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Effect of some biotic and abiotic factors on soft-rot bacteria (*Pectobacterium carotovorum* subsp. *carotovorum*) on Potato plants

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ABSTRACT

The effectiveness of some biocontrol agents (*Pseudomonas fluorescens* and *Trichoderma viride*) and organic acids (acetic acid and boric acid) against soft rot bacteria *Pectobacterium* sp. were studied under laboratory and field conditions. Two soft rot bacterial isolates *Pectobacterium carotovorum* subsp. *carotovorum* Pcc and *Pectobacterium carotovorum* subsp. *carotovorum* Pcc160 (Acc. No. LN811442) were used in this study. Pcc isolate was isolated from Potato tuber (Burn variety), Pcc and Pcc160 isolates caused soft rot symptoms of potato. PCR was used to identify the Pcc isolate molecularly using universal primers for 16S rRNA. The PCR products' DNA sequence, BLAST analysis, and Genbank data revealed that the Pcc isolate belonged to *Pectobacterium carotovorum* sub sp. *carotovorum*. According to the phylogenetic tree based on the DNA nucleotide sequences of the 16s rRNA gene, the Egyptian isolate resembles the Chinese isolate (OQ727369) closely. *In vitro* acetic acid showed the strongest effect against Pcc and Pcc160 isolates with inhibition zones 41.33 and 33.67 mm and MIC 50 and 100 µg/mL respectively. Scanning electron microscope showed distinct inhibitory effect of acetic acid and *T.viride* on the growth and survival of *P.c.* subsp. *carotovorum* (Pcc). *In vivo* experiment *T. viride* treatment was the most effective, where it caused a reduction of disease severity index (DSI). Acetic acid and *T. viride* were evaluated for their efficacy in inducing defense enzymes, total soluble protein, phenols and salicylic acid (SA) content in potato against *P. c.* subsp. *carotovorum* (Pcc). The activity of defense enzymes [peroxidase (POD) and phenylalanine ammonia lyase (PAL)], total soluble protein content, polyphenols content and salicylic acid (SA) content were found to be increased in *T. viride* treated potato plants in both seasons compared to other treatments. The results of this study demonstrate that *T. viride* showed effective inhibitory activity against Pcc, thus *T. viride* could have potential application in controlling *P. c.* subsp. *carotovorum*.

Keywords: Biocontrol agents, soft rot, *Pectobacterium* sp., Organic acids, 16s rRNA, salicylic acid and defense enzymes.

INTRODUCTION

One of the most significant foods and crops in the world is the potato (*Solanum tuberosum* L.), whose output has grown at a pace of 2.8% each year in emerging nations (Salem and Abd El-Shafea, 2018). One of the most prevalent and destructive soft rots in potatoes is caused by *Pectobacterium carotovorum*, which spreads throughout the plant and its tubers via vascular veins. The bacterium may also enter the plant through natural openings or wounds. The bacteria have the ability to release enzymes that break down the plant cell wall, including protease, cellulase, pectate lyase, polygalacturanase, and pectin-methyl esterase (Naas *et al*., 2018; Azaieza *et al*., 2018; Abdel-Gaied *et al*., 2020; Abd El Khair *et al*., 2021; Hossain *et al.,* 2023). The bacterium can be found in the largest variety of economically significant crops and ornamentals, such as tomato, squash, sweet potatoes, cabbage, carrots, cucumbers, garlic cloves, onion bulbs, pepper, potato tubers, and radish roots (Opara and Asuquo 2016).

Numerous plant reactions are triggered by stress, including changed gene expression, modifications to cellular metabolism, growth rate alterations, changes in crop yields, etc. Abiotic stress and biotic stress are the two main types of stress that affect plants. While biotic stress that crop plants are exposed to comes from biological entities like diseases, insects, etc., abiotic stress that is imposed on plants by their surroundings can be either physical or chemical (Gull *et.al*., 2019; Tekiner *et.al*., 2019). Application of biotic or chemical agents strengthened plant defenses against disease, whereas resistance induction—applying inducers via foliar application or optimized soil management techniques—improved plant health status against disease (Song *et al.,* 2013 and Abdel-Gaied *et al.,* 2020).

Utilizing organic acids and bio-control agents could be crucial as secure substitute methods for managing soft rot infection. *Trichoderma viride, Peudomonas fluorescens, and B. subtilis* combined with chitosans, respectively, showed the strongest antagonistic activity against *E. carotovora subsp. carotovora*. All three treatments were also able to inhibit the development of soft rot until after 20 weeks of storage (El-Khair and Haggag 2007; Abdel-Gaied *et al*., 2020; Osei *et al.,* 2022). *In vitro* experiments demonstrated the bactericidal action of boric acid or acetic acid against *P. carotovorum subsp. carotovorum*. The infection rate and weight loss were both markedly reduced by all the therapies. Compared to acetic acid, boric acid was the most successful in preventing the soft rot disease that affects potatoes stored in storage (Rahman *et al*., 2017).

The objective of this study was to assess the biotic and abiotic stress of two biological agents, namely *Trichoderma viride* and *Pseudomonas fluorescence*, as well as two organic acids, including boric acid and acetic acid, against the causative agent of potato soft rot, *Pectobacterium carotovorum subsp. carotovorum*.

MATERIALS AND METHODS

Bacterial strains:

Two pathogenic *Pectobacterium* isolates were used in this study, *Pectobacterium carotovorum* subsp. *carotovorum* [Pcc160(LN811442)] isolate was provided by the Laboratory of Bacteriology, Faculty of Agriculture, Alexandria University, Egypt (Ashmawy *et al.,* 2019) and Pcc isolate was isolated from naturally infected potato (*Solanum tuberosum*, L.) tubers (Burn variety) exhibiting soft decay signs. During the 2020–2021 growing season, potato tubers were obtained from various places inside the El-Behaira Governorate, Egypt. Glycerol agar medium was used for maintenance of the tested bacterial organisms.

Identification of Pcc isolate using 16S rRNA gene:

Genomic DNA extraction:

The protocol for extracting bacterial genomic DNA was (Yahiaoui-Zaidi *et a*l., 2003).

16S rRNA gene amplification by PCR:

Using the universal primers P0 (F) (5'GAAGAGTTTGATCCTGGCTCAG3') and P6 (R) (5'CTACGGCTACCTTGTGTTACGA3'), the entire 1550 bp 16S rRNA gene was amplified from the isolate of *Pectobacterium sp*. (Pcc). A total of 50 µL was used for the PCR amplification, which included 5 µL of 10 x buffer, 4 µL of 25 mM MgCl2, 4 µL of 2.5 mM dNTPs, 2 µL of 10 pmol forward primer, 2 µL of 10 pmol reverse primer, 2 µL of 50 ng of bacterial genomic DNA, and 0.4 µL (5 units/µL) of Taq DNA polymerase (Promega, Germany) (Sambrook *et al*., 1989). The thermal cycler (Techne, UK) used for the PCR amplification was programmed to run one cycle at 95ºC for five minutes, then 34 cycles with 45 s at 95ºC for denaturation, Two minutes at 72ºC for elongation and one minute at 50ºC for annealing. For the last extension, the reaction mixture was incubated for 10 minutes at 72ºC. The PCR products underwent separation on a 1.5% agarose gel in TBE buffer (Maniatis *et al*., 1982), ethidium bromide staining, and ultraviolet (UV) photography. The amplified products of the 16S rRNA gene were purified using the QIAquick PCR purification kit (Qiagen, Germany).

