedlings were obtained in the different inoculations' methods (foliar and root). The results showed that the protein concentration of the seedlings pre-inoculated with *R. aquatilis* 17 only in both experiments (RF and RR) were highly significant and the concentrations were 10.79 and 18.17µg BSA/µL, respectively comparing with the uninoculated pepper seedlings (SWF and SWR) where the concentrations were 8.12 and 11.36µg BSA/µL, respectively. In foliar application method, the least protein concentration (6.88µg BSA/µL) was recorded for pepper seedlings pre-inoculated with *P. carotovorum* Pep3B only (PF). However, in root inoculation, the least protein concentration (9.55µg BSA/µL) was recorded in the pepper seedlings pretreated with *R. aquatilis* 17 firstly and then treated with *P. carotovorum* Pep3B (RPR) as shown in Table 4.

Discussion

The bacterial disease known as soft rot affects fruits, vegetables, and ornamental plants. It is caused by numerous species of *Pectobacterium* and *Dickeya* bacteria. Rapid development of disease symptoms for complete maceration of plant tissue is possible. Under warm and humid conditions, the disease may rapidly spread and cause significant economic losses for the crop. Biological control utilizing beneficial microorganisms is a promising strategy for managing soft rot disease as opposed to the application of toxic bactericides. Therefore, in the current study, the antagonistic soil bacteria *R. aquatilis* 17 and 55 were screened for their antagonistic activity against 9 soft rotting bacterial strains of different species. The obtained results revealed that *R. aquatilis* 17 and 55, were found to be efficient, on nutrient agar plates, against nine soft rotting *Pectobacterium* and *Dickeya* strains of different species. In a previous study *R. aquatilis* 17 and 55 inhibited the growth of different strains of *Bacillus cereus*, *B. subtilis*, *Erwinia carotovora*, *Escherichia coli*, *Pseudomonas syringae*, *Serratia marcescens* and *Xanthomonas campestris*, on solid medium (El-Hendawy et al., 2003), which indicate that *R. aquatilis* 17 and 55 are able to antagonize a wide range of bacterial species including plant pathogenic bacteria.

Other studies reported the antagonistic activity of *R. aquatilis* strains against different plant pathogenic bacteria such as: *Erwinia amylovora, Agrobacterium vitis, Clavibacter* spp,

Pectobacterium spp*, Dickeya* spp*., Pseudomonas* spp*, and Xanthomonas* spp (Laux et al., 2002; Chen et al., 2007, 2009; Li et al., 2020b).

