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Molecular detection of the causative agent of the potato soft rot, *Pectobacterium carotovorum*, in Egypt and essential oils as a potential safe tool for its management

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Abstract

Forty-two bacterial isolates were collected from soft-rotted potato tubers originating from four governorates in Egypt. Their phenotypic and genetic characteristics were studied. The phenotypic identification resulted in grouping the studied isolates into five different species and/or genera including *Pectobacterium* sp., *Bacillus* sp., *Dickeya* sp., *Pseudomonas* sp. (1), and *Pseudomonas* sp. (2). The molecular identification of the 10 isolates of them were pathogenic to potato tubers and other hosts. More specific identification of the later 10 isolates, using two specific primers for *P. carotovorum* subsp. *carotovorum* (Pcc) and the reference strain PccY46 (Acc. No. KP187511.1), revealed that all these isolates had yielded 220–272-bp DNA fragments identical to the 16S rRNA gene of PccY46. Phylogenetic analysis showed sequence similarity ranging from 87 to 98%, which confirmed the genetic variation among the 10 tested strains of Pcc. The isolates were distributed in four major clusters, each subdivided into a few sub-clusters. In another experiment, two different essential oils (peppermint and clove oils) plus a nano-Cu-based fungicide (Tango®), in comparison to the two different antibiotics and the two copper fungicides, were evaluated for their potential management on the three most severe bacterial isolates (Pcc1, Pcc5, and Pcc10). Results of in vivo experiment showed that clove oil was the most effective, where it caused a reduction of disease severity (0.0%) on potato tubers, 4 days post artificial inoculation.

Keywords: *Pectobacterium carotovorum*, Molecular detection, Essential oil, Nano-cu, Safe control, Potato

Background

In terms of food crops such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.), potato (*Solanum tuberosum*) has so far been ranked fourth in the world, and regarding its remarkable nutritive value, its total world acreage gets far beyond any other cultivated crop (Douches et al. 1996).

In 2016, Egypt was ranked 14th in the world, with 5.0 million tons of potatoes produced (FAO 2016). The blackening of potato tuber in field and the tuber soft rot in storage, transit, and field are all caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), which causes a great loss (Nabhan et al. 2011). Perombelon and Kelman (1980) referred the main causes of tuber bacterial soft rot to either unsuitable environmental conditions during growth or a bad storage state or to both. Among the members of the species *Pectobacterium*, which infect a wide range of vegetable crop hosts, *Pectobacterium atrospeticum* was identified as the main potato pathogen (Gardan et al. 2003).

Many strains of *Pectobacterium atrosepticum* have been subjected to genetic analyses, which suggested that almost all were, to a great extent, quite close to the other species, *Pectobacterium atrosepticum* (Avrova et al. 2002). Ma et al. (2007) reported that despite the close resemblance, any good reason(s) to explain how the *Pectobacterium* spp. members uniquely infect different

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vegetable crops still has not been genetically figured out yet.

Controlling bacterial potato bacterial soft rot is a real challenge for producers. Many control strategies have been developed and applied. Using, for example, protective chemicals make ware tubers unsafe for consumption. In addition, Crépin et al. (2012) stated that although biological control techniques have recently progressed so far to control to some extent disease severity, they argued that their use, however, is still limited. Though certified, tubers are subject to the occurrence of soft rot during later tuber handling, which leads to severe disease infection loss. So, it is quite crucial to measure, in these tubers, both disease severity and incidence (Czajkowski et al. 2011).

The application of plant resistance elicitors, as a complementary option to trigger preventive defense reactions, might aid in controlling the disease; however, this control strategy is not a top priority, since plants lack highly efficient defense elicitors (Deravel et al. 2014). Using chemical resistance, inducer technique has been proved effective in controlling *P. carotovorum* and other bacterial species, which cause soft rot disease (Farrar et al. 2009). Bokshi et al. (2003) showed that acetyl-salicylic acid (ASA) enhanced resistance against *E. carotovora* subsp. *carotovora* which causes the disease.

In addition, there are other compounds that can inhibit plant pathogens and/or suppress toxin production by pathogens, for example, organic and inorganic salts (Olivier et al. 1998). Benzoic acid and sodium benzoate at the rates of 1.0, 5.0, and 10.0 mM, as reported by Saleh and Huang (1997), relatively minimized soft rot disease infection. Acetic and boric acid and bleaching powder are also effective against onion soft rot, *Burkholderia cepacia* (Rahman et al. 2013). Some essential oils and hemp flower water extract were tested against *Erwinia carotovora*, a potato bacterial pathogen (Krebs and Jaggir 1999). Simeon and Abubakar (2014) recommended aqueous extracts of each of the lemon grass (*Cymbopogon schoenanthus*), Aloe vera (*Arabian peninsula*), and neem (*Azadirachta indica*), as well as borax salt, as all had inhibitory effects on *Pectobacterium* spp. and could be used for managing the tuber soft rot.

