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Activity assessment of potato soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum* under certain metallic salts effect

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Abstract: Ten isolates of *Pectobacterium carotovorum* subsp. *carotovorum* were isolated from naturally soft rot tubers of potato plants collected from Assiut and Sohag governorates, Egypt. All the tested isolates infect potato tubers with different degrees of disease severity, isolate No. 3 showed the highest disease severity followed by isolate No. 5 and the lowest one was isolate No. 2. The growth assessment of soft rot pathogen of potato tubers was carried out in the presence of metallic salts "silver nitrate ($AgNO_3$), chromium III sulfate hydrate ($Cr_2(SO_4)_3.12H_2O$) and zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)" under laboratory conditions. The three selected salts revealed inhibiting activity against the pathogen using disc diffusion Kirbey-Bauer method. A clear zones at minimum inhibitory concentration made by silver nitrate, chromium III sulfate hydrate and zinc sulfate heptahydrate were 9.45mm / 150 µg/ml, 9.28mm / 200 µg/ml and 7.18mm / 400 µg/ml respectively, which indicate the bacterial growth reduction with percentage 21.2%, 20.8 % and 16.1 %, respectively. Further study needs to explain the soft rot disease progress made by *P. carotovorum* subsp. *carotovorum* in the presence of the three salts effect under storage conditions.

Keywords: Potato soft rot, metallic salts, antibacterial effect, *P. carotovorum* subsp. *carotovorum.*

1.Introduction

Potato (*Solanum tuberosum* L.) is categorized among the most valuable tuber crops worldwide for many reasons such as local consumption, economic and exportation value **[1]**. Also, potato is considered very important source of different necessary components that needed for health like protein, carbohydrates and fats [**2**]. Potato represents strategic crop not only for local demands but also for the whole world. Potato production is negatively affected by various infections such as bacterial soft rot that originated by member of enteriobactericeae family *Pectobacterium carotovorum* subsp. *Caroto-vorum* (*Pcc*). It is very destructive potato tuber disease under the favorable conditions of environment that needed for the microbial growth [**3,4**]. Though the amount of antibiotics used against plant pathogens is skimpy, still the antibiotic resistant plant pathogens have emerged which further make more difficult their management, also further antibiotics on the plant surfaces loses activity against plant pathogens swiftly [**5**]. Antibiotic resistance was one of the world's most essential problem for public which can confer a huge loses in the future, by microbes being resistant to antibiotic treatment [**6,7**]. Scientists have used alternative methods for soft rot pathogen control such as avoiding contamination and reliance on seed certification schemes with widely use but with partially successful. Also, *Streptomyces sioyaensis* as a biocontrol and cinnamon oil were used for growth inhibiting of potato soft rot pathogen [**8**].

Recently, several workers suggested that metallic salts are alternative and safety antimicrobial agents for different bacterial pathogens. Metallic salts treatment used to

control plant pathogens because these are simple salts contain metal ions such as silver, chromium and zinc that have high cytotoxicity against different microbes, [**9-12**]. This research focused the methods required for isolation of bacterial potato tubers soft rot agent within naturally diseased potato tubers, these tubers were collected from different localities in Assiut and Sohag Governorates with making complete microbial identification. The main point was the bacterial growth assessment of the pathogen under the lab conditions by metallic salts application such as silver nitrate, chromium III sulfate hydrate and zinc sulfate heptahydrate as antibacterial compounds.

2.MATERIALS AND PROCEDURES

Isolation procedures of soft rot pathogen:

For the isolation process, forty eight different bacterial isolates were obtained from naturally infected potato tubers as the main source for sot rot pathogen. These infected tubers were obtained from different isolation areas in Assiut Governorate such as Sedfa, Abu-Tij, Abnub, Manfalout and Assiut city city (with the GPS coordinates of 27°10'51.46"N and 31°11'1.25"E) and Sohag Governorate such as Tahta, El-Manshah, Girga, El-Balyana and Sohag city (with the GPS coordinates of 26°33'32.6664"N and 31°41'44.4156"E), Egypt. They were sterilized by immersing its surface in 1% sodium hypochlorite solution (NaOCl) for 2 min in sterilized H_2O, they were washed twice, small part of diseased tissues macerated in sterilized H_2O in Petri dishes. After that, one loop of the resulting suspension was streaked over the surface of the enriched and suggested solid medium of nutrient sucrose agar (NSA) in clean and sterilized Petri-dishes **[13]**. Then incubated at 27±2^{o}C for 48 h, and examined for the colony development. For pure cultures preservation, single colony technique was used.