The 16S rRNA gene's sequencing:

Using a Big Dye terminator cycle sequencing kit, the amplified product (1550 bp) of 16S rRNA was sequenced. The ABIPRISM model 310 automated DNA sequencer at Sigma Scientific Services Company was used to resolve the sequencing products after they had been purified using Centri-Sep spin columns.

Phylogenetic analysis and alignment:

CLUSTALW (1.82) http://www.ebi.ac.uk/clustalw was used to align DNA sequences both pairwise and many times (Thompson *et al*., 1994). Bootstrap neighbor-joining tree using CLUSTALW alignment produced with MEGA version 11 (Tamura *et al.*, 2021). Using BLASTN searches at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov), comparisons with sequences in the GenBank database were made.

Antibacterial susceptibility test of bioagents and organic acids:

Disc diffusion method:

Two organic acids namely, acetic and boric acid produced by Sigma Chemical Company and two bio agents; Biocure-B 1.75% WP (*Pseudomonas flourecenes*) and biocure-T (*Trichoderma viride*) by Trade line corporation company India were tested for their ability to suppress the growth of the isolated *Pectobacterium sp*. *in vitro* and *P. c.* subsp. *carotovorum* (Pcc160) at the concentrations 1.5, 2.5 and 4.0 mg/mL. Testing effect of these compounds on growth of the bacterial isolates was studied by determination of inhibition by agar disc diffusion method according to (NCCLS, 1997). Three replicates were used for each treatment.

Minimum inhibitory concentration (MIC) estimation:

Tested compounds were dissolved in dimethyl sulfoxide (DMSO). Appropriate volumes of the stock solution (50, 100, 200, 250, 300, 500, 1000, 1500, 1750 and 2000µg/mL) recommended by European Society of Clinical Microbiology and infection Disease (ESCMID, 2000).

Scanning electron microscopy (SEM) examination:

Examination of *Pectobacterium* cells treated with acetic acid:

The most effective acetic acid treatment resulted in the greatest reduction of bacterial growth, and this result was chosen for additional investigation by SEM analysis. The *Pectobacterium* sp. (Pcc) culture was cultivated in nutritional broth media. The cell suspension was then incubated at 37 °C for 60 minutes at 120 rpm, with 4 mg/ml concentration of acetic acid. The bacterial cells were then extracted. Tahmasebi *et al.* (2015) reported on the scanning electron microscopy analysis.

Evaluating the efficacy of antagonists against *Pectobacterium* sp:

Trichoderma viride tested against *Pectobacterium* sp. (Pcc isolate) for microscopic visualization of the inhibition, the scanning electron microscopy analysis was according to Tahmasebi *et al.,* (2015).

Field Experiments:

Two field trials were designed to study the efficacy of *Trichoderma viride* as biotic agent and acetic acid as abiotic agent for controlling the soft rot disease caused by *Pectobacterium sp*. in potato (*Solanum tuberosum, L.)* The field tests were carried out at El-Sabahya Research Station in Alexand ria throughout the course of the growing seasons of 2021 and 2022. Potato tubers were planted in September 2021 and the soil texture was sand y clay. The potato variety was Burn (B) that obtained from local market. This cultivar is a promising addition to Egypt's potato crop, having just been introduced. A complete randomized block design with four replications was used. The treatments *T. viride* and acetic acid at their recommended rate and were sprayed on both upper and lower surface of leaves as well as stems were well covered at 6 days before inoculation. Before adding treatment solution materials, the spray tank was completely cleaned. Potato plants inoculated with *Pectobacterium sp. (Pcc)* were served as a positive control, where other ones treated with sterile distilled water were served as a negative control. Streptomycin (Streptomycin sulfate) was used as a bactericide standard. All plots received traditional agricultural practices such as irrigation and fertilization. Potatoes were harvested after 110 days from planting date. The severity of infection was recorded three weeks after inoculation**.**

Disease severity Index (DSI):

Disease severity index (DSI) was recorded by estimating the leaf lesions on a scale from 0 to 4 suggested by DSI = ∑ ((n*c)/N*df) Where DSI. = Disease Intensity, n = Number of infected leaves per category, c = Category number and N = Total number of leaves and df= degree of freedom (Cohen *et al*., 1994).

Total Soluble Phenol Content:

Total soluble phenol content of potato leaves determined according to (Slinkard *et al*., 1997) µg tannic acid /gm fresh weight = (((A/K)*(20/0.2)/1)), Where: A= absorbance at 765 nm, K= the extension coefficient =0.016898 µg/ml. by using a spectrophotometer (Tuner, model 390).

Total protein assay:

Total protein was determined according to method described by Dixon (1985), with slight modification proposed by Lowry et al. (1951). mg protein/g fresh weight = ((A/K)*100)/0.25), K(BSA) protein=0.029 mg/ml, A= absorbance at 700 nm.

Determination of enzymes activities:

Peroxidase (POD) activity:

Peroxides activity (expressed as % Activity), was determined according to Murage and Masuda (1997) with some modification. Measured at 470 nm determined.

PAL activity:

Phenylalanine ammonia-lyase (PAL) was measured at 290 nm according to El-Shora (2002).

Quantification of salicylic acid:

Extraction of free salicylic acid:

Free SA was extracted from potato leaves according to the modified method of Malamy and Klessing (1992).

Determination of salicylic acid by HPLC

Separations by HPLC (agilent technology infinity 1260 (Germany) equipped with an agilent variable wavelength ultraviolet detector) are performed on ZORBAX Eclipse Plus C18 analytical column (250 × 4.6 mm id, 5 µm particle size). Mobile phase Acetonitrile: Methanol (30:70) Injection volume

=25uL, Flow rate = 1 mL/min, Wavelength = 232 nm, column Tem = 30 C° Express SA as ng (SA) per gram of fresh weight of plant as shown by Quentin *et al*. (2016).

Statistical analysis:

All experiments were set up in a Randomized complete block design (RCBD). Data were subjected to analysis of variance (ANOVA), using the Statistical analysis system (SAS Institute Inc**.,** 2004). Means were compared with L.S.D. test at P< .05 levels**.**

RESULTS

Molecular identification of Pcc soft rot bacterial isolate:

Identification through 16s rRNA gene:

Approximately 1550 bp region of 16S rRNA gene was amplified for Pcc bacterial isolate.

