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INFLUENCE OF SOME FACTORS ON SUSCEPTIBILITY OF TWELVE VIRULENT ISOLATES OF *RALSTONIA SOLANACEARUM* TO FIVE NANOPARTICLES

Amira Rabea[1*] ; E. Naeem[2] ; Naglaa M. Balabel[1,3] ; M.S. Hanafy [1,3] and Ghadir E. Daigham[2]

[1]Bacterial Disease Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt

[2]Department of Botany and Microbiology, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, Egypt

[3]Potato Brown Rot Project, Ministry of Agriculture, Dokki, Giza, Egypt

[*]**E-mail-amirasun86@yahoo.com**

ABSTRACT

Overall objective of the present study was to evaluate presence of pathogenic *Ralstonia solanacearum* isolates, the causative agent of wilt disease, which isolated from different sources in four Egyptian Goverovernorate. In addition, optimizing the environmental conditions necessary for the antibacterial activities of five nanoparticles *In Vitro* trials against the isolated pathogen isolates. The results indicated that, the highest bacterial incidence (87.2 %) was detected in the samples of El-Behira Governorate, while, the lowest (26.7 %) was recorded in samples collected from El- Ismaelia Governorate. Only twelve isolates were virulent and subjected to further studies. The highest severity of disease incidence was recorded with (R9) isolate. Identification of the isolates from soil, irrigation water, tubers and weeds were carried out by immunofluorescent antibody staining of the pathogenic isolates which showed short rod shaped stained as bright green fluorescent in IF test confirmed that these isolates belong to *R. solanacearum*. Results of Polymerase Chain Reaction (PCR) technique on the twelve isolates, which gave the highest virulence showed that the samples visualize specific 718 bp PCR product under UV light pointing to the very close similarity between the twelve isolates tested of *R. solanacearum*. No variation could be detected as well in (*Taq*-Man) determination, *Taq*-Man is more specific test used to detect races and biovars of *R. solanacearum* by using reference bacteria (standard known bacterial isolates) and no variation could be noticed among the isolates, confirmed that these isolates belong to race 3 biovar2. Influence of different exposure times, temperature and pH values on *R. solanacearum* susceptipility to CuO-NPs and MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration were carried out and the exposure of *R. solanacearum* to NPs for 2, 3 and 4 days demonstrates their pathogenicity efficacy and all NPs were superior for minimizing growth

of *R. solanacearum* at 35°C. Also it was found that, Ag/CsNC was more inhibitive for *R. solanacearum* at low temperature (less than 25°C) and high temperature (45°C). Ag/CsNC compeletly inhibited *R. solanacearum* growth when cultivated in growth medium with pH ranging from 5.6 to 8 than other NPs.

INTRODUCTION

In Egypt potato is the second most economically important crop after citrus. *R. solanacearum* has contributed to catastrophic bacterial wilt, which has resulted in the world-wide reduction of potato production. According to EU regulations for exportation, this bacterium is listed as a quarantine organism. Egypt is the largest potato producer in Africa and potato is an important crop in Egypt for local consumption and exportation **(Mahgoub *et al*., 2015).** In 2020, the total cultivated area of potato in Egypt was 208980.47 hectares producing 6,427,124 tons of tubers with an average yield of 30.75 tones/hectares **(FAOSTAT, 2020).** In fact, bacterial wilt is considered the single most destructive bacterial plant disease because of its extreme aggressiveness, wide geographic distribution, and unusually broad host range **(Perea Soto *et al.,* 2011)**. *Ralstonia solanacearum* caused losses on a number of crops, especially solanaceaous crops in many countries in Sub-Saharan Africa. Potato tuber brown rot or bacterial wilt of potato plants, caused by *Ralstonia solanacearum*, ranked globally as the second most important bacterial plant pathogen after late blight caused by *Phytophthora infestans* in tropical and sub-tropical regions of the world **(Messiha *et al*., 2019).**