Disease severity test (Pathogenicity):

The disease severity test (pathogenicity test) was applied to forty eight bacterial isolates and defined as the ability of pure bacterial pathogens to make a disease under suitable conditions. The bacterial suspension needed for pathogenicity was prepared as followed: a pure bacterial suspension from early log-phase cells was obtained by growing single and pure colony in nutrient broth, incubation for 48 h/27±2^{o}C on a rotary shaker at 150 rpm. Centrifugation of the resulted suspension for 8 minutes at 10.000 x g, then the pellet re-suspended in sterilized distilled water. The cell density adjusted to be $5x10^8$ cfu/ml [**14**], corresponding to optical density (0.2) using Spectronic 20D spectrophotometer instrument at wavelength 620 nm. For the pathogenicity tests, healthy potato slices and tubers were used. Firstly, the test on healthy slices carried out on healthy Burren cultivar as followed: from healthy tuber with sterilized surface, one centimeter of potato tuber were cut into fine slices and placed in sterilized Petri dishes supplemented with moist sterilized filter paper. A 100 µl ($5x10^8$ cfu/ml) of 48 h-old cultures of pathogenic bacteria planned over the surface of slices. Incubation of plates at 27±2^{o}C with examination of rotting daily for 72 h. Secondly, pathogenicity test on healthy tubers was proved within surface sterilized with 1% sodium hypochlorite. One cm in depth and half cm in width, a cavity was made in each healthy tuber by sterilized cork-borer and 100 µl of adjusted suspension 48 h-old cultures ($5x10^8$ cfu/ml) of tested isolate injected inside each cavity, then covered with removed potato plugs. Tested tubers were kept in clean and sterilized plastic boxes each contained a sterile damp cotton and incubated for 96 h at 27±2 ^{o}C. Five tubers were used as replicates for each isolate and the experiment was repeated twice.

Disease severity %:

Disease severity % was determined after 96 h of inoculation according to equation used for disease severity percentage % estimation **[15]**:

$$\textbf{Disease severity (\%)} = \frac{\textbf{TW1}-\textbf{TW2}}{\textbf{TW1}} \textbf{ X 100}$$

Where: TW1, TW2 are the Total weight of tuber with and without rotting tissue.

Characterization of soft rot bacterial pathogen:

The pathogenic property of the tested bacterial isolates was mathematically investigated by the percentage of disease severity within visual soft rot symptoms onto treated potato slices and tubers. Also, the microbiological identification of pathogenic isolates were identified according to the morphological, cultural and physiological properties according to the volume II of

Bergey's Manual of Systematic Bacteriology 2nd edition, the *Proteobacteria*, part B *Enterobacteriaceae* [**16**].

Application of metallic salts against *Pectobacterium carotovorum* subsp. *carotovorum* (*In vitro* studies):

Three different salts mainly are, silver nitrate ($AgNO_3$), chromium III sulfate hydrate ($Cr_2(SO_4)_3.12H_2O$) and zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) were used in this study are of fine analytical grade and purchased from Alpha Chemika and Oxford Lab chem.(Mumbai, India). The antibacterial impacts of these salts are applied within 5mm discs soaked in solutions with concentrations range from 50 µg/ml to 4000 µg/ml against potato soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum* isolate No. 3 causing the highest disease severity.

The assessment of *P. carotovorum* subsp. *carotovorum* growth activity under metallic salts effect through disc diffusion method:

The activity of soft rot pathogen was evaluated in the presence of metallic salts under laboratory conditions within disc diffusion method [**13**]. For preparation of bacterial suspension, highly pathogenic isolate of *P. carotovorum* subsp. *carotovorum* isolates No. 3 was grown in 100 ml of nutrient sucrose broth (NSB) medium at 250 ml Erlenmeyer flasks. Then incubation for 48 h at 27±2 °C on a rotary shaker at 150 rpm after that centrifugation step of pathogenic cell suspension at 10.000 x g/8 min and the pellet re-suspended in sterilized distilled H_2O. The optical density of suspension adjusted to be $5x10^8$ cfu/ml that correlated to (OD 0.2) at wavelength 620 nm [**14**].