This present study aimed to isolate the potato soft rot disease isolates found in Egypt and to identify these isolates through morphological, physiological, and molecular (PCR) techniques, as well as to test some essential oils as natural safe materials to control the disease.

Materials and methods

Isolation and purification of bacteria associated with soft-rotted potato tubers

Isolation of the causal bacterium was done to potato tubers (*Solanum tuberosum*) showing symptoms of the soft rot disease, cultivated in four different farms located in Sinai, Sharqia, Gharbia, and Beheira governorates, Egypt. The infected tuber samples with bacterial lesions were picked and washed with tap water to get rid of adherent soil and then were air-dried. The samples were cut into small slices, with 1–2 cm thickness each; each one was surface-sterilized for 2 min with 2% sodium hypochlorite solution, rinsed three times in sterile distilled water, and then dried in-between two sterilized filter papers. Then, each sample was transferred onto nutrient agar medium (NA) plates. The inoculated plates were incubated at 30 °C for 48 h. Randomly, initiated bacterial colonies were picked and transferred to new NA medium plates for purification (Barker 2005). The resulting bacterial colonies were further purified and sub-cultured repeatedly, until pure cultures were obtained (Yuan et al. 2004).

Identification of the pathogenic bacteria

Morphological, nutritional, and physiological identification

A primary identification of the bacterial isolates was conducted on the bases of their morphological, nutritional, and physiological characteristics according to the schemes suggested by Murray and Holt (2001).

Preparation of bacterial inoculum

Under laboratory conditions, bacterial suspensions of the aforementioned purified bacterial isolates were prepared as described by Desender et al. (2006) with some modifications. Each of the tested bacterial isolate was grown in and sub-cultured on King's B medium for 24 h at 30 °C and then suspended in sterile distilled water and centrifuged at 3000 rpm/min for 30 min. The pellets were re-suspended in distilled water and adjusted turbidimetrically (O.D. at 580 nm) to approximately the density of 10^8 CFU/ml, using a spectrophotometer. The bacterial concentration was estimated from $O.D_{580}$ data using a standard curve.

Pathogenicity test

Potato tubers had been flame antisepticised prior to cutting into 1-cm-thick slices using a sterile knife. Each slice was placed on a moistened sterilized filter paper in a Petri dish. A volume of 0.50 ml of bacterium inoculum of each isolate was pipetted into the center of the five slices to induce artificial infection. Distilled water was used as the control treatment. All treatments were incubated at 30 °C for 4 days, then pathogenicity was examined on a daily basis. According to Lelliott and Stead (1987) disease infection ranking of rotted potato slices was recorded on a scale of negative (−), low (+), moderate (++), and high (+++) infection.

Host range

Tubers, fruits, bulbs, and corms, for 30 different host plants, were inoculated with each of the 10 tested bacterial isolates. Equal slices of each tested plant material were inoculated with 0.5 ml of each isolate at a rate of 10^8 CFU ml^{-1} by pipetting into the center of each of five slices. For the control, sterilized water was pipetted as well into the center of five slices. Disease rating, as explained, was measured following all treatment incubation at 30 °C for a 4-day period.

Molecular identification of the pathogenic bacteria

DNA extraction

DNA was extracted from 24-h-old pure bacterial cultures of 12 bacterial isolates grown on NA medium at 37 °C. These isolates were primarily identified as *P. carotovorum* (*Pc*). Pure bacterial colonies were picked by a sterile loop and mixed in 4 ml of nutrient broth media in a sterile labeled culture tubes (Velp, Italy) and incubated overnight at 37 °C, with shaking at 150 rpm.

DNA was extracted, using DNeasy Blood and Tissue Kit (Qiagen, China). The protocol was performed according to the manufacturer's instructions. The quantity and quality of the extracted DNA were measured, using the UV-spectrophotometer (T80 plus, PG Instruments Limited, England), and DNA was visualized by electrophoresis (Cleaver, power PAC300) in 1% agarose gel in 1X Tris-Acetate-EDTA (TAE) (Promega, Madison, Wisconsin) buffer stained with ethidium bromide (0.5 μg/ml) (Sambrook 2001). The DNA was stored at − 20 °C for further PCR work.

The primers used in this study were synthesized by Thermo Fisher Scientific Inc., as described by Yuan et al. (2004).