Disease is a complicated challenge due to loss of resistant in potato cultivars in addition to latent infection in tubers harding the detection of the pathogen **(Karim and Hossain 2018)**, in addition to the hazardous environmental impact due to excessive use of chemicals used during disease management with traditional control methods. The limitations and less efficiency of these traditional methods have led to the development of new and recent methods for better management of this important challenge. Nanotechnology is the best possible way if keeping their properties in mind, its support plant pathology in many sides, such as detection, identification of plant pathogens and management of plant diseases. Using nanoparticles (NPs), nanocapsules, and nanocrystals, which show a better control with a lower dose and reduce environmental contaminations that has become the need of the hour **(Elmer and White, 2018)**. Due to their antibacterial activities, nanoparticles represent an effective solution for overcoming bacterial resistance **(Rai *et al.,* 2009)**. The emergence of nanotechnology has come with the promising broad spectrum NP-antimicrobial agents due to their vast physiochemical and functionalization properties. In fact, NP-antimicrobial agents are able to

unlock the restrictions experienced by conventional antimicrobial agents **(Wang *et al.,* 2017)**.

The antimicrobial activities of chitosan, magnesium oxide, silver, copper oxide NPs have been previously reported. Recently**,** CsNPs applied successfully to control diseases and increase crop production in many crops **(Borines *et al***., **2015; Abd El-Aziz *et al*., 2019; Esyanti *et al*., 2020).** It is used for controlling plant pathogenic bacteria which has been extensively explored *Erwinia carotovora* and *Agrobacterium tumefaciens* causing bacterial soft rot and crown gall **(Mohammadi *et al*., 2016). Khairy *et al*., (2022)** reported the role of eco-friendly application of nano-chitosan at a concentration of 200 mg/mL for controlling potato and tomato bacterial wilt caused by *Ralstonia solanacearum*.

Nanoparticles of silver (AgNPs) are known to exhibits an efficient antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* **(Kim *et al*., 2011),** *Bacillus subtilis* **(Li *et al.,* 2013),** *Pseudomonas aeruginosa* **(Yan *et al.,* 2018).** Also, it can be widely used in agriculture sector for several purposes especially in the plant protection and management of different plant diseases against some plant pathogenic bacteria in a relatively safer method compared to synthetic pesticides **(Pestovsky and Martinez-Antonio 2017; Rivas-Cáceres *et al*., 2018).**

Cai *et al*., (2018) studied on antibacterial of MgO nanoparticles against *R. solanacearum In Vitro* and *In Vivo*. The results demonstrated that MgO nanoparticles possessed statistically significant concentration-dependent antibacterial activity. However, little is known about the antimicrobial properties of MgO nanoparticles toward plant pathogenic bacteria **(Cai *et al*., 2018).**

Copper in several formulations has been in use to control plant pathogens as documented by **Vanathi *et al*., (2016)** who reported that spherical CuO nanoparticles (28 ± 4 nm in diameter) could exhibit antifungal activity against phytopathogens that decreased in the following order: *Fusarium culmorum* > *Aspergillus niger* > *Fusarium oxysporum* > *Aspergillus flavus* > *Aspergillus fumigatus*.

One more advantage of the nanocomposite also possesses good antimicrobial and biosensing activity **(Sanpui *et al*., 2008)**.

The antibacterial capacities of nanoparticles against plant pathogenic bacteria, however, are little understood **(Cai *et al*., 2018)**. Recently, antibacterial activity of silver and MgO nanoparticles against bacterial wilt were conducted for the first time by **Chen *et al.,* (2016); Imada *et al*., (2016) and Cai *et al*., (2018)**. Also, **Khairy *et al*., (2022)** used *In Vitro* and *In Vivo* nano-chitosan for controlling potato and tomato bacterial wilt. MgONPs exhibited antifungal activity against some plant pathogenic fungi including *Alternaria alternata, Fusarium oxysporum,*

Rhizoctonia stolonifer and *Mucor plumbeus* **(Wani and Shah, 2012)**. CuONPs exhibited antifungal activity against *Alternaria alternata, Rhizoctonia solani* and *Botrytis cinerea* **(Al-Dhabaan *et al*., 2017)** and against *Penicillium digitatum* and *Fusarium solani* on citrus fruit **(Youssef *etal*., 2017).**

The use of nanoparticles could provide a harmless alternative for treating potato tubers brown rot, which are presently one of the main challenge faced by the potato industry in Egypt.