Twenty ml of molten sterilized nutrient sucrose agar (NSA) poured into sterilized Petri-dishes and left to stand and cool. In each of the Petri-dish, one loopful of adjusted bacterial suspension streaked on medium surface, four sterilized discs 5 mm in diameter previously damped in each solution with concentration range 50 µg/ml to 4000 µg/ml and put in equal distance. In each plate, only one concentration used with incubation for 48 h/27±2^oC after that, inhibition zones in mm were measured of each metallic salt and correlated to MIC/MBC value "Minimum Inhibitory Concentration/Minimum Bactericidal Concentration". For each treatment, four replicates were applied and twice.

Statistical analysis:

Data was subjected to statistical analyses of variance was carried out using SAS computer program version 9.0 [**17**]. Means were compared using Duncan`s multiple rang test at level of $P \leq 0.05$ [**18**]

EXPERIMEMENTAL RESULTS

Isolation and disease severity results of soft rot pathogen:

From naturally infected potato tubers, only ten / forty eight pure isolates of bacterial causal pathogen of soft rot disease were obtained as shown in **Fig.** (**1**). Severity degree of soft rot disease caused by these pure isolates was investigated by healthy potato tubers and slices as revealed in **Figs.** (**2A,B**) and **Figs.** (**3A,B**). Results were manifested in **Fig.** (**4**), the results exhibited that the ten isolates were able to cause soft rot symptoms with various degree onto the tubers Burren cultivar. Isolate No. 3. with 75.22% as the highest disease severity, isolate No. 5 came next with disease severity 49.66% then the ranking of disease severity made by isolates No. 7,8,9 & 10 with 42.18%, 39.36%, 35.8% and 31.58%, respectively. The intermediate disease severity showed in isolates No. 6,1 & 4 with values 17.18%, 14.38% & 11.4%, respectively. Lowest disease severity exhibited by isolate No. 2 with value 4.06%.

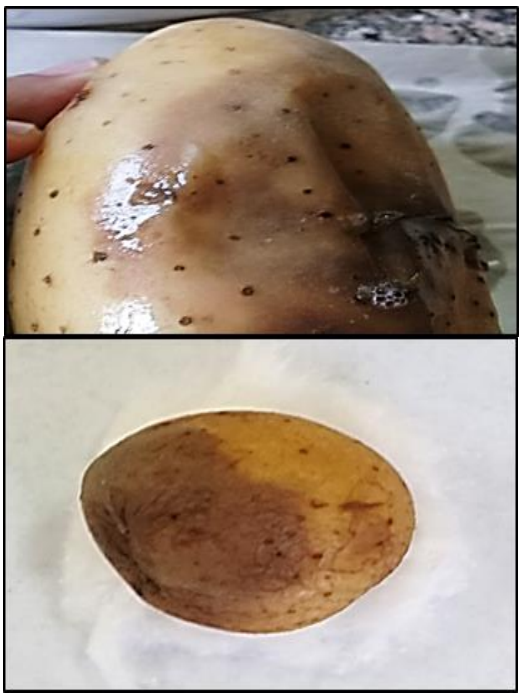

Fig. (1): Naturally infected potato tubers with soft rot symptoms

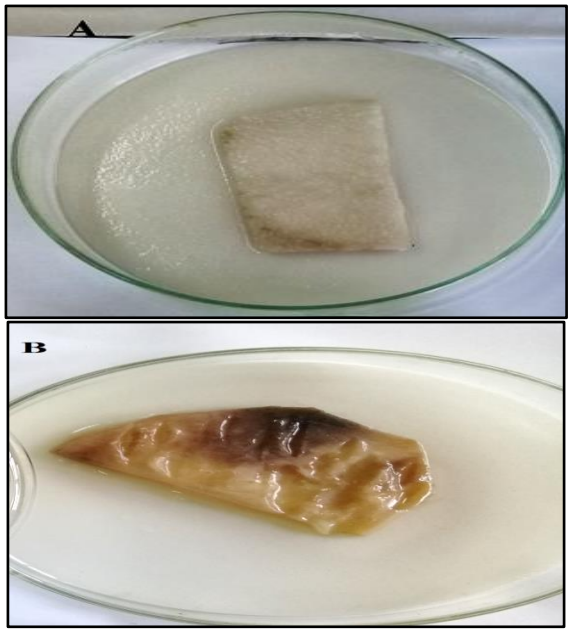

Fig. (2):- Pathogenicity test:- (A): Control potato slice. (B): Positive soft rot