The PCR specific primers (Fs1: CAGAGTTTGATCCTGGCTCAG) and (Rs1: AAGGAGGTGATCCAGCC) used herein were designed based on the region of the 16S ribosomal RNA gene of *P. carotovorum* subsp. *carotovorum* (Pcc) strain Y46 (Acc. No. KP187511.1). The primers amplified a 220 to 272-bp PCR amplicon unique to *P. carotovorum*, compared to all known DNA sequences in the available databases, and showed homology only to *P. carotovorum* subsp. *carotovorum.*

DNA amplification was performed according to the non-conventional method (De-Boer and Ward 1995), and the PCR reaction mix (25 μl) contained 5.0 μl 5X Crimson Taq buffer, 1.1 μl 25 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 0.13 μl 5 U/μl Taq polymerase, 1.25 μl 10 μM of each primer, and 2.0 μl DNA template finalized to 25 μl by adding 13.77 NFW. PCR was performed in a thermal cycler TProfessional (Biometra, Germany) using the following protocol: denaturation (94 °C, 5 min), followed by 35 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 1 min) with a final extension (72 °C, 7 min), and adjusted as needed.

DNA sequencing and phylogenetic analysis

PCR product was recognized, and nucleotide sequencing was performed in each direction by Macrogen Korea (Seoul, Rep. of Korea). The similarity search of the PCR product was performed with a Basic local alignment searching tool (BLAST) at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A Blast search was performed for nucleotide, using BLASTn. Biological evolutionary trees for the data were reconstructed, using MEGA (Kumar et al. 2001), using the Neighbor-Joining (NJ) method.

In vitro antibacterial assay of tested essential oils

Antibacterial activity was in vitro assayed for the three most virulent *P. carotovorum* isolates Pcc1, Pcc5, and Pcc10 (the causal pathogen of potato tubers rot) for two essential oils, two antibiotics, and three fungicides, as well as for a systematic fungicide. The two oils were (peppermint, *Mentha piperita*, and clove, *Syzygium aromaticum*) each at a rate of 1.5, 3.0, and 5.0% (vv^{-1}). The two antibiotics were tetracycline and erythromycin, using rates of 100, 200, and 500 ppm for each. The two fungicides were Galben-Cu (Benallxyl Cu 54% WP) and Copper oxychloride (48% WP), using rates of 250, 500, and 750 ppm for each. In addition to the above, the systematic fungicide Tango® 23% (copper sulfate 8% + sulfur 28%) was tested. All treatments were tested using nutrient agar (NA) plates. For the control treatment, paper disks were immersed in distilled water. For all treatments, the NA plates were inoculated with 0.5 ml of the tested pathogenic bacterial suspension of 10^8 CFU dilution for 24 h. For each bacterial isolate, four 5-mm filter paper disks, for each treatment, were placed onto the surface of each inoculated plate and later on all plates were incubated at 28 °C for 72 h. Within the inhibition zone surrounding disks, the bacterial growth was an indication of treatment effect (Fatmi and Schaad 1988).

In vivo antibacterial assay of tested essential oils in potato tubers

Seven treatments were tested in this experiment using the concentration which gave the highest inhibition zone; two essential oils (peppermint and clove) each at a rate of 5%, two antibiotics (tetracycline and erythromycin) each at a rate of 500 ppm, and three fungicides (Copper oxychloride, Benalaxyl-Cu, and systemic fungicide Tango®) each at a rate of 750 ppm. The effects of these materials were relatively compared on the three virulent isolates of bacterial potato tubers' rot (Pcc1, Pcc5, and Pcc10 as mentioned above). Five potato tubers

cv. Spunta, previously inoculated with the pathogenic strains, were treated with each treatment in a completely randomized design (CRD). Nine (30 × 40 × 10 cm) boxes were used. In the first three-box set, each box contained five tubers treated with each of the two essential oils. In the second set, five tubers were treated with each of the two antibiotics. In the last set, five tubers were treated with the three fungicides. In addition to another five inoculated tubers used as controls. All boxes incubated at 28 ± 2 °C where soft rot disease severity was estimated after 2 and 4 days based on a 0–5 ranking scale (Bdliya and Langerfeld 2005) where 0 = no symptom, 1 = 1–15% rotten tuber, 2 = 16–30%, 3 = 31–45%, 4 = 46–60%, and 5 ≥ 61%. The severity was then computed, using the following formula:

$$Disease\ Severity = \frac{\sum nv}{5N} \times 100$$

where n = number of infected tubers in each category, v = numerical values of each category, N = total number of the infected tuber, and 5 = highest score on the severity scale.