R. aquatilis inhibited *P. carotovorum* Pep3B growth indicating that it released antibacterial compounds which appeared to damage and disintegrate the cell envelops specially plasma membrane leading to leakage of the cytoplasmic contents as shown by TEM. Similar results were obtained from *Dickeya dadantii* treated with the cell free culture supernatant of *Paenibacillus polymyxa* ShX301 (Hossain et al., 2023). However, the inhibition of bacterial growth by *R. aquatilis*, could be attributed to: competition for sucrose, which was suggested as one mechanism of the strain Ra39 against Ea7/74 (Laux et al., 2002), siderophores production (El-Hendawy et al., 2003; Jafra et al., 2009) which are useful as biological control, production of pyrroloquinoline quinone (PQQ) or glucose dehydrogenase (GDH) (Guo et al., 2009), production of antibacterial compound that inhibits RNA and protein synthesis (Chen et al., 2009). Also, Tao et al. (2021) showed that *R. aquatilis* strain L103, which was isolated from the soil near the mycelia of mushrooms, produced antibacterial protein with a wide antibacterial spectrum against both Gram-positive and Gram-negative bacteria as well as a substantial antioxidant capacity. However, future work is planned to investigate the mechanisms of bacterial inhibition by *R. aquatilis* 17 and 55. Results of the biological control experiment indicated that seedlings inoculated with the pathogen (Pep3B) only showed a reduction in the evaluated vegetative parameters. *R. aquatilis* strains were found to be capable of protecting pepper seedlings against *P. carotovorum* infection. This was demonstrated by the reduced percentage of infection in seedlings pre inoculated through leaves with *R. aquatilis* strain compared to control seedlings inoculated with *P. carotovorum*. However, reduction of infection by plant pathogenic bacteria as a result of pretreatment with antagonistic bacteria has been observed in some diseases. For example, *R. aquatilis* strains reduced bacterial spots of tomato caused by *Xanthomonas campestris* (El-Hendawy et al., 2005). Another study reported that pretreatment of melon cotyledons with viable *Pseudomomas fluorescens* cells reduced soft rot disease symptoms caused by *Erwinia carotovora* subspecies *carotovora* up to 50% (El-Hendawy et al., 1998). However, excision of root tips might enhance pepper seedling resistance, this could explain the absence of infection when the pathogen was inoculated into pepper seedlings whose roots were excised before transplanting compared to seedlings from foliar treatments and this could be attributed to the direct inhibition of the pathogen by the antagonist as they come in contact in the same leaf. Some differences in the seedling length between the different sets were visible and the results recorded a remarkable shortening in the length of seedlings inoculated with *P. carotovorum* Pep3B (PF and PR) relative to the controls seedlings length (SWF and SWR) in both inoculation methods (foliar and root), as well as a significant reduction in fresh and dry weights of pepper seedlings was observed. Reduction in fresh and dry weight of tomato leaves as a result of inoculation with *Xanthomonas campestris* pv. *Vesicatoria* has been reported (El-Hendawy et al., 2005). Reduced photosynthesis in the advanced stages of some diseases would lead to decreased growth, which ultimately lead to a loss in the plant's fresh and dry weight (Agrios, 2005). On the other hand, fresh and dry weights of pepper seedlings inoculated with *R. aquatilis* 17 only and with both *R. aquatilis* 17 and *P. carotovorum* Pep3B showed significant increase in both the foliar and root applications. Previous studies have reported that *R. aquatilis* can act as plant growth-promoting rhizobacteria (PGPR), which can stably colonize the rhizosphere of plants or the soil environment and provide nutrients for plant development, play a significant role in promoting crop growth and enhancing food quality (Yuan et al., 2020). Plant disease suppression and mineral phosphate solubilization by *R. aquatilis* could be attributed to the glucose dehydrogenase cofactor and pyrroloquinoline quinone (PQQ) (Li et al., 2014). Production of siderophores by *R. aquatilis* 17 has been reported by El-Hendawy et al. (2003) which might help in improving the seedlings growth and increasing fresh and dry weights. However, it has been reported that PGPR have a higher affinity for binding iron and can survive at far lower concentrations of iron (Ouf et al., 2023). Kong et al. (2020) reported that when purified siderophores were added to seedlings cultivated hydroponically with weakly soluble iron (III) oxide, the maximum plant height, root length, leaf length, and fresh weight of camphor seedlings were noted. In addition, microbes that produce siderophores have been utilized to protect camphor from iron deficiency and yellowing. The application of *R. aquatilis* JZ-GX1 to treat the

increasingly serious iron deficiency chlorosis in *Cinnamomum camphora* was successful (Kong et al., 2020). The inoculation with JZ-GX1 significantly increased the chlorophyll content of *C. camphora*, which promoted the redistribution of active iron in roots and leaves, increased the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), and thus reduced membrane damage in iron-deficient *C. camphora* caused by reactive oxygen species (Kong et al., 2020). In addition, Li et al. (2021) reported that *R. aquatilis* JZ-GX1 promotes the growth of maize directly by secreting IAA and indirectly by secreting phytase. Furthermore, *R. aquatilis* AZO16M2, was characterized for its phosphate solubilization capacity to improve the establishment and survival of *Musa acuminata* var. *Valery* seedlings under *ex vitro*-acclimation (Landa-Acuña et al., 2023). Additionally, Abdel-Latif et al. (2021) stated that the inoculation of plants with PGPR *Bacillus subtilis* improved the growth of salt-stressed barley plants primarily by preserving the integrity of the cellular membranes, elevating nitrate reductase and glutamine synthetase activities, and providing the cultures with the growth hormone indole-3-acetic acid/indole acetic acid (IAA). Also, the inoculation reduced the generation of ethylene under salt stress by secreting the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which may enhance nutrient uptake and growth.