Fig. (3): Pathogenicity test:- (A): Control potato tuber. (B): Positive soft rot

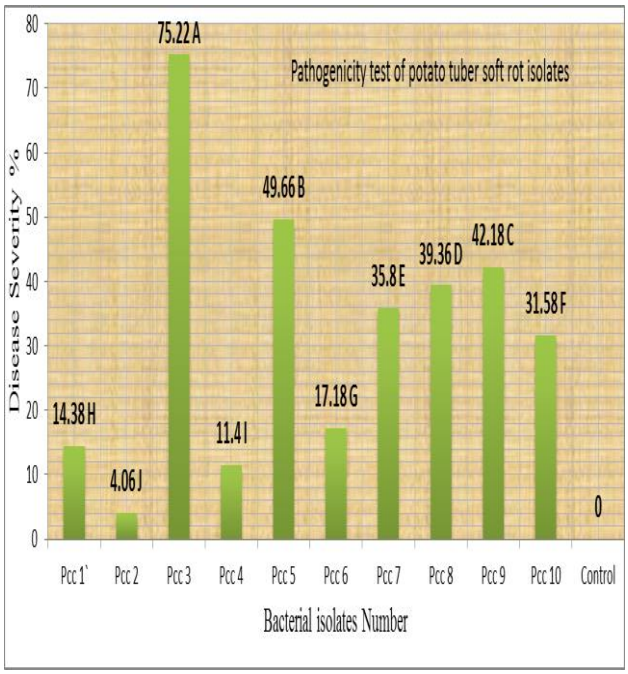

Fig. (4): Pathogenicity test of soft rot bacterial causal isolates on potato tubers.

* Means with the same letter vertically are not significantly different according to Duncan's multiple range test at level of ($p \leq 0.05$).

Characterization of soft rot bacterial pathogen:

Microbial characterization of bacterial soft rot pathogen isolated from infected potato tubers was carried out according to morphological and physiological properties. The morphological properties included: the cell shape, motility, Gram staining and spore forming as well. Meanwhile, the physiological properties included several tests such as starch hydrolysis test, hydrogen sulfide production test, catalase test, Voges-Proskaur test, citrate hydrolysis test, methyl red test, reducing compounds from sucrose test, gelatin liquefaction, casein hydrolysis and fermentation process carried out within different carbon sources such as glucose, fructose, sucrose, lactose, mannitol, sorbitol, dextrose and dextrin.

The microbial characterizations of the tested bacterial isolates were manifested in **Table** (**1**). Results exhibited that the ten tested isolates showed the following properties: rod-shaped, motility, Gram negative staining, non-spore forming, positive catalase test (gas bubbles forming), negative H_2S production test, negative starch hydrolysis test, negative Voges-Proskaur test, positive gelatin liquefaction test, positive methyl-red test, able to hydrolyze casein, positive citrate hydrolysis test and did not able to form reducing compounds from sucrose. Fermentation results revealed that all tested isolates have the tendency to utilize carbon source glucose, fructose, sucrose, lactose, mannitol, sorbitol, dextrose and dextrin and produce acid with variety in gas generation. According to the microbial characterization results of the tested isolated that were agreed with several authors who identified as *Pectobacterium carotovorum* subsp. *carotovorum* [**3, 16, 19]**. The rode-shaped of bacterial cell causal pathogen of soft rot was

identified through transmission electron microscope (TEM) and the colony shape of bacterial causal pathogen on (NSA) media was circular and white near to creamy **Figs.** (**5, 6**).

Fig. (5): Colony shape on NSA medium

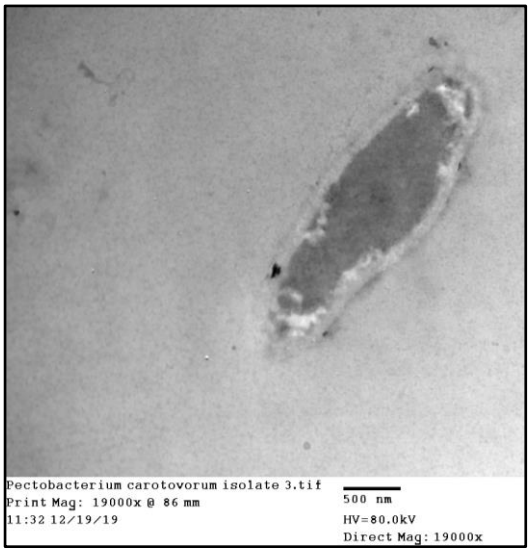

Fig. (6): Rod-shaped cell by (TEM).