Seqnencing of 16s rRNA gene:

A partial sequence for *Pectobacterium* sp. isolate Pcc was acquired when a large (1550 bp) purified portion of the 16s rRNA gene was sequenced using an automated DNA sequence, the ABI PRISM model 310 at Sigma Company. The BLAST search (http://www.ncbi.nlm.nih.gov) revealed that the sequence corresponding to the bacterial isolate Pcc was almost identical to that of *Pectobacterium carotovorum* subsp. *carotovorum*. The homology of the Egyptian *Pectobacterium* isolate to the GenBank strains reached 100%. The sequence was submitted to GenBank with accession number as illustrated in Table 1.

Table 1. Accession number and isolate code of partial 16s rRNA gene of *Pectobacterium carotovorum* subsp. *carotovorum* isolate in the GenBank.

Bacterial isolate	Isolate code	Accession no.
Pectobacterium carotovorum* subsp. *carotovorum	Pcc	OP565055

Alignment and phylogenetic analysis:

Alignment of *Pectobacterium carotovorum* subsp. 16s rRNA sequence. *carotovorum* (Pcc) isolate with the 16s rRNA sequence of other *Pectobacterium* strains collected from the GenBank was carried out using CLUSTAL W (1.82) (http://www2.ebi.ac.uk/clustalw,Thompson et al.,1994) from which MEGA version 11 (Tamura et al., 2021) was used to generate the Bootstrap neighbor-joining tree (Fig. 1). According to the phylogenetic tree based on the DNA nucleotide sequences of the 16s rRNA gene, the Egyptian isolate resembles the Chinese isolate (OQ727369) closely.

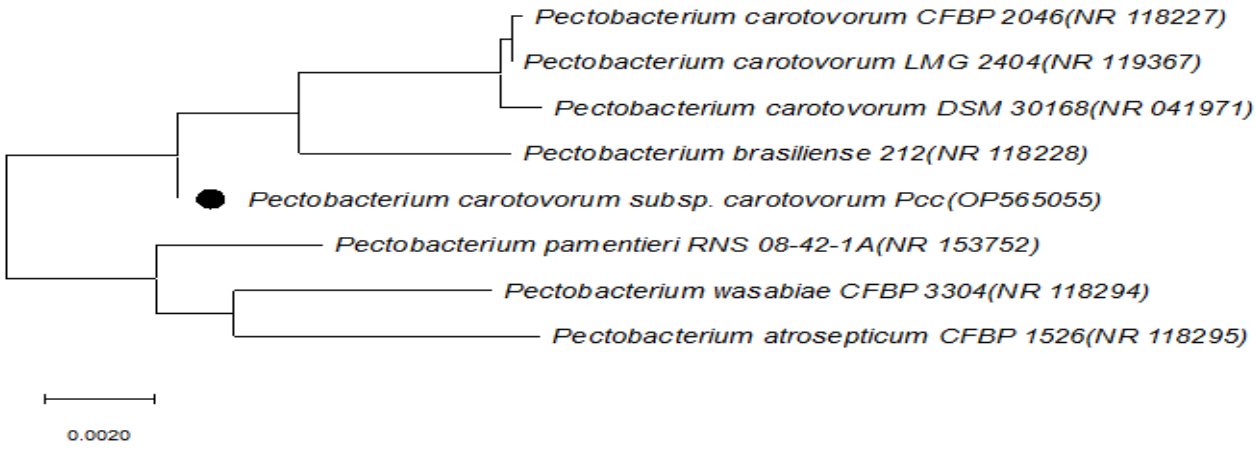


Fig. 1 . phylogenetic tree constructed upon bootstrap neighbor-joining tree method based on 16S rRNA gene partial sequence of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolate. The scale at the bottom indicates linkage distance**.**

Antibacterial activity test of bioagents and organic acids:

Disc diffusion method:

The antibacterial activity of the bioagents *Pseudomonas fluorescens* and *Trichoderma viride*, as well as organic acids namely, acetic acid and boric acid have been investigated at concentration of 1.5, 2.5 and 4 mg/ml on the growth of two bacterial isolates of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc and Pcc 160), with disc diffusion method (Table 2). DMSO alone was served as a control treatment. The obtained results exhibited that acetic acid was the most effective treatment against *P. carotovorum* subsp. *carotovorum* (Pcc) with inhibition zone at range of 17.67 to 41.33 mm, followed by *T. viride, P. fluorescens* and boric acid with

inhibition zone at range of 2.33 to 4.67 mm, 1.33 to 1.67 mm and 0.00 mm, respectively. Furthermore, the bacterial isolate of *P. carotovorum* subsp. *carotovorum* (Pcc 160) showed inhibition with acetic acid (from 4.67 to 33.67 mm), *T. viride* (1.33 mm with all concentrations), *P. fluorescens* (1.00 to 1.33 mm) and boric acid (0.00 mm with all concentrations). Both tested isolates (Pc and Pcc160) were sensitive to acetic acid, *T. viride* and *P. fluorescens* at 1.5, 2.5 and 4 mg/ml concentrations and resistant to boric acid treatment. The most effective concentration was 4mg/ml. Also, acetic acid treatment at various concentrations recorded significant differences with both isolates (Pcc and Pcc160) of *P. carotovorum* subsp. *Carotovorum.*

Table 2. Antibacterial activity of bioagents and organic acids against *Pectobacterium carotovorum* subsp. *carotovorum* isolates using disc diffusion method.

Treatments		Bacterial isolates		The main effect of concentrations	The main effect of treatments
		Pcc	Pcc 160		
Control	Conc.mg/ml	Inhibition zone (mm)			
	0	0.0000 i	0.0000 i	0 (0.0) B	
Acetic acid	1.5	17.67 *e	4.67 f	1.5mg/ml (0.35) B	1.88 A
	2.5	31.33 c	21.67 d		
	4	41.33 a	33.67 b		
Boric acid	1.5	0.00 i	0.00 i	2.5mg/ml (0.76) A	0.00 B
	2.5	0.00 i	0.00 i		
	4	0.00 i	0.00 i		
Pseudomons fluorescens fluorescens	1.5	1.33 hi	1.00 hi	4mg/ml (1.05) A	0.10 B
	2.5	1.67 h	1.33 hi		
	4	1.67 h	1.33 hi		
Trichoderma viride viride	1.5	2.33 gh	1.33 hi	-	0.18 B
	2.5	3.33 fg	1.33 hi		
	4	4.67 f	1.33 hi		
The main effect of bacterial isolates		0.66 A	0.43 A		

*Values, in each column, or rows (inhibition zone) followed by the same litter (s) are not significantly different at (P = 0.05); LSD of treatments = 0.38; LSD of bacterial isolates = 0.27; LSD of concentration = 0.38; LSD of interaction of all = 0.14

Minimum inhibitory concentration (MIC) estimation:

Table (3) indicates the result of treatments acetic acid, boric acid, *Pseudomonas fluorescens* and *Trichoderma viride* concentrations (50, 100, 200, 250, 300, 500, 1000, 1500, 1750 and 2000 µg/mL) Comparing to controls to evaluate (MIC) of these compounds on the growth of 2 different bacterial isolates under study *P.c.* subsp. *carotovorum* Pcc and Pcc160. All concentrations of the previous compounds had variable inhibitory effect on all bacterial strains. The MIC of acetic acid was reached at concentration of 50 µg/mL that completely (100%) inhibited Pcc isolate while MIC was attained at 100 µg/mL for Pcc160 isolate. The MIC of boric acid was reached at concentration of 1500 µg/mL that completely (100%) inhibited Pcc isolate while MIC was attained at 2000 µg/mL for Pcc160 isolate. The MIC of *P. fluorescens* was reached at concentration of 2000 µg/mL that completely (100%) inhibited Pcc isolate while MIC was attained in 1750 µg/mL for Pcc160 isolate. The MIC of *T. viride* was reached at concentration of 300 µg/mL that completely (100%) inhibited both of bacterial isolates Pcc and Pcc160.

Table 3. Antibacterial activity of bioagents and organic acids against *Pectobacterium carotovorum* subsp. *carotovorum* isolates using minimum inhibitory concentration (MIC) method.

Treatments	Bacterial isolates MIC(µg/ml)	
	Pcc	Pcc160
Acetic acid	50	100
Boric acid	1500	2000
Pseudomonas fluorescens	2000	1750
Trichoderma viride	300	300

Scanning Electron Microscopy (SEM) examination:

Morphological examination of *Pectobacterium* cells exposed to acetic acid:

Analyse *P. carotovorum subsp. carotovorum* (Pcc) cell morphology using a scanning electron microscope (SEM) following acetic acid treatment at 4 mg/ml conc. Control cells (Fig. 2d) showed cell wall deformations and cell damage since they were not exposed to acetic acid. The current investigation shows that the treated *P. c*. subsp. *carotovorum* (Pcc) surface changes due to a remarkable 4mg/ml concentration of acetic acid. When cells were treated with 4 mg/ml concentration of acetic acid, the images clearly showed that the treated bacterial cell forms showed

significant structural changes compared to the untreated bacterial cells; the treated cells were elliptical rods with rough cell surfaces (Fig. 2a). The bacterial cells of *P. c.* subsp. *carotovorum* (Pcc), after treatment, control cells were not treated with acetic acid (Fig.2), The surface of the membrane created by the control, which displayed a bright and smooth surface devoid of any apparent imperfections (Fig. 2d), appeared to swell, enlarge in size, and experience surface roughening (Fig. 2b) and explode (Fig. 2c).

Evaluation the efficacy of antagonists against *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc):

The effect of *Trichoderma viride* against *P. c.* subsp. *carotovorum* (Pcc) was analyzed using scanning electron microscope illustrates the clear inhibitory effect on *P. c.* subsp. *carotovorum* (Pcc) growth and survival. The findings showed that the pathogenic cells (*P. c.* subsp. *carotovorum*) grew more slowly or restrictedly in or towards the region of *T. viride* growth as compared to the region away from the growth of antagonist, and that the *Pectobacterium* cells that were inoculated in the plate during the cotton swab were unable to grow in the zone where *T. viride* was growing. The images from Figure 3 reveal that most of the cells near the growth region of *T. viride* were distorted in shape, the tested bacterial cells of *P. c.* subsp. *carotovorum* appeared swell, enlarged in size and explode (Fig.3a) and surface roughening and explode (Fig.3b) compared to the region 4 cm far from the growth of *T. viride* as the *P. c.* subsp. *carotovorum* is a rod-shaped bacteria (Fig.3c). Control (*P. c.* subsp. *carotovorum* without *T. viride*) tended to spread, which confirms the spreading pattern of *P. c.* subsp. *carotovorum* (Fig.3d).

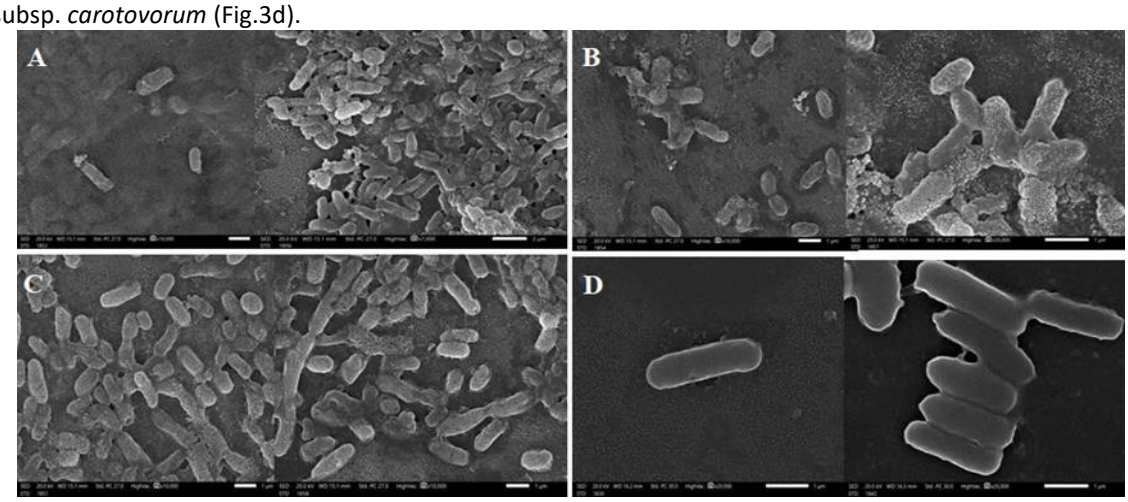


Fig. 2. Scanning electron microscopic micrographs of *P. carotovorum* subsp. *carotovorum* Pcc isolate treated with acetic acid. (a) Bacteria treated with acetic acid at 4mg/ml conc. were surface roughening, (b) swell and enlarged, (c) explode and (d) non treated stand ard cell morphology (control).