Results for chlorophyll content showed an obvious difference between the chlorophyll content for bacterial application through foliar and root inoculation. There was a significant reduction of chl a, chl b, and carotenoids in pepper seedlings inoculated with *P. carotovorum* Pep3B only in root in comparison with the chlorophyll content of other seedlings. On the other hand, the chlorophyll content (chl a, chl b, and carotenoids) of the seedlings inoculated with *R. aquatilis* 17 only showed significant increase. The inoculation of *R. aquatilis* 17 to Pep3B inoculated seedlings had mitigate the reduction of the chlorophyll content. This observation agreed with Hafez et al. (2018), as they reported that the highest chlorophyll content was observed in squash plants inoculated with *Bacillus pumilus*, *Trichoderma viridi,* and *Bacillus subtilis* leading to enhanced growth. Root inoculation of *Pisum sativum* with bacterial endophytes isolated from *Ocimum sanctum* Linn mitigated the decrease in chlorophyll content caused by root rot pathogen *Fusarium oxysporum* (Gupta et al., 2022). Sugarcane plants with fungal infections that caused red rot disease had much lower amounts of carotenoids, chl a, and chl b, while plants inoculated with *Bacillus xiamenensis* had higher levels of these compounds, which served to decrease the negative consequences of fungus infection (Amna et al., 2020). The pathogenic infection negatively influences a group of physiological processes such as photosynthesis, stomatal conductance, transpiration rate, and efficient water use. The pathogenic infection inhibits the photosynthetic pathway by interfering with stomatal conductance during transpiration and reducing the activity of mesophyll cells and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) (Mishra et al., 2018). Plants produce a large number of small molecules with antimicrobial action, and at least some of these antimicrobial compounds target the pathogenicity pathways of diseases (Joshi et al., 2015). Polyphenols and phenolic acids are secondary metabolites have important roles in plant defense against pathogens, herbivores, and abiotic stress (Joshi et al., 2015). In the current study, the results revealed that the amount of total phenolics and flavonoids compounds in the seedlings inoculated with *R. aquatilis* 17 and the phytopathogen *P. carotovorum* Pep3B significantly increased in both root and foliar applications in comparison to their controls. *R. aquatilis* inoculation to bean plants caused significant increase in the content of phenolic compounds relative to control bean plants (Sallam, 2011). Accumulation of phenolic compounds at the infection site has also been linked to a restriction of the pathogens growth because these substances are toxic and cause changing in the pH of plant cell cytoplasm. In tobacco and potatoes, the phenol salicylic acid increased resistance to *Dickeya solani* and *Pectobacterium carotovorum subsp. carotovorum* (Joshi et al., 2015). *Pectobacteria* secrete numerous exoenzymes, which work together to break down plant cell walls and induce the symptoms of soft rot, while as the majority of these enzymes belong to the pectin-degrading family. Exoenzymes such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), pectin lyase (Pnl), and proteases (Prt) are secreted by the type II secretion system (T2SS) under the direction of quorum sensing, and together they exert the pathogenicity of bacteria (Su et al., 2022). These enzymes cause the development of disease symptoms such moist lesions, the maceration of plant tissue,

and ultimately the decomposition of plant organs (Khadka et al., 2020). Previous studies observed that phenolic compounds appear to inhibit synthesis of *Pectobacterium* exoenzymes (Joshi et al., 2015). Catalase enzyme activity of pepper seedlings was significantly increased for Pep3B inoculated seedlings whether alone or in combination with *R. aquatilis* 17 in foliar application. These results are in agreement with Amna et al. (2020), who noted that the highest level of antioxidant activities of superoxidase dismutase, peroxidase, and catalase enzymes increased in sugarcane plants inoculated with both *Bacillus xiamenensis* and *Colletotrichum falcatum* in comparison to controls. Furthermore, in response to various stresses (biotic or abiotic), plants store huge amounts of a variety of appropriate solutes. Proline, sucrose, polyols, trehalose, and quaternary ammonium compounds (QACs) are examples of these solutes. These solutes provide protection to plants from stress by regulating cellular osmotic pressure, eliminate ROS, safeguard membrane integrity, and stabilize enzymes and proteins (Hayat et al., 2012). The amino acid proline is greatly beneficial to plants under varied stress conditions. Proline serves as a better osmolyte and also performs three other essential roles under stress, including metal chelation, antioxidant defense, and signaling molecule (Hayat et al., 2012). Differences in proline content were detected between seedlings of foliar and root treatments in this study. In foliar inoculated seedlings, an overall significant increase was detected for seedlings inoculated by *R. aquatilis* 17 and/or *P. carotovorum* Pep3B although, was more pronounced for *R. aquatilis* 17 inoculated seedlings. *Bacillus xiamenensis* and red rot diseased sugarcane plants had greater proline levels than controls. *Bacillus xiamenensis* may increase proline synthesis in plants to help them withstand pathogen infection (Amna et al., 2020). Loutfy et al. (2022) reported that, when there is a drought, proline buildup indicates plant damage brought on by stress. *R. aquatilis* 17 inoculations to pepper by foliar or root application caused a significant increase in total protein content while a significant reduction was associated with *P. carotovorum* inoculation by foliar inoculation. Inoculation of *R. aquatilis* 17 had alleviated the reduction in protein content caused by Pep3B inoculation. Level of protein content in pepper seedlings in root treatment is overall higher than those observed by foliar treatment. This may be a response to wound which introduced to facilitate the bacterial inoculation to root. The wounding may lead to switch on several defense mechanisms for healing wounded tissues and protecting them from the subsequent invasion of pathogens. One of these methods is suberization, which inosculated the cell wall (Woolfson et al., 2022), enhancing the biosynthesis of phenylpropanoids (Perincherry et al., 2021), cell wall constituents crosslinking (Rui & Dinneny, 2020) and the local or systemic induction of a set of proteins involved in defense (Kaur et al., 2022). As final gene products, proteins reflect the expression profile within plants in spatial order. Synthesis and accumulation of pathogen related proteins have been reported to play an important role in plant defense (Manikandan & Raguchander, 2015). No variation in protein profiling pattern of different treatments is being observed. Protein profiling is very unwavering excluding the fluctuation arises from different environmental factors. Suggested no formation of new protein and rather than affecting the level of expression of the already formed proteins which is witnessed by changing protein content in response to different inoculation.