Results and discussion

Frequency of the isolated bacteria from potato tubers showing soft rot symptoms

Isolation trials from potato tubers showing bacterial soft rot, collected from Sinai, Sharqia, Gharbia, and Beheria governorates, yielded 42 bacterial isolates. The isolates were distributed among five species, belonging to four genera grouped as *Pectobacterium* sp., *Bacillus* sp., *Dickeya* sp., *Pseudomonas* sp. (1), and *Pseudomonas* sp. (2) at the frequency of (10, 13, 5, 8, and 6 isolates), respectively. Different governorates did not show much difference in the frequency of the isolated species, being 10, 13, 11, and 9 isolates from Sinai, Sharqia, Gharbia, and Beheira, respectively. Van der Wolf and De Boer (2007) reported that the main bacteria causing potato tuber soft rot were *Pectobacterium atrosepticum*, *P. carotovorum* subsp. *carotovorum*, and *Dickeya* spp. (Table 1). Potato seed lots were tested for the soft rot pathogen and found impure with *Dickeya* spp. and *P. carotovorum* subsp. *carotovorum*, but not with *P. atrosepticum*. High concentrations of both *Dickeya* spp. and *Pcc* were found in the stolon ends, whereas relatively low densities were found in the peel and in deeper located potato tissue (Czajkowski et al. 2009). *Pseudomonas syringae* van Hall was found only as a species of Gram-negative bacteria isolated from rotted potatoes, collected from clamps in England in 1945–1947 (Jones and Dowson 1950). The isolates of *Bacillus* spp. are thought to be saprophytic rather than pathogenic, as Bacilli are not formerly reported to be the primary causal agents of soft rots. Lund and Wyatt (1979) highlighted that potato tubers may harbor pectolytic saprophytic bacteria (*Bacillus* spp., *Clostridium* spp., *Flavobacterium* spp., and *Pseudomonas* spp.) which, if given the opportunity, can also cause rotting.

Pathogenicity and host range of isolated bacteria

The pathogenicity of obtained isolates was examined on diverse plant hosts (Table 2). Results showed that each of the tested isolates was pathogenic on potato tubers. Out of them, solely three isolates (1, 5, and 10) caused extremely severe symptoms of soft rot on potato tubers; meanwhile, isolates 2, 3, 7, 8, and 9 showed moderate severity and isolates 4 and 6 showed low severity on potato tubers once tested for their pathogenicity. On the other hand, on all tested host plants, except sugar beet, isolates 1, 5, and 10 were extremely pathogenic, while isolates 2, 3, 4, 6, 7, and 8 showed low to moderate severity.

Obtained results indicate that there was a correlation between the host plant and the disease severity of some bacterial isolates as potato tubers were highly vulnerable to *Pcc*1, *Pcc*5, and *Pcc*10 and squash fruits were lowly vulnerable to most isolates, while sugar beet was not vulnerable to the majority of them. The present results are in harmony with those reported by Frampton et al. (2012) who confirmed that *P. carotovorum* was the main causal agent of soft rot disease on potato tubers.

Table 1 Frequency of the isolated bacteria from potato tubers showing soft rot collected from four governorates

The isolated bacteria	Frequency of the isolated bacteria				Total	% Frequency
	Sinia	Sharqia	Gharbia	Beheira		
Pectobacterium sp.	2	3	3	2	10	23.8
Bacillus sp.	3	4	3	3	13	30.9
Dickeya sp.	2	2	1	2	7	11.9
Pseudomonas sp. (1)	3	3	2	1	9	19.1
Pseudomonas sp. (2)	2	1	2	1	6	14.2
Total	12	13	11	9	42	

Table 2 Severity of soft rot caused by confirmed isolates of *Pectobacterium carotovorum* on different hosts

Host species	Organ	Bacterial isolates									
		1	2	3	4	5	6	7	8	9	10
Potato (*Solanum tuberosum)*	Tuber	+++	++	++	+	+++	+	++	++	++	+++
Sweet potato (*Ipomoea patats*)	Root	+++	+	++	++	+++	++	++	+	++	+++
Carrot (*Dacus carrot*)	Root	+++	+	+	++	+++	+	++	++	++	+++
Squash (*Cucurbita pepo*)	Fruit	+++	+	++	+	+++	+	+	+	++	+++
Sugar beet (*Beta vulgaris*)	Root	+	–	–	–	+	–	–	–	–	+

Severity of soft rot symptoms was assessed visually according to Lelliott and Stead (1987) as follows: (–) negative infection, (+) low infection, (++) moderate infection, and (+++) high infection

Identification of isolated bacteria

Morphological, physiological, and biochemical identification

Morphological, physiological, and biochemical assays by traditional identification of the 10 strains of the presumptive pathogen *Pectobacterium* spp. isolates confirmed that all of them were *P. carotovorum,* as they showed the following features: creamy, short rods, and Gram negative, and did not have the ability to produce any pigment in Kings B medium. All the tested isolates were positive for motility, starch hydrolysis, growth at 37 °C, growth in 5% NaCl and 7% NaCl, catalase activity, pectate degradation, fried egg in PDA, and relation O_2 but negative for KOH 3% and pigment production. The strains also induced typical bacterial soft rot symptoms on tuber slices of cv. Spunta (Table 3).