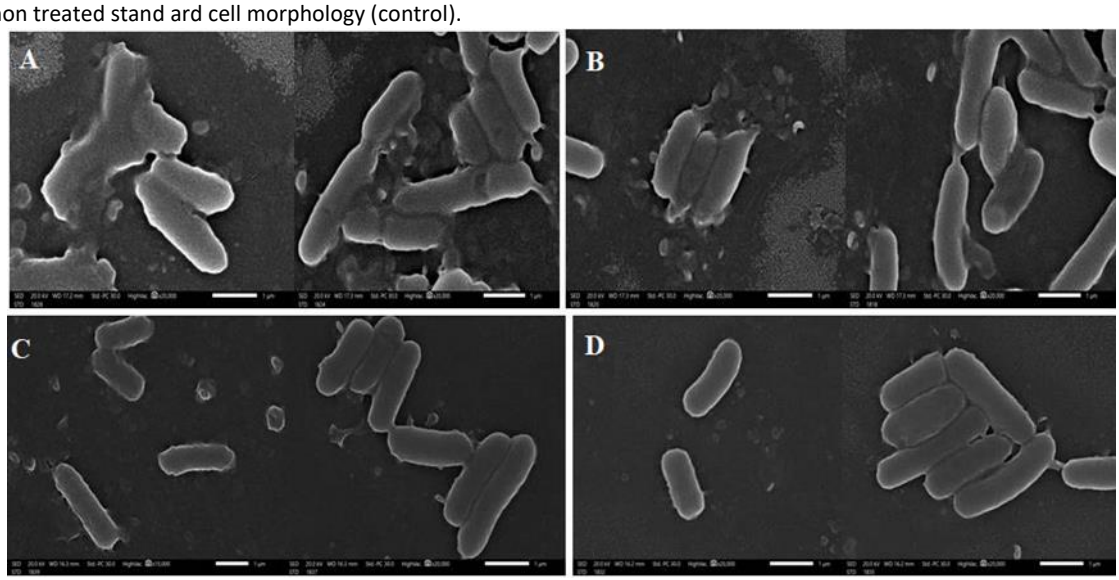


Fig. 3. Scanning electron microscopic micrographs of *P. carotovorum* subsp. *carotovorum* Pcc isolate affected by *T.viride* (a) bacterial cells were swell, enlarged in size and explode, (b) surface roughening and explode, (c) rod-shaped bacterial cells in the region 4 cm far from the growth of *T. viride* and (d) non tested stand ard cell morphology (control).

Field experiments:

Efficacy of acetic acid and *Trichoderma viride* on disease severity index against potato soft rot.

In two successful growing seasons, 2021 and 2022, the effects of the five treatments—acetic acid, *Trichoderma viride*, and streptomycin sulfate as the reference—as well as the treated control with soft rot disease and the untreated control as measured by the disease severity index (DSI) of potato plants were displayed in table 4. The study revealed that there were no significant differences in the disease severity index (DSI) between the first and second seasons for any of the treatments, including acetic acid and *T. viride*. However, the DSI values were highly significant when compared to the positive control, with the mean DSI values ranging from 18.41 to 9.94. But significant decreased between treatments (acetic acid and *T. viride*) in two seasons DSI ranged 10.74 and 5.35 compare with positive control DSI ranged 56.19. It is notable that *T. viride* treatment was the best reduction in DSI with a value of 9.94 in the first season and 4.65 for the second one with general mean 5.35. Concerning the sprays, continued reduction moral clear and obvious until the third one. We can also note that acetic acid gave an excellent control rate in the two seasons.

Table 4. Efficacy of *Trichoderma viride* and acetic acid on disease severity index against potato soft rot [*P. c.* subsp. *carotovorum* (Pcc)].

% Diseases severity index (DSI)							
TRT	**Season1**			**Season2**			
	1week	Two week	Three weeks	1 week	Two weeks	Three weeks	**Means**
Negative control (H2O)	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	0.0	0^{d}
Positive control	43.88^{b}	51.08^{ab}	58.03^{a}	58.06^{a}	62.46^{a}	63.60^{a}	56.19^{a}
Acetic acid	14.86^{c}	18.41^{c}	18.41^{c}	1.89^{cde}	4.05^{de}	6.83^{e}	10.74^{b}
Trichoderma viride	5.33^{de}	8.89^{cde}	9.94^{cde}	0.56^{e}	2.72^{de}	4.65^{de}	5.35^{c}
Strptomycine sulfate	0.00^{e}	0.48^{e}	0.92^{e}	0.56^{e}	1.16^{e}	1.72^{e}	0.81^{cd}
LSD	**12.9**						**5.34**

Impact on some factors related to plant defense response against soft rot disease:

Impact of *Trichoderma viride* and acetic acid on total soluble phenol content of potato leaves:

Applying acetic acid and *Trichoderma viride* treatments—by spraying on the foliage of potato leaves—increased the amount of polyphenols in the leaves relative to the positive control. This increase was particularly noticeable when treating *T. viride* 9593.68 (µg tannic acid /g f.wt)and using Streptomycin sulfate as the reference, which gave 9495.08 (µg tannic acid /g f.wt)compared to the positive control, whose leaves contained 7410.8 (µg tannic acid /g f.wt),we noticed the acetic acid 7301 (µg tannic acid /g f.wt) decreased non-significant compared positive control (Table 5).

Table 5. Effect of biotic and a biotic agents on total soluble phenols (µg tannic acid /g f.wt) on potato leaves.

Total soluble phenol			
µg tannic acid /g f.wt			
TRT	**season1**	**season2**	**Means**
Negative control (H2O)	6454.28^{bc}	5881.80^{b}	6168.04^{c}
Positive control	7807.29^{b}	7014.31^{bc}	7410.80^{b}
Acetic acid	7608.14^{b}	6993.87^{bc}	7301.00^{b}
Trichoderma viride	9526.82^{a}	9660.53^{a}	9593.68^{a}
Strptomycine sulfate	9480.96^{a}	9509.20^{a}	9495.08^{a}
LSD	**1606.8**		**1055.7**

Effect of *Trichoderma viride* and acetic acid on total soluble protein content of potato leaves:

Table 6 demonstrates that the application of *Trichoderma viride* greatly increased the protein content of potato leaves in both the first and second seasons, while the second treatment—acetic acid—significantly decreased the protein content with values of 131.86 and 78.84 mg protein/g f.wt for the mean of two seasons, respectively, compared to the positive control and stand er bactericide which had a protein content 91.40 and 115.19 mg protein/g f.wt for means of two seasons, respectively.

Table 6. Effect of biotic and a biotic agents on total soluble protein (mg protein/g f.wt) of potato leaves**.**

Total soluble protein			
mg protein/g f. wt			
TRT	**season1**	**season2**	**Means**
Negative control (H2O)	73.95bc	47.44c	60.70c
Positive control	111.94b	70.85bc	91.40b
Acetic acid	88.37b	69.30c	78.84cb
Trichoderma viride	128.84a	134.88a	131.86a
Strptomycine sulfate	113.80a	116.59a	115.19a
LSD	**23.07**		**18.92**

Efficacy of acetic acid and *Trichoderma viride* on peroxidase activity of potato leaves:

Data of Table (7) demonstrated that the treatment of *Trichoderma viride* enhanced the activity of (POD) dramatically in the two seasons either in the first season or the second one compared with positive control treatment, Mean activation for the two seasons was at its greatest point, reaching about 135**%.**

Table 7. Effect of biotic and abiotic agents on peroxidase (POD) of potato leaves**.**

Peroxidase activity			
% Activity			
TRT	**Season1**	**Season2**	**Means**
Negative control (H2O)	74.63c	63.84c	69.23c
Positive control	100.00b	100.00b	100.00b
Acetic acid	97.09b	101.37b	99.23b
Trichoderma viride	132.67a	135.19a	133.93a
Strptomycine sulfate	123.87a	129.02a	126.45a
LSD	**18.624**		**12.502**

Effect of acetic acid and *Trichoderma viride* on Phenylalanine ammonia Lyase of potato leaves:

Data in table (8) showed that phenylalanine ammonia lyase (PAL) activity was found to be high in inoculated plants treated with *Trichoderma viride* (138.65%) and treated inoculated plants with acetic acid improved the PAL% (105.3%) when compared to their corresponding positive control, all treatment significantly increased when compared with negative and positive control. Results in two seasons e no significantly increased but showed highest activity of PAL when treatment with *T. viride* and acetic acid in two seasons.