Table 1: Microbial characteristics of the ten pathogenic isolates of potato tuber soft rot disease.

Property	The isolated pathogenic bacterial isolates No.										
	STD isolate*	1	2	3	4	5	6	7	8	9	10
Cell shape	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)
Motility	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)	(M)
Gram staining	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Spore forming	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
H_2S production	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Methyl-red	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Voges-Pros.	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Gelatin liqu.	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Citrate test	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Casein hydro.	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Reducing From	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
sucrose	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Catalase	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Starch hydro.	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Fermentations	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Sucrose	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Glucose	A/G	A	A	A	A	A/G	A	A/G	A/G	A	A
Fructose	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Lactose	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Mannitol	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Sorbitol	A/G	A/G	A/G	A	A/G	A	A/G	A	A/G	A/G	A/G
Dextrose											
Dextrin											

*STD: Standard identified *Pectobacterium carotovorum* subsp. *carotovorum* isolate [**16**].
(+) = Positive reaction (-) = Negative reaction R = Rod M = Motile A= Acid G= Gas

Activity assessment of *Pectobacterium carotovorum* subsp. *carotovorum* under metallic salts "$AgNO_3$, $Cr_2(SO_4)_3.12.H_2O$ and $ZnSO_4.7H_2O$"

***In vitro* studies:**

Data in **Table** (**2**) showed that all three metallic salts have inhibition activity against the growth of *P. carotovorum* subsp. *carotovorum* isolate No. 3 *in vitro* with relative difference in its antibacterial activity that measured by the size of clear zone. The clear zone indicated the bacterial growth reduction with preferable result made by silver nitrate followed by chromium III sulfate hydrate and zinc sulfate heptahydrate.

Table 2: Table of inhibition zone (mm) of the metal salts a) $AgNO_3$, b) $Cr_2(SO_4)_3.12.H_2O$ and c) $ZnSO_4.7H_2O$ against *Pectobacterium carotovorum* subsp. *carotovorum* isolate No. 3.

Conc. (µg/ml)	Inhibition Zone diameter (mm)		
	a)	b)	c)
50	0.00 U*	0.00 U*	0.00 S*
100	0.00 U	0.00 U	0.00 S
150	9.45 T	0.00 U	0.00 S
200	10.08 S	9.28 T	0.00 S
250	10.43 R	9.78 S	0.00 S
300	11.05 Q	10.28 R	0.00 S
400	12.28 P	11.13 Q	7.18 R
500	13.35 O	12.38 P	8.05 Q
700	15.65 N	14.48 O	10.25 P
900	17.83 M	16.43 N	12.38 O
1000	19.15 L	17.65 M	13.38 N
1200	21.45 K	19.78 L	15.48 M
1400	23.73 J	21.88 K	17.43 L
1600	26.03 I	23.83 J	19.68 K
1800	28.48 H	26.08 I	21.78 J
2000	30.65 G	28.18 H	23.85 I
2200	33.03 F	30.15 G	25.85 H
2400	35.35 E	32.23 F	28.08 G
2600	37.65 D	34.13 E	30.15 F
2800	39.83 C	36.25 D	32.13 E
3000	42.35 B	38.38 C	34.30 D
3300	44.60 A	41.50 B	37.40 C
3600	44.60 A	44.60 A	40.45 B
4000	44.60 A	44.60 A	44.60 A
Control	0.00	0.00	0.00

*Means with the same letter vertically are not significantly different according to Duncan's multiple range test at level of ($p \leq 0.05$).

DISCUSSION

The bacterium, *P. carotovorum* subsp. *carotovorum* causes serious damage to potato tuber causing marked losses in Assiut and Sohag Governorates, Egypt. The problem of soft rot occurs under different conditions such as in the field, during the storage and marketing. The present research was carried out to obtain some investigations that could be useful as a new application required for the growth inhibition of bacterial soft rot pathogen *P. carotovorum* subsp. *carotovorum* the causal agent of potato tuber soft rot disease.