The studies suggest that NPs can be used as intellectual agents to inhibit pathogen *In Vitro* and it is important to optimize the environmental conditions necessary for the antibacterial activities of these nanoparticles.

MATERIALS AND METHODS

1. Samples collection for isolating *Ralstonia solanacearum*:

Samples of soil, potato tubers, irrigation water and weeds developed in the site of sampling soil, were collected from different Governorates in Egypt , namely El-Sharkia (Zagazig, El-Molak and El-Salhia), El-Dakhlia (Mansoura and Meet-Ghamr), El-Ismaelia (Ezz-Eldin and El-Qassasin) and El-Behira (El-Khatatba and El-Nobaria) during seasons of 2019 and 2020. Samples were transported to the laboratory for isolation.

2. Isolation of *Ralstonia solanacearum* from different habitats:

2.1. Isolation from Potato tubers:

Potato tubers exhibited brown rot symptoms were washed in running tap water, surface sterilized by flaming and dipping in ethyl alcohol 70%, and then the ends were aseptically removed. Cores of 5-10 mm in diameter and 5 mm thick, containing main vascular and cortical tissues were macerated in 10 mL sterile phosphate buffer (M, 0.01and pH 7.0) in sterile plastic bags. The macerate was allowed to stand for 30 minutes then the resulting supernatant was streaked on Semi Selective Media of South Africa (SMSA medium) and incubation was done at 28°C as described by **Wenneker *et al*., (1999)**. Fluidal, slightly raised irregular, white or white with pink centers colonies, typical for virulent colonies of *R. solanacearum*, were selected and inoculated on glucose nutrient agar medium at 28°C for 48 hours.

2.2. Isolation from stems of potato plants:

Stems exhibited wilt symptoms were used to isolate the pathogen. These were washed with running tap water and surface disinfected by dipping in ethyl alcohol 70% and flaming. Thin sections (5 mm) were aseptically macerated in 1 mL sterile phosphate buffer (0.01 M and pH 7.0) in small sterile plastic bags and allowed to stand for 30 min. The supernatant was plated (1mL/plate) on SMSA medium and observation of colonies growth was carried out as described above.

2.3. Isolation from irrigation water:

Water samples were collected from the central irrigation system. Three replicates of 50 mL/sample using sterile bottle, ten minutes after a brief course of irrigation then labeled, placed in an ice box then transported to the lab and used directly for isolation. The samples were centrifuged at 10.000 rpm at 15°C for 15 minutes, by using Micro 200 RS Hettich, Germany Centrifuge. The supernatant was discarded and pellet was re-suspended in one mL phosphate buffer (0.01 M and pH 7.2), vortexed for homogenization and plated on SMSA medium **(Wenneker *et al.*, 1999)**. Observation of pathogenic colonies were carried out as previously described above.

2.4. Isolation from soil:

Three samples for each location (100 g) were taken from 30 cm depth, using a sampling auger. Samples were mixed in the laboratory for homogenization then isolation was made on SMSA **(Wenneker *et al.*, 1999)**. Dillutions of the obtained samples were made in 90 mL sterile phosphate buffer (0.05 M and pH 7.2) then shaked for two hours at 15°C. Sterile Petri dishes with SMSA media were inoculated and incubated at 28°C for 3-6 days. The resulted pathogenic colonies were observed as described above.