Table 8. Effect of biotic and abiotic agents on phenylalanine ammonia lysae of potato leaves**.**

PAL Activity			
% Activity			
TRT	**Season1**	**Season2**	**Mean**
Negative control (H2O)	74.63^{e}	85.80^{de}	80.21^{c}
Positive control	100^{cde}	100^{cde}	100^{bc}
Acetic acid	97.09^{cde}	113.51^{bcd}	105.30^{b}
Trichoderma viride	132.67^{abc}	144.62^{a}	138.65^{a}
Strptomycine sulfate	123.87^{abc}	155.18^{a}	139.53^{a}
LSD	**37.471**		**24.866**

Effect of acetic acid and *Trichoderma viride* on salicylic acid (SA) content of potato leaves:

The retention time was approximately 2.37 minutes for the SA stand ard which measured by High Performance liquid Chromatography (HPLC) (Fig.4). Salicylic acid content was recorded two weeks after inoculation with *P.carotovorum* subsp. *carotovorum* (Pcc). Results were presented in Table (9) and illustrated in Fig.5.

Season1:

The highest SA level from *Trichoderma viride* treated inoculated potato plants (Burn variety) (Fig.5) fowolled by inoculated plants with tested bacteria were 1924.3 ng/g^{-1} and 1149.3 ng/g^{-1}fresh weight respectively. The lowest SA level from acetic acid treated inoculated plants was 728.0 ng/g^{-1}fresh weights. There are significant differences were recorded in SA content between *T. viride* and acetic acid treatments and between *T. viride* and streptomycin (1014.5 ng/g^{-1} fresh weight) treatments. No significant differences were recorded in SA content between inoculated plants with *P.c.* subsp. *carotovorum* (Pcc) (1149.3 ng/g^{-1} fresh weigh) and control plants (961.2 ng/g^{-1} fresh weight).

Season2:

The highest SA level was obtained from *T. viride* treated inoculated potato plants (Burn variety) (Fig.5) fowolled by streptomycin treated inoculated potato plants were 2739.3 ng/g^{-1} and 2340.3 ng/g^{-1} fresh weigh. The lowest SA level from acetic acid treated inoculated plants was 1145.5 ng/g^{-1}fresh weights. There are significant differences were recorded in SA content between *T. viride*, acetic acid and streptomycin treatments. No significant differences in SA content between acetic acid treatment, inoculated plants by tested bacteria (1541.1 ng/g^{-1} fresh weight) and control plants (1147.6 ng/g^{-1} fresh weight). The best mean intervals of SA content in potato plants were detected season2 than seasson1after inoculation.

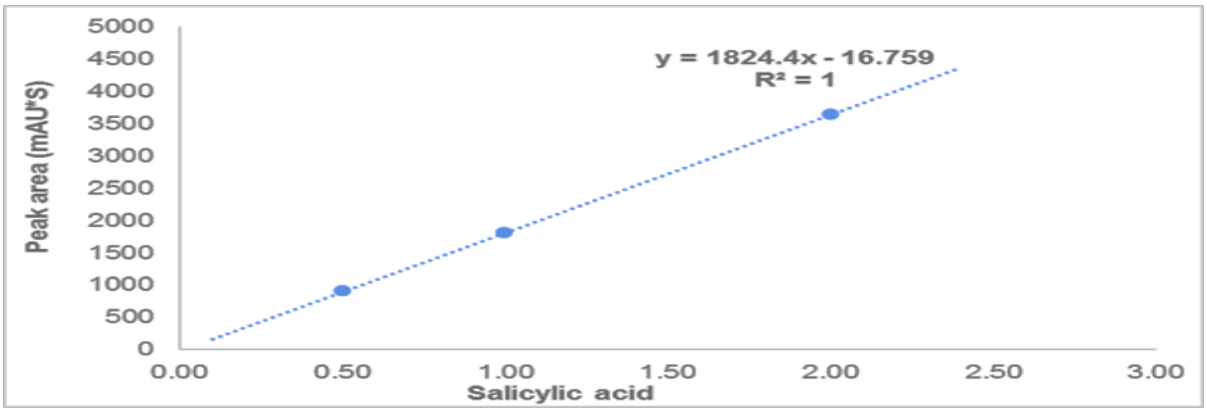


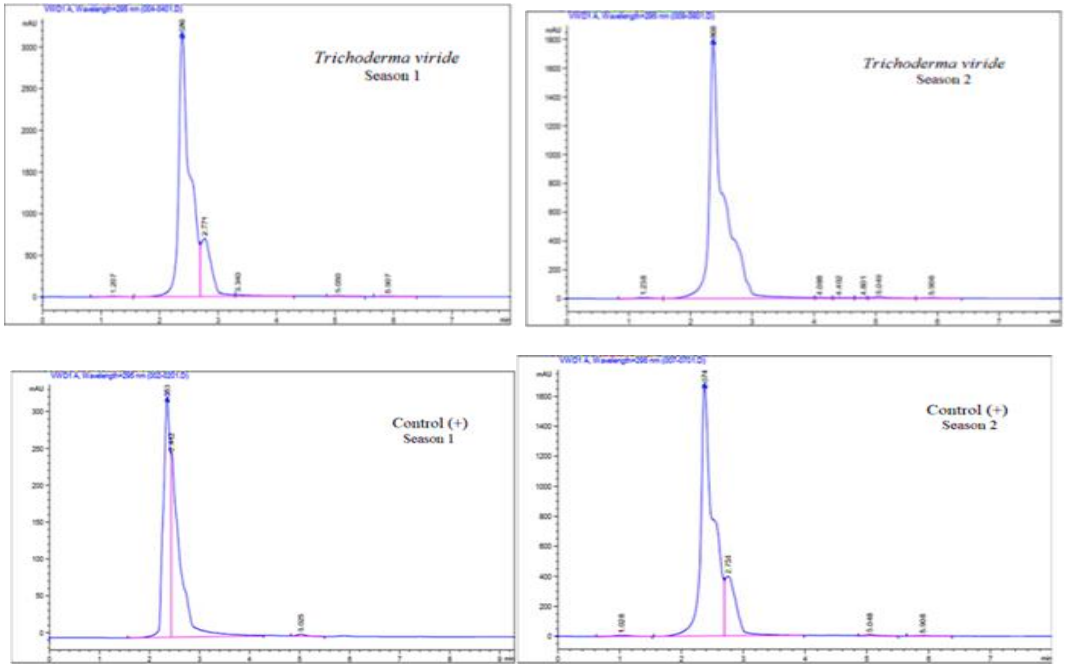
Fig. 4. Stand ard curve of salicylic acid (SA).