Conclusion

In this study, the antagonistic *R. aquatilis* 17 and 55 inhibited the growth of nine soft rotting strains of *P. carotovorum* and *D. chrysanthemi*. Transmission electron micrographs of *P. carotovorum* cells inhibited *with R. aquatilis* 17 showed deformed cells and disintegrated cell envelopes which might indicate that the antagonist released antibacterial compound(s) caused damage of pathogens cells. Additionally, *R. aquatilis* mitigated the effect of *P. carotovorum* on pepper seedlings and promoted seedlings growth indicating that it has high probability of being an effective biocontrol agent and plant growth promoter.

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Authors' contributions: HE Proposed the research topics, supplied the bacterial strains, designed the experiments. HE, ES and MH contributed to data curation, writing, reviewing, and editing of the manuscript. KA, performed the practical work and contributed to writing of the initial draft of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate: The plant related work was undergoing in accordance with the institutional, national, and international guidelines and legislation.

Availability of data and materials: All data produced in the current study is included within the article

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المكافحة الحيوية لمرض العفن الرخو البكتيري للفلفل المتسبب عن بكتيريا *Pectobacterium carotovorum* باستخدام بكتيريا *Rahnella aquatilis*

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تعد أنواع بكتريا*Pectobacterium* و *Dickeya* من العوامل الرئيسية المسببة لمرض العفن الرخو البكتيري الذي يؤثر سلبًا على الفواكه والخضروات و المحاصيل المختلفة، مما يؤدي إلى خسائر اقتصادية كبيرة. تعتبر المكافحة الحيوية باستخدام الكائنات الحية الدقيقة النافعة بديلاً واعداً لمبيدات البكتريا الخطرة. لذلك، تم تقييم النشاط المضاد لسلالتين بكتيريتين مختلفتين من *Rahnella aquatilis* في المختبر و في الوسط الحيوي ضد تسع سلالات بكتيرية تسبب مرض العفن الرخو. بكتيريا التربة المستخدمة كمكافح حيوي *R. aquatilis* سلالات 17 و 55 قيدت نمو تسع سلالات بكتيرية تسبب مرض العفن الرخو على أطباق أجار، (7 سلالات *Pectobacterium carotovorum* و سلالتان *Dickeya chrysanthemi*). أظهرت الصور المجهرية الإلكترونية النافذة لخلايا *P. carotovorum* Pep3B المضادة لسلالة *R. aquatilis* 17، خلايا تالفة مع غشاء بلازمي مدمر يحرر المحتويات الخلوية. و لدراسة ما إذا كان *R. aquatilis* 17 يصلح للاستخدام كعامل مكافحة حيوية فعال لمرض عفن الفلفل الرخو البكتيري، تم إجراء تطبيقين. تمت معالجة شتلات الفلفل قبل ظهور العامل الممرض بـ*R. aquatilis* 17 من خلال الأوراق والجذور. أظهرت جميع الشتلات المعالجة بالسلالة المضادة *P. carotovorum* pep3B انخفاض الحساسية تجاه ، وزيادة أوزان البادرات الطازجة والجافة وارتفاع طولها مقارنة بالبادرات المعالجة. لقد أثرت معالجة البادرات بواسطة*R. aquatilis* 17 إيجابيا على العوامل الفسيولوجية التي تم دراستها، مثل محتوى الكلوروفيل، والكاروتينات، والفينولات، والفلافونويدات، وتركيز البروتين وكذلك تركيز البرولين. كما بينت النتائج أن *R. aquatilis* 17 قد خففت من تأثير *P. carotovorum* على بادرات الفلفل وعززت من نموها، مما قد يدل على إمكانية استخدام هذه السلالة البكتيرية في تطبيقات للمكافحة الحيوية لتعزيز نمو النبات والتحكم في الأمراض البكتيرية التي تصيبه.