Molecular identification of the pathogenic isolates

Detection of bacterial 16S rRNA gene

Amplifications with the Fs_1 and Rs_1 primers were positive in all strains also the Fs_1 and Rs_1 primers discovered *P. carotovorum* subsp. *carotovorum* DNA in the 10 tested strains (Fig. 1). A sequence similarity was undertaken by using the Blast server at the National Center for Biotechnology Information. It was found that PCR product was 220–272 bp long, the same size as the region on the *P. carotovorum* subsp. *carotovorum* 16S rRNA gene between the two primers. An alignment of the nucleotide sequence of the eventual PCR product and the region confined with the two primers of the *P. carotovorum* 16S rRNA gene showed an exact size and typical sequence match. Results presented in Table 4 showed the

Table 3 Morphological, physiological, and biochemical characters of isolated bacteria (*Pectobacterium* sp.)

Identification tests	1	2	3	4	5	6	7	8	9	10
Gram reaction	–	–	–	–	–	–	–	–	–	–
KOH 3%	–	–	–	–	–	–	–	–	–	–
Shape	R	R	R	R	R	R	R	R	R	R
Size	S	S	S	S	S	S	S	S	S	S
Motility	+	+	+	+	+	+	+	+	+	+
Growth 37 ° C	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Tolerance NACL 5%	+	+	+	+	+	+	+	+	+	+
Tolerance NACL 7%	+	+	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+	+	+
Pigment production	–	–	–	–	–	–	–	–	–	–
Pectate degradation	+	+	+	+	+	+	+	+	+	+
Sucrose reduction	–	–	–	–	–	–	–	–	–	–
Fried egg in PDA	+	+	+	+	+	+	+	+	+	+
Relation O_2	An.	An.	An.	An.	An.	An.	An.	An.	An.	An.
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+
Acid prod. gas lactose	+	+	+	+	+	+	+	+	+	+
Acid prod. gas glucose	+	+	+	+	+	+	+	+	+	+
Name	Pcc1	Pcc2	Pcc3	Pcc4	Pcc5	Pcc6	Pcc7	Pcc8	Pcc9	Pcc10

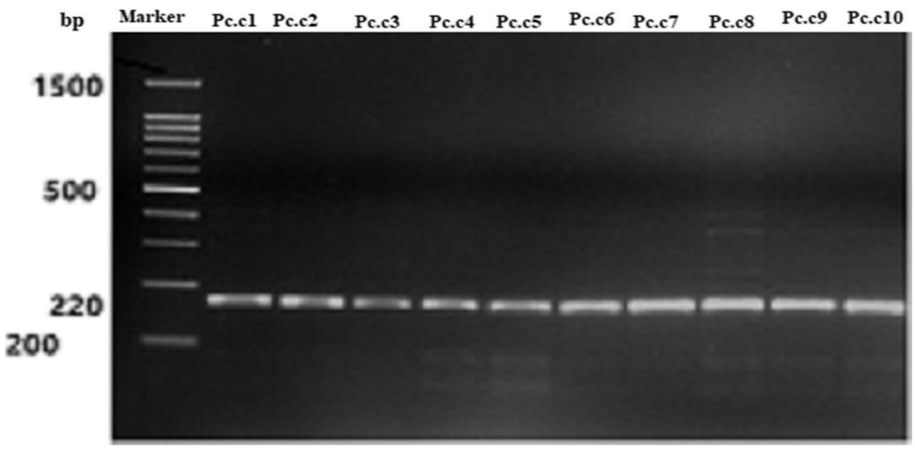

Fig. 1 PCR amplified DNA in 10 indigenous isolates of *Pectobacterium carotovorum* (*Pcc*), using Fs1 and Rs1 primers

sequence of this 210–250 bp PCR amplicon. The latter PCR amplicon was obtained by the phenotypically identified *P. carotovorum* subsp. *carotovorum* strains only, but in the other examined strains, the PCR products were not observed. These results agree with those obtained by Weisburg et al. (1991) who reported that for definitive identification of *P. carotovorum* strains, 16S rRNA gene analysis was carried out, using two PCR primers for the specific 16S rRNA gene of *P. carotovorum* (GenBank Accession No. KP187511.1). The obtained nucleotide sequence of the 16S rRNA PCR product was in alignment with that of *P. carotovorum* 16S rRNA gene sequence. These results are in harmony with those obtained by Toth et al. (2001) who proved that the 16SrRNA analysis was used to identify the isolated bacterial strains at the molecular level. Yap et al. (2004) had similar results and identified a single amplicon produced by specific primers; however, Zhu et al. (2010) obtained four amplicons produced by the designed 16S rRNA gene primers.

Phylogenetic analysis

Searching nucleotide database, using a nucleotide query (BLASTn) of all isolates' sequences, obtained from 10 isolates that amplified with Fs1 and Rs1 set of primers, showed a high similarity with different strains of *Pcc* deposited in the GenBank (Accession No. KP187511.1). The nucleotide sequence similarity percentage ranged from 87 to 98%, which confirms a variation among the 10 isolates (Fig. 2a–d).