Table 1. Salicylic acid concentration (ng/g FW) in potato plants treated with acetic acid, *Trichoderma viride* and streptomycin**.**

SA content ng/g- fresh weight (FW)			
TRT	**1st Season**	**2nd Season**	**Mean of treatments**
Negative control (H2O)	961.2* fe	1147.6 de	1054.4 CD
Positive control	1149.3 de	1541.1 cd	1345.2 BC
Acetic acid	728.0 f	1145.5 de	936.7 D
Trichoderma viride	1924.3 c	2739.3 a	2331.8 A
Streptomycin sulfate	1014.5 fe	2340.3 b	1677.4 B
Mean of intervals	**1155.5 B**	**1782.8 A**	

*****values within the table are means of interaction between treatments and intervals of SA determination. LSD of treatments = 369.4;

LSD of seasons = 233.63.



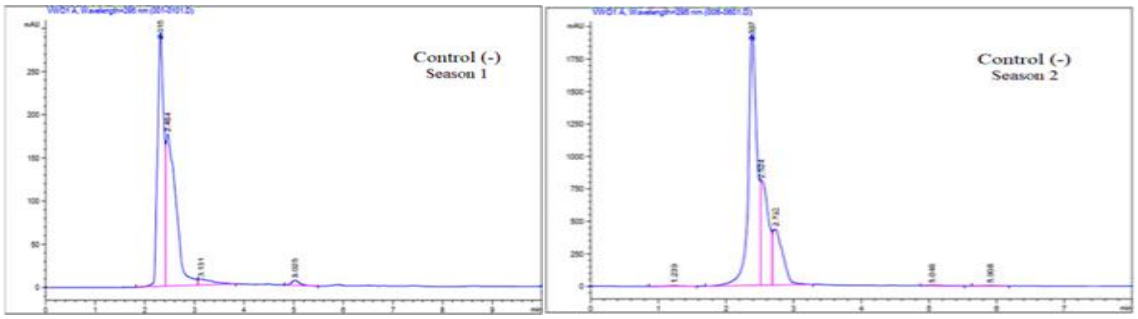


Fig. 5. Salicylic acid (SA) content in potato plants inoculated with *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) after treatment with *Trichoderma viride* comparing with positive and negative control.

DISCUSSION

The results of the molecular techniques (16S rRNA gene sequence) of the isolated potato bacterial isolate revealed that *P. carotovorum* subsp. *carotovorum* was associated with soft rot disease (Oskiera *et al.,* 2017). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker 16S rRNA gene was used to identify the tested isolate. One band with the correct predicted molecular length was found in the tested isolate's results. The examined isolate's DNA sequences showed that they belonged to *P. carotovorum* subsp. *carotovorum*. Such findings agreed with data obtained by **(**El-habbak and Refaat 2019; Loc *et al.,* 2022).

The antibacterial activity of the tested bioagents and organic acids was revealed that acetic acid was the most effective treatment against *P. carotovorum* subsp. *carotovorum* (Pcc), the present results agree with Rahman *et al.,* (2017**)** and Abdalla *et al.,* (2019**)** who clarified that acetic acid was effective in management of soft rot disease. The bioagent *Trichoderma viride* (biocure-T) was effective against Pcc isolate at concentration 4mg/ml which agrees with Abd-El-Khair *et al.* (2021). Also, *Pseudomonas flourecenes* (Biocure-B, 1.75% WP) was less effective against both tested isolates, which in contrast with Abd-El-Khair *et al.* (2021**)** who proved that *P. flourecenes* was highly effective against *P. c.* subsp. *carotovorum*.

In the current investigation, it was discovered that the organic acids and biocontrol agents employed inhibited the soft rot bacterial isolates, *P. c.* subsp. *carotovorum* Pcc and Pcc160, to varying degrees. *Trichoderma viride* was determined to be the most effective, after acetic acid. It has previously been documented that bacteria are susceptible to acetic acid and that *T. viride* controls bacteria **(**Abdalla *et al.,* 2019; Sulaiman *et al.,* 2020**)**. Our findings indicate that acetic acid was most effective in preventing *P. c.* subsp. *carotovorum* from growing and producing soft rot disease (Park *et al.,* 2023).

According to the results obtained by Scanning Electron Microscope (SEM) showed that acetic acid caused damage in the cells of *P. c*. subsp. *carotovorum* (Pcc) and deformations of cell wall which led to the bacterial cells were apparent swell, enlarged in size, surface roughening and explode. These findings were approved with Grower *et al.* (2004) and Sin Mei *et al.* (2015) results. *P. c*. subsp. *carotovorum* (Pcc) cells affected with *Trichoderma viride* (biocure-T) were distorted in shape and appeared swell, surface roughening and explode. *T. viride* secrets different compounds against bacteria which hinder the growth of *P. c*. subsp. *carotovorum* (Pcc). These results in agreement with Yan and Khan, (2021). Pretreatment of potato plants with acetic acid before inoculation with *P. c.* subsp. *carotovorum* gave an excellent control rate,Our results were compatible with (Rahman *et al.,* 2017) since they found that Acetic acid, showed bactericidal activity against soft rot bacteria, our study agree with (Sulaiman *et al.,* 2020) According to their suggestions, soft rot in potatoes caused by *P. c.*subsp. *carotovorum* may be managed biologically by using *Trichoderma* spp. We concurred with the findings of several studies (Harman, 2006; Bhattocharrya and Purohit 2008) regarding *T. viride* has ability to inhibit the growth of various plant pathogens, as well as the findings of Abd El Khair *et al.* (2021) which demonstrated the potential significance of *T. viride* application in the management of bacterial soft rot disease in vegetables.

It is well known that many plants phenolic compounds have antibacterial properties, act as signal molecules or as building blocks for structural polymers like lignin (Hammerschmidt, 2005). According to Abo-Elyousr *et al.* (2009), there was a good correlation between the level of plant resistance against the pathogens and a significant rise in phenolic content. In our study treatment with *T. viride* increased the amount of polyphenols in the leaves, Our results were in agreement with (Daayf *et al.,* 1995 and 1997; Aly *et al.,* 2003) They established that treatment with *T. viride* increased the amounts of soluble phenols in plant leaves and

caused their build up to occur earlier. This increase in total phenolic may have attributed to increase in the defense capability of plants to infection disease and development. Either McQuilken and Gemmell (2004) and Al-Jarah *et al.,* (2013) agree with us, they demonstrated how *Trichoderma* can develop from direct parasitism on *Pectobacterium* cells, which is followed by cell penetration and the production of enzymes that break down the host cell wall, ultimately resulting in the death of the cell. According to reports, some fungi enter host cells by use of specific enzymes including chitinase, B-1, 3-glucanase, and protease.