2.5. Isolation from weeds associated with potato fields:

Weeds associated with potato fields, during the crop development seasons, were collected and isolation was carried out from stems. Sterilized stems were cut to of 5-10 mm and macerated in 10 mL sterile phosphate buffer (0.05 M and pH 7.0), allowed to stand for 30 minutes. The resulting supernatant was plantingon Semi Selective South Africa as described by **Pradhanang *et al.* (2000)**. Pathogenic colonies were observed as described above.

3. Pathogenicity of isolates

Pathogenicity tests were conducted in greenhouse of Bacteriology Department, Plant Pathology Institute. Potato seed-tubers cvs, used for planting in this study, were kindly provided by the Potato Brown Rot Project (PBRP), Agric. Res. Center, Giza, Egypt.

Positive isolates were tested for pathogenic potential(s) via inoculation into healthy potato plants, grown in pots filled with a mixture of sandy and clay soil (1:1, v/v) collected from potato districts in El-Sharkia Governorate, Egypt. Each pot contained 6 kg of soil, and one uniform disease-free Sponta cv tuber was planted in each pot under green house conditions, by the stem puncture technique **(Janse, 1988 and Wenneker *et al.*, 1999)**. Injection was made at the leaf axis after 30 days from planting by a needle laden with the 10^6 CFU /mL of the pathogen suspension. Controls were prepared by using sterile water instead of bacteria. The inoculated seedling was covered with polyethylene bags at

30°C for three days, at high humidity, then bags were removed and pots were irrigated daily. According to Koch's postulates, the inoculated bacterium was reisolated from plants showing wilt syndromes typical of brown rot disease. Re-isolation was made as mentioned above and the most pathogenic ones were selected. The disease severity was determined according to the key proposed by **Kemp and Sequeira (1983)** describing the wilt symptoms in the plant as follow:

1 = no symptoms; 2 = 1-25% of the plant was wilted; 3 = 26-50% of the plant was wilted; 4= 51-75% of the plant was wilted and 5 = more than75%plant was wilted by using the formula as disease index (%) as given below:

$$\text{DI (disease index)} = \frac{\sum(\text{No. of wilted plants in treatment} \times \text{wilt grade})}{\text{Total No of plants} \times \text{highest grade}} \times 100$$

The selected cultures were serologically examined in PBRP using immunofluorescent antibody staining (IFAS) to confirm *R. solanacearum* identity.

4. Potato cultivars response to the most virulent *R. solanacearum* isolate (R9):-

Sponta, Kara, Draga, Famosa and Mundial cultivars were kindly provided by the Potato Brown Rot Project (PBRP), Agric. Res. Center, Giza, Egypt.They were subjected to the most virulent isolate (R9) as described above and percentage of disease incidence % was considered as previously mentioned.

5. Identification and characterization of the pathogen:

5.1. Pigments production:

5.1.1. Plating on Semi selective Medium of South Africa (SMSA) medium:

Plating on SMSA medium suggested by **Engelbrecht (1994)** and modified by **Elphinstone *et al.*, (1996)**, was used for detection the possible variations in morphology of *R. solanacearum* colonies. A loopfull of a slightly turbid bacterial suspension obtained from different sources prepared on nutrient agar for three days old culture, were streaked on SMSA agar medium **(Pradhanang *et al.*, 2000)** then incubated at 28°C for 3-5 days. The developed colonies were examined with a hand lens. Kites of SMSA were obtained from Potato Brown Rot Project (PBRP).

5.1.2. Plating on TZC medium:

Tetrazolium chloride medium (TZC) can help to differentiate between colonies of virulent or avirulent type, which are irregularly shaped white or cream-coloured, and highly fluidal, from colonies of the mutant or non-virulent type which appear uniformly round, smaller, and dry. It is important to note that pathogenic *R. solanacearum* is a slow-growing microbe even on this rich medium. Colonies that were visible in

less than 36hrs at 28°C are not *R. solanacearum*. Often there is a brown discoloration of the medium around the colonies. It is best to incubate at 28°C to increase pigment production **(Denny, 2006).**

5.1.3. Plating on king's B medium:

King's B medium was used to confirm if *R. solanacearum* produced soluble fluorescence stain or not.