Isolates *Pcc*1, *Pcc*2, *Pcc*3, and *Pcc*4 clustered closely to reference strains Y46 (Acc. No. KP187511.1) with 87 to 99% bootstrap value, whereas isolates *Pcc*6 and *Pcc*8 clustered individually and were closer to the *Pcc* strain Y46 (Acc. No. KP187511.1) with 100% bootstrap value. Moreover, isolates *Pcc*5 and *Pcc*7 clustered to the reference strain Y46 (Acc. No. CP001657.1) and *Erwinia carotovorum* (Acc. No. KP405846.1) with 90 to 92% and 95 to 99% bootstrap value, respectively.

In the present study, PCR was carried out for all DNA extracts of bacterial soft rot isolates that were identified by biochemical tests as *Pcc*. The results of the 10 isolates

Table 4 Homology search (BLAST) results of all sequenced samples

No.	Primer set	Isolate*	No. of bases	*E* value	Maximum similarity hit
1	Fs1/Rs1	Pcc1	250 bp	5e−124**	93–98%
2		Pcc2	240 bp	6e−92**	94–98%
3		Pcc3	240 bp	7e−110**	87–92%
4		Pcc4	238 bp	7e−97**	96–99%
5		Pcc5	240 bp	8e−109**	90–92%
6		Pcc6	210 bp	4e−30**	99–100%
7		Pcc7	210 bp	1e−93**	95–99%
8		Pcc8	210 bp	2e−104**	98–99%
Acc. No. KP187511.1		Pcc9	*P. carotovorum*	4e−106	100%
Acc. no. KP405846.1		Pcc10	*Erwinia carotovorum*	6e−16	100%

*The type of product that have been sequenced (PCR products) and ** significance

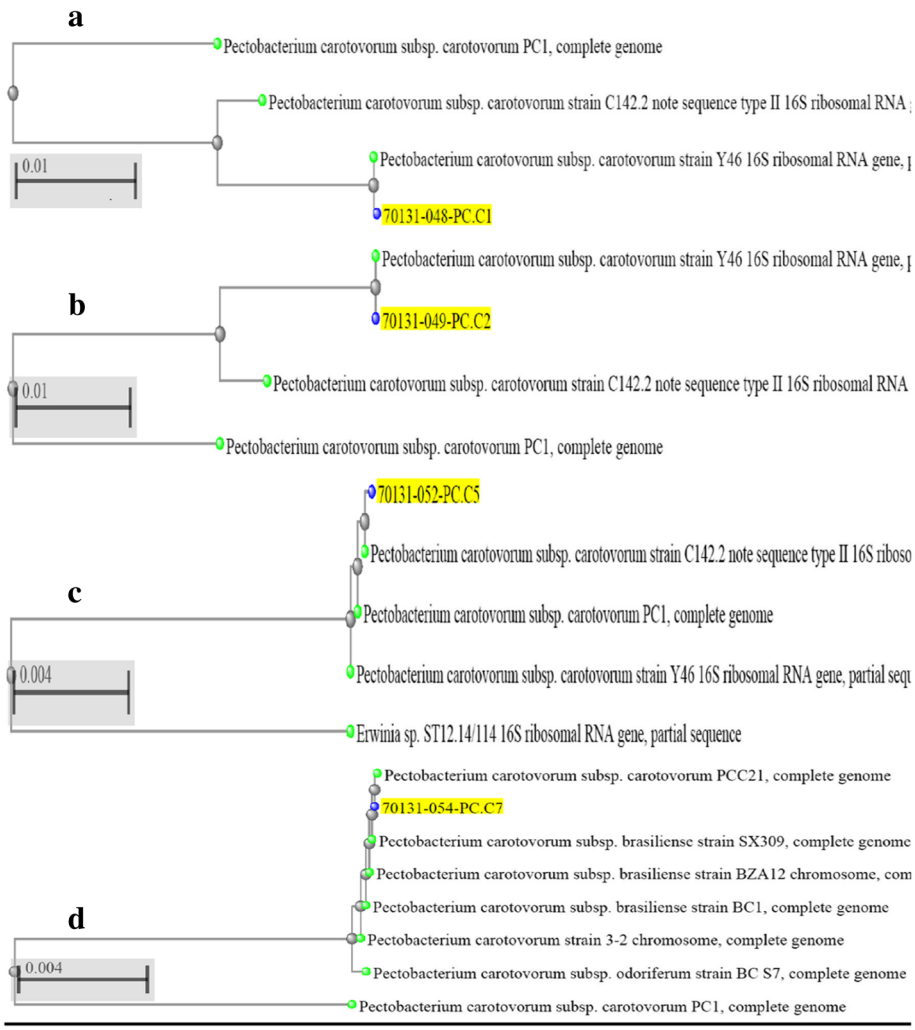

Fig. 2 Phylogenetic analysis of nucleotide sequences of *Pectobacterium carotovorum* isolates (*Pcc*) detected, based on sequences of Pc.c1 = 250 bp (**a**), Pc.c2 = 240 bp (**b**), Pc.c5 = 240 bp (**c**), and Pc.c7 = 210 bp (**d**) and aligned on different regions of the 16S ribosomal gene, with close species. The branching pattern was generated by the Neighbor-Joining method; stability of the tree was assessed by 1000 bootstrap replication