Total soluble proteins are essential for plant defense because of their antibacterial activity, capacity to inhibit the development of symptoms, and quick and early accumulation, which is frequently linked to incompatibility (Wang *et al.,* 2005). In the present study application of *T. viride* greatly increased the protein content of potato leaves in both the first and second seasons, while —acetic acid—significantly decreased the protein content.

Plant defense mechanisms against pathogen invasion rely heavily on peroxidase (POD), as there is a significant correlation between enzyme activity and the development of resistance in plants. Applying biotic or abiotic agents strengthened plants' defenses against disease, while resistance induction—applying inducers via foliar application or optimized soil management techniques—improved plants' health status against disease (Zeller 2006; Tamma *et al.,* 2011; Song *et al.,* 2013).

In our study the treatment of *T. viride* enhanced the activity of (POD) in potato leaves dramatically in the two seasons, These findings agree with those reported by Caruso *et al*. (2001) and Nawar and Kuti (2003), They stated that there are positive correlations between enzymatic activity and the development of plant resistance, and that the bio-control agent accumulation of particular enzymes, such as POD and PPO, is crucial to plant defense mechanisms against pathogen infection. In treated bean plants, *Trichoderma* spp. also raised POD and PPO enzyme activity levels (Abd-El-Khair and Khalifa, 2010).

Phenylalanine ammonia lyase (PAL) activity was found to be high in inoculated plants treated with *T. viride* and acetic acid.

Salicylic acid (SA) was known to be an important signal molecule of infection. SA measurement in inoculated potato plants with tested pathogenic isolate after treated with some biotic and abiotic agents revealed that the endogenous SA levels increased in *T. viride* (biocure-T) treated inoculated plants in season1 and season 2, this result are in agreement with (Segarra *et al.,* 2007; Bhat *et al.,* 2017**)**.The presence of high SA concentrations in leaves subjected to biotic or abiotic stress is attributed to its role as a signaling compound in the induction of acquired systemic resistance. Applying biocontrol agents beforehand can effectively stop disease attacks, make plants induce systemic resistance, encourage plant growth, and boost the number of secondary metabolites the biocontrol agent secretes (Yendyo *et al.,* 2018). Therefore, we advise farmers to consistently use biocontrol agents in their fields to establish systemic resistance in plants and protect them from pathogen invasion.

CONCLUSION

It has been documented that treating sites with a range of agents, both biotic and non-biotic, can induce resistance in plants, which is defined by a reduction of the development of disease symptoms and a restriction of pathogen growth. Our results indicate that Trichoderma viride as biotic agents to control the soft rot disease in potato leaves, also found that stimulation of the defense response in plant and accumulation the salicylic acid in potato leaves. Then, it came to light that acetic acid was a cheap and effective agent for organic acids, which have long been utilized in the food sector and agriculture due to their antibacterial and antifungal properties.

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تأثير بعض العوامل الحيوية واللاحيوية على بكتيريا العفن الطري (*Pectobacterium carotovorum* subsp. *carotovorum*) على نباتات البطاطس

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فعالية بعض عوامل المكافحة الحيوية (*Pseudomonas Fluorescens* و *Trichoderma viride*) والأحماض العضوية (حمض الأسيتيك وحمض البوريك) ضد بكتريا العفن الطري في البطاطس *Pectobacterium* sp. تمت دراستها في ظل الظروف المعملية والحقلية. عزلتان من بكتيريا العفن الطري *Pectobacterium carotovorum* subsp. *carotovorum* Pcc و *Pectobacterium carotovorum* subsp. *carotovorum* Pcc160 (Acc.No. LN811442) تم استخدامهم في هذه الدراسة. تم عزل عزلة Pcc من درنات البطاطس (صنف Burn)، وتسببت عزلات Pcc و Pcc160 في ظهور أعراض العفن الطري على البطاطس. تم تعريف العزلة Pcc جزيئيًا عن طريق مضاعفة الجين 16srRNA فى تفاعل البوليميريز المتسلسل بى سى آر(PCR) بأستخدام بوادئ عالمية. تتابع الحمض النووي DNA لنواتج ال PCR، وتحليل BLAST، وبيانات Genbank أظهرت إن عزلة Pcc تنتمي إلى البكتيرة بكتوباكتيريم كاروتوفورم *Pectobacterium carotovorum* sub sp. *carotovorum*. طبقا لشجرة القرابة الوراثية المبنية على التتابعات النيوكلوتيدية للحمض النووي DNA لجين 16s rRNA، فإن العزلة المصرية تشبه العزلة الصينية (OQ727369) بشكل وثيق. معمليا أظهر حامض الخليك أقوى تأثير ضد عزلات Pcc و Pcc160 بمناطق تثبيط 41.33 و 33.67 ملم و MIC 50 و 100 ميكروغرام/مل على التوالي. أظهر المجهر الإلكتروني الماسح تأثيرًا مثبطًا واضحآ لحمض الأسيتيك و*T.viride* على نمو وبقاء البكتيرة بكتوباكتيريم كاروتوفوروم *P.c.* subsp. *carotovorum* (Pcc) ، فى التجربة الحقلية كان أستخدام *T. viride* هو الأكثر فعالية، حيث تسبب في انخفاض مؤشر شدة المرض (DSI). تم تقييم حمض الأسيتيك و*T. viride* لفعاليتهما في تحفيز إنزيمات الدفاع، البروتين الكلي الذائب، محتوى الفينولات وحمض الساليسيليك (SA) في البطاطس ضد البكتيرة بكتوباكتيريم كاروتوفوروم *P. c.* subsp. *carotovorum* (Pcc) وجد أن نشاط الإنزيمات الدفاعية [البيروكسيديز (POD) وفينيل ألانين أمونيا لياز (PAL)] والمحتوى الكلي من البروتين الذائب ومحتوى البوليفينول ومحتوى حمض الساليسيليك (SA) يزداد في نباتات البطاطس المعاملة بـ *T. viride* في كلا الموسمين مقارنة بمعاملات أخرى. أظهرت نتائج هذه الدراسة أن *T. viride* أظهر نشاطًا مثبطًا فعالًا ضد Pcc، وبالتالي يمكن أن يكون لـ *T. viride* تطبيق محتمل في مكافحة *P. c.* subsp. *carotovorum*.

الكلمات المفتاحية: عوامل المكافحة الحيوية، العفن الطري، بكتوباكتيريم، الأحماض العضوية 16s rRNA، حمض الساليسيليك ،الإنزيمات الدفاعية.