5.2. Immunofluorescence Antibody Stain (IFAS):

IFAS is a serological method for rapid detection and presumptive identification of bacteria as defined by **Janse, (1988)**. The procedure was conducted at Potato Brown Rot Project (PBRC), Agric. Res. Center, Giza- Egypt.

5.3. Polymerase Chain Reaction (PCR):

PCR procedure was conducted at Potato Brown Rot Project (PBRC), Agric. Res. Center, Giza- Egypt. PCR is based on the method described by **Pastrik *et al.* (2002)**. Polymerase Chain Reaction (PCR) is one of the high sensitive methods used for verification of identity of *R. solanacearum* isolates from different sources **(Pastrik *et al*, 2002)**. Under the following reaction conditions the expected amplicon size from *R. solanacearum* template DNA is 712 bp using *R. solanacearum* specific oligonucleotide primer OLI-1 (5` GGG GGT AGC TTG CTA CCT GCC3`) and non-specific primer Y-2, (5` CCC ACT GCT GCC TCC CGT AGG AGT 3`).

The steps of this technique were conducted as follows:

1. Extraction of DNA:

Crude DNA of *R. solanacearum*, of twelve isolates which proved to be virulent, were extracted by heating 100 µl aliquots of cell suspension (10^6 CFU/mL) to 100°C for 5 minutes then stored at (-20°C) until use.

2. DNA amplification:

2 µl from each isolate were added to 23 µl reaction mixtures [(16.106 µl, Sterile Ultra Pure Water (SUPW); 2.5 µl of 10X PCR buffer; 1.5 µl MgCl2; 0.125 µl of each d-ATP, d-CTP, d-GTP and d-TTP; 1.25 µl primer OLI-1; 1.25 µl primer Y-2 and 0.1 µl Taq polymerase)]. Different PCR cycles were performed (1 cycle of 5 min at 95°C to denaturate template DNA; 35 cycles of 30 seconds each at 68°C for annealing of primers and 35 cycles of 45 seconds each at 72°C for extension of copy and final extension cycle of 5 min at 72°C **(Pastrik *et al.*, 2002)**.

3. Analysis of the PCR product:

PCR fragments were detected by using agarose gel electrophoresis and stained with ethidium bromide according to **Pastrik *et al.* (2002)**. The positive control and distilled water as a negative control, were mixed gently then loaded into the wells of the gel. An appropriate DNA marker was included as reference in at least one well. Gel was run by applying 80 V voltage at 400 mA (8 v/1cm) until the front of tracking indicator being within 1 cm from the end then the power supply was switched off. Gel was removed carefully and soaked in the

eithidium bromide solution (0.5 µg per mL) for 30-45 min. A specific PCR product of 718 bp was visualized under UV trans-illumination at 355 nm and photographed (**Pastrik *et al*., 2002**).

5.4. Real-time PCR (Taq-Man) assay:

5.4.1 Bacterial suspension preparation and DNA extraction:

Twelve isolates were grown on Casamino Acids Peptone Glucose (CPG) agar for 24 h at 27°C**.** DNA extraction was carried out according to **Weller *et al*. (2000),** where a single colony was transferred to sterile DNA-RNA free eppendorf tubes containing 100 µl of sterile nucleic acid-free water, vortexed, heated to 100°C for 5 min and cooled rapidly on ice. Samples were finally diluted in 900 µl of the sterile DNA- RNA free water and stored at (-20°C) until required. Automated DNA extraction was carried out using Biosprint 15, Qiagen company as cleared in **Table (1)**

Table (1): Reagents used for DNA extraction in automated machine:

Well number	Reagent Name	Reagents volume (µL)
1	Isopropanol	200
1	Magnetic attract suspension G	20
1	Sample	200
2	Buffer RPW	500
3	Ethanol 96 %	500
4	Elution buffer	80

5.4.2 DNA amplification:

DNA amplification was performed in 25-mL volumes using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (Applied biosystems) for each well. All reagents were obtained from Applied Biosystems.