Characterization results discussed herein showed that the ten tested isolates recovered from naturally infected potato tubers with soft rot symptoms collected from "Abu-Tij, Sedfa, Manfalout, Abnub as well Assiut city" within Assiut Governorate and "Tahta, El-Manshah, Girga, El-Balyana as well Sohag city" within Sohag Governorate were able to cause soft rot disease for healthy tubers and slices. These ten isolates were identified as *P. carotovorum* subsp. *carotovorum*. The morphological and physiological results revealed that the ten bacterial isolates were characterized by the following, rod- shaped, gram-negative staining, motile, non-spore forming, positive catalase test, positive Methyl-Red (MR) test, negative Voges-Proskaur (VP) test, not able to produce hydrogen sulphide (H_2S), not able to produce reducing compounds from sucrose, not able to hydrolyze starch, positive casein hydrolysis test, positive gelatin liquefaction test and able to hydrolyze citrate, in addition to the fermentation process of different carbon sources and produce acid and variable gas production. All these results are agreed with those demonstrated by several authors [**3, 19**] and according to volume II of "Bergey's Manual of Systematic Bacteriology" 2nd edition, the *Proteobacteria*, part B *Enterobacteriaceae* [**16**].

From our first findings, there were direct effects of the three chemicals silver nitrate, chromium III sulfate hydrate and zinc sulfate heptahydrate towards the pathogen, *in vitro*, through the clear zone as an indicator of growth reduction of the pathogen made by the chemicals. The assessment studies serve as an important allusion of the sensitivity of *P. carotovorum* subsp. *carotovorum* towards these salts to control the vitality of the soft rot pathogen. These conclusions are the first application on *P. carotovorum* subsp. *carotovorum* and agreed with other reports [**12,20**] who found that inhibitory effect caused by silver nitrate ($AgNO_3$) against *Escherichia coli* by using different methods of assay at minimum inhibitory concentration value 12.5 µM (at equivalent Ag concentrations) and 130 ppm, respectively.

Chromium III sulfate hydrate ($Cr_2(SO_4)_3.12H_2O$) salt possessed antibacterial activities against *Pectobacterium carotovorum* subsp. *carotovorum* as the first application against *P. carotovorum* subsp. *carotovorum*. These result makes in line with the findings [**11**] who reported and proved that chromium III salt at least possessed antibacterial activity against Gram+ve bacterium *Bacillus cereus* growth with reduction value 79% and 76.6% at Cr^{3+} salt concentrations 0.1 and 0.5 mM/L, respectively.

Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) salt against *Vibrio fischeri* exhibited antibacterial activity as mentioned [**10,21**] who studied anti-bacterial activity of zinc sulfate heptahydrate salt against Gram -ve *Vibrio fischeri* with minimum inhibitory concentration value 10 mg/l. The antibacterial activity of zinc sulfate heptahydrate is investigated [**22**] who assessed minimum inhibitory concentration value on the *Vibrio cholera* growth at 2 values (0.5 mg/l and 1mg/l).

The three chemicals exhibited various antibacterial activities according to its metallic toxicity towards the bacteria within the effective ions Ag^+, Zn^{2+} and Cr^{3+}. The results of silver nitrate on the first activity ranking due to the toxicity nature of silver that allows to produce reactive oxygen species (ROS) and makes oxidative stress to bacterial cell components **[23]**. Another effect of silver ions was the existence of the Na^+-translocating NADH:quinone oxidoreductase, as a particular target for sub-micromolar Ag^+, indicating that the other Ag^+- modified membrane proteins (or perhaps the Ag^+-modified phospholipid bilayer itself) could cause the H^+ leakage, thus explaining the wide spectrum of the antimicro-bial activity of silver ions, by making collapse the proton motive force [**24**]. Chromium III sulfate hydrate on the second ranking according to the toxic effect of Cr^{3+} as mentioned by scientists [**25,26**] who stated toxic effect of Chromium III ions on crop growth too. Zn II ions also have the tendency of binding to the membranes of the micro-organisms, conseq-uently prolonging the lag phase of the growth cycle and increasing the generation time of the organisms needed to make a complete cell division as demonstrated by [**22, 27**].

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