examined detected the presence of the desired DNA fragments of 1530 bp, using the 16S rDNA set of primers Fs1 and Rs1 resembling the positive DNA extract of the reference strain *Pcc* Y46 (Acc. No. KP187511.1), which gave a product size of 1530 bp. The 16S rDNA sequences were conserved with stable copies, and their analysis were characteristic than other ribosomal regions. In general, 16S rDNA was amplified and sequenced by universal primers to identify species and subspecies (De Boer et al. 2012).

In the homology search, BLASTn of *Pcc* isolates sequenced on the bases of the 16S rDNA showed high similarity, with diverse reference strains at the GenBank (Acc. No. KP187511.1 and Acc. No. KP405846.1) that were closely related to the sequences of other bacterial rotting causal agents such as *Pseudomonas* spp., *Bacillus* spp., *Serratia* spp., and *Enterobacter* spp. In fact, the Fs1 and Rs1 primers that were used in the study were general primers, which can detect different bacterial causal agents (Shrihari and Negi 2011) and could be used as an exploratory step in bacterial identification.

In the majority of the cases, genotypic isolates were more carefully related than those isolated from different geographical regions. However, the similarity among the groups of isolates separated from several regions suggested the same genetic origin of these isolates.

Soft rot disease is widely common in different potato-growing areas in Egypt, and the results of biochemical and physiological experiments confirmed that the main causative agent of bacterial soft rot in Egypt is *P. carotovorum* subsp. *carotovorum*, while using the PCR primer pair Fs1/Rs1 was authoritative in detection and identification of all the soft rot Egyptian isolates of *Pcc*. DNA sequencing was found to be the most

reliable way in specific detection and affirmation of the causal agent of soft rot. Furthermore, studies to assay soft rot disease etiology and epidemiology are needed. Compared to diversified DNA sequence analysis, used in this study, biochemical experiments were able to identify most isolates but misidentified others.

Effect of essential oils, antibiotics, and fungicides on the P. carotovorum in vitro

Assays of potential management materials in vitro and in vivo were carried out on the three most virulent isolates of *Pcc* (*Pcc*1, *Pcc*5, *Pcc*10), as confirmed by the pathogenicity test. The different isolates varied in their sensitivity to the examined materials. Notably, there is a positive correlation between the growth inhibition and the concentration increase of all examined materials. At the highest concentrations, most of them positively inhibited the growth of the pathogen (Fig. 3). Peppermint oil showed the greatest inhibitory effect among all the examined materials. Surprisingly, the effect caused by the highest concentration of the nano-copper-based fungicide Tango was worthless. Among the tested antibiotics, tetracycline showed higher inhibitory effect than erythromycin that equals nearly a twofold increase. Preferable control of *E. carotovora* by erythromycin and tetracycline and partial control with copper compound were also reported by Mills and Hurta (2006).

The antibiotics tetracyclines and oxytetracycline stimulate their bactericidal effect by the inhibition of protein synthesis; present studies also approved the bactericidal efficiency of such chemicals against *P. carotovorum* subsp. *carotovorum*. Among the examined fungicides, Galben-Cu was nearly had equal effect as copper oxychloride. Tango showed three- to fourfold decrease in inhibition than the former two fungicides (Fig. 3). Concerning safety, peppermint oil is considered the preferable treatment for controlling *Pcc* isolates in vitro.

In vivo management trials of Pcc soft rot disease on potato

The results showed that the effect of the same examined compounds in vivo behaved differently than they did in in vitro assays when tested on the same isolates. The results in Table 5 indicate that percentages of disease severity (DS) and disease incidence (DI) were significantly affected by tested treatments. The disease severity values obtained by peppermint oil reached 0.0 to 6.5%, while the values of disease incidence reached 0.0 to 17.5%, recorded as the highest significant decrease as compared to control and depending on the isolate. Meanwhile, clove oil in this experiment reduced the disease severity and disease incidence significantly to be 0.0%. As for the two antibiotics, tetracycline reduced the disease severity significantly to be 0.0% as compared to control, which was better than the effect of erythromycin that reached 0.0 to 1.5% without any significant differences in-between them. In respect of the disease incidence, the same trend was noticed. The tested commonly used copper fungicides equalled in their effect and reduced the soft rot severity (0.0 to 1.5%) as compared to control. Contrary to its effect in vitro, the nano-Cu fungicide Tango reduced the disease severity to 0.0% than in the control. Gakuubi et al. (2016) obtained a strong antibacterial activity with the essential oil of Mexican marigold (*Tagetes minuta* L.) against bacterial plant pathogens, i.e., *P. savastanoi* pv. *phaseolicola*, *Xanthomonas axonopodis* pv. *phaseoli*, and *X. axonopodis* pv. *maanihotis*. In another study, *T. minuta* also showed antibacterial activity against *P. carotovorum* in potatoes grown in the field and in storage (Al Abbasy et al. 2015). The antibacterial activity in the essential oils of several medicinal plants has been related to the attack on the phospholipids in the cell membranes of the microbes, which causes