The PCR mixture was prepared as described by **Weller *et al*. (2000),** but optimized for real-time (Applied biosystems,7500) by manufacture company (Applied biosystems) which consists of 12.5 µL from ready universal PCR master mix, 1 µL of forward primer RS-I-F GCA TGC CTT ACA CAT GCA AGT C, 1 µL of reverse primer RS-II-R GGC ACG TTC CGA TGT ATT ACT CA, 1 µL of probe RS-P AGC TTG CTA CCT GCC GGC GAG TG, 7 µL of DNase free water and 2.5 µL from DNA (sample).

Real-time 7500 detection system (Applied biosystems) was used for amplification and fluorescence measurement. All cycles began at 50°C for 2 min and then went to 95°C for 10 min, followed by 40 two-step cycles of 10 second at 95°C and then 1 min at 60°C.

6. Influence of some factors on the susceptibility of *R. solanacearum* to nanoparticles:

6.1. Influence of different exposure times on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

All nanoparticles, included (CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC) were previously prepared and characterized by the author **Rabea**

***et al.* (2023).** Silver, chitosan, copper nitrate, polyvinylpyrrolidone (PVP), sodium hydroxide, and magnesium sulfate, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Louis, MO, USA).

This experiment was done to obtain the optimum incubation time for growth of the bacterial isolate *R. solanacearum* at 200 µg/mL concentration of the selected nanoparticles (CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC) using pour plate method (**Bonev *et al.*, 2008).** In this experiment prepared plates were incubated at 30°C for different incubation periods 24, 48, 60, 72, 84 and 96 h. After each incubation period the number of colony forming units CFU /mL was detected.

6.2. Influence of different exposure temperature on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

This experiment was done to obtain the optimum incubation temperature for growth of the bacterial isolate *R. solanacearum* at 200 µg/mL concentration of the selected NPs using pour plate method. In this experiment plates were incubated at different incubation temperatures 15, 25, 30, 35, 40, 45°C for 48 h. After incubations the number of colony forming units CFU /mL was determined.

6.3. Influence of different pH values on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

This experiment was conducted to obtain the optimum pH for growth of the tested organism *R. solanacearum* at 200 µg/mL concentration of NPs using pour plate method. Nutrient agar medium was adjusted to different pH values 5.6 using acetate buffer and 6.5 to 8 using phosphate buffer. The plates were incubated at the optimal previous conditions. Then CFU /mL was determined for each plate.

RESULTS

1. Isolation, purification and identification of the causal organisms:-

Samples of potato tuber showed brown rot symptoms, irrigation water, soils and weeds were taken from different localities in Egypt, namely El-Sharkia (Zagazig, El-Molak and El- Salhia), El-Dakahlia (Meet –Ghamr and Mansoura), El-Ismaelia (Ezz-Eldin and El-Qassasin) and El-Behira (El-Khatatba and El-Nobaria) for isolation of bacterial pathogens. **Table (2)** illustrated and **Figs. (1&2)** show percentage of twelve isolates isolated from the aforementioned localities.

2. Pathogenicity tests for isolates:

Data in **Table (2)** and **Fig. (1)** proved that the tested *R. solanacearum* isolates were significantly varied in inducing different criteria of disease incidence. The highest incidence was induced by isolate R9 (87.2%), followed by isolate R6 (86.7%), meanwhile isolate R2 (34.3%) and R10

(26.7%) recorded the lowest disease incidence when compared with the control (0.0%).