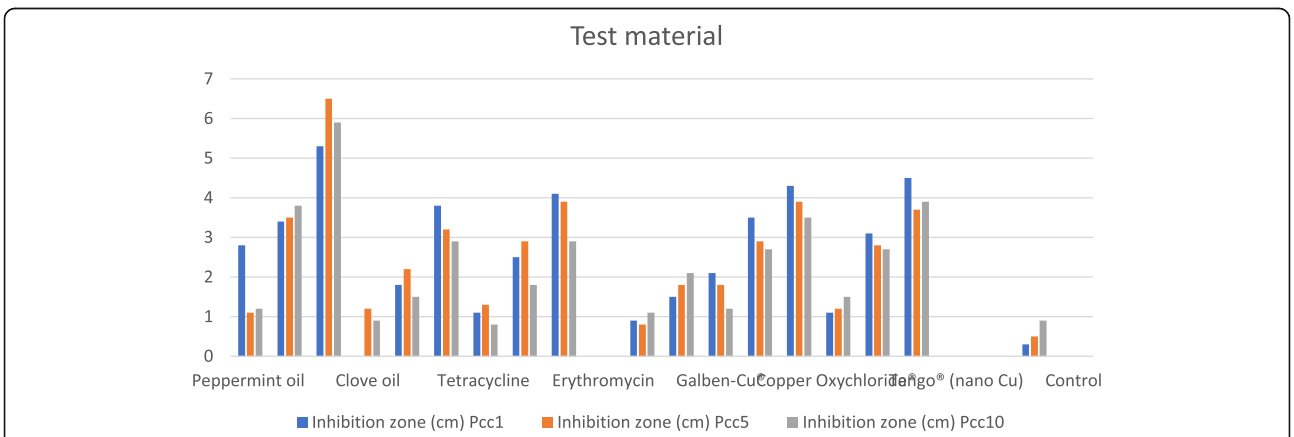

Fig. 3 In vitro inhibitory effect of essential oils, antibiotics, and fungicides on the growth of *P. carotovorum* isolates, 2 days after incubation at 28 ± 2 °C

Table 5 Effect of different materials on soft rot severity on potato tubers 2 and 4 days after storage

Treatment	*Pcc*1				*Pcc*5				*Pcc*10			
	2 days		4 days		2 days		4 days		2 days		4 days	
	DS	DI	DS	DI	DS	DI	DS	DI	DS	DI	DS	DI
Peppermint oil	4.5	17.5	6.5	20.5	0.0	0.0	1.5	4.7	1.5	4.7	4.5	17.5
Clove oil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Erythromycin	0.0	0.0	1.5	4.7	0.0	0.0	1.5	4.7	0.0	0.0	1.5	4.7
Tetracycline	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
Galben-Cu	0.0	0.0	1.5	4.7	0.0	0.0	1.5	4.7	0.0	0.0	1.5	4.7
Copper oxychloride	0.0	0.0	1.5	4.7	0.0	0.0	1.5	4.7	0.0	0.0	1.5	4.7
Tango	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	27.9	53.3	65.5	86.7	37.8	60	61.3	80	33.8	66.7	67.7	93.3

DS disease severity%, *DI* disease incidence%

increased permeability and leakage of cytoplasm thereby killing the bacteria.

Conclusions

Studies of 10 isolates of *P. carotovorum* (soft rot) showed pathogenicity to potato tubers and other hosts. *P. carotovorum*, in the reference strain Pcc Y46 in PCR experiments and the sequencing that followed, revealed that all the isolates yielded 220–272-bp DNA fragments identical to the 16S rRNA gene of Pcc Y46. Potential management against the disease was evaluated by testing two essential oils, i.e., peppermint and clove, and a nano-Cu-based fungicide, i.e., Tango, which were compared to two different antibiotics and two different commonly used copper fungicides. In vitro assays, peppermint oil at the highest tested concentration was the best treatment, giving higher inhibition zones than the antibiotics and copper fungicides. PCR-based assay made it easy and accurate to identify Egyptian isolates of the soft rot causative agent *P. carotovorum* subsp. *carotovorum* from diseased potato tubers. Clove essential oil was a new safe material for the management of soft rot disease of potato tubers in storage.

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