Table (2): Pathogenicity test for different isolates on Sponta cv measured as percentage of disease incidence

Governorate	Location	Isolate No	Isolate Source	Disease incidence %
Sharkia	El-Molak	R1	weed	63.6
	El-Salhia1	R2	water	34.3
	El-Salhia2	R3	soil	37.8
Dakahlia	Meet –Ghamr	R4	tuber	70.0
	Mansoura	R5	soil	54.0
Behira	El-Khatatba	R6	water	86.7
	Nobaria 1	R7	weed	76.7
	Damanhor	R8	soil	65.6
	Nobaria 2	R9	tuber	87.2
Ismaelia	Ezz-Eldin	R10	soil	26.7
	El- shabab	R11	water	46.3
	Qassasin	R12	tuber	63.3
Control (without infestation)		0.0		

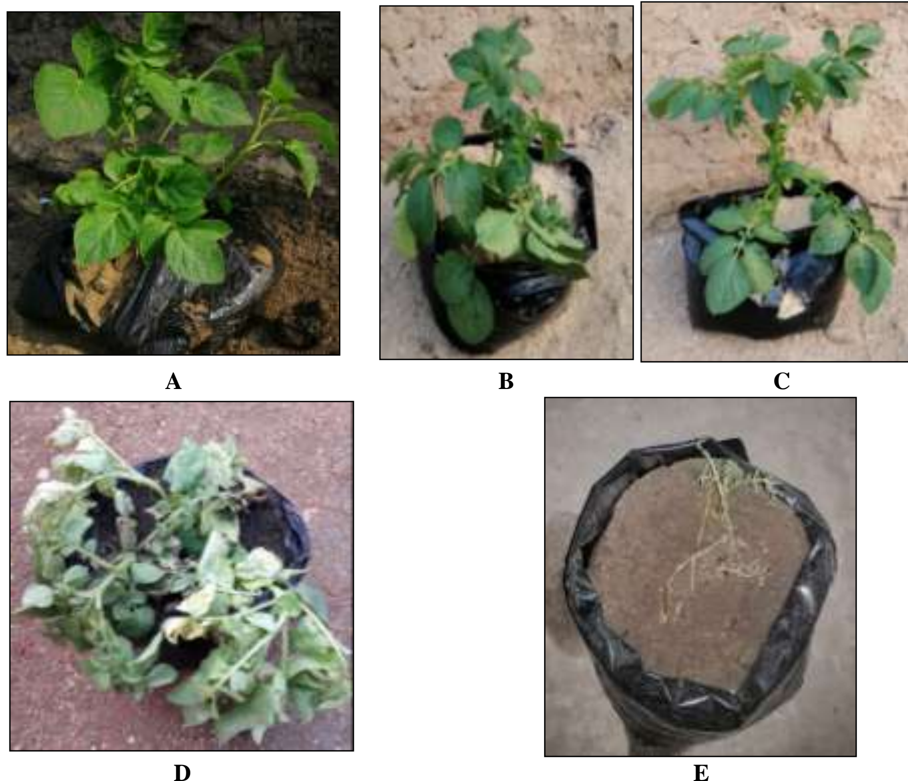

Fig. (1): (A, b and c): Photographs showing sponta cvs healthy plants, (D and E) Wilt symptoms on potato plants infected by *Ralstonia solanacearum* isolate (R2 and R 9).

3. Potato cultivars response to *R. solanacearum* (R9):-

Sponta, Kara, and draga cultivars were the most susceptible ones scoring 87.1, 83.8 and 75.4% of disease severity caused by isolate (R9), respectively, where percentage disease severity was 63.3 and 60.45 in Famosa and Mundial, respectively considered as moderately resistant as shown in **Table (3).**

Table (3): Potato cultivars response to *R. solanacearum* (R9).

CV	Disease incidence %
Sponta	87.1
Kara	83.8
Draga	75.4
Famosa	63.3
Mundial	60.45

4. Pathogen identification:

a. Plating on the SMSA medium:

The typical colony morphology was the main type on SMSA isolation as shown in **Fig. 2.**

Fig. (2): Typical colonies of *R. solanacearum* on SMSA medium.

b. Immunofluorescent antibody staining test :

It is clear from the obtained results that the cells morphology of the tested bacteria had short rod shape stained evenly as bright green fluorescent as clear in **Fig. (3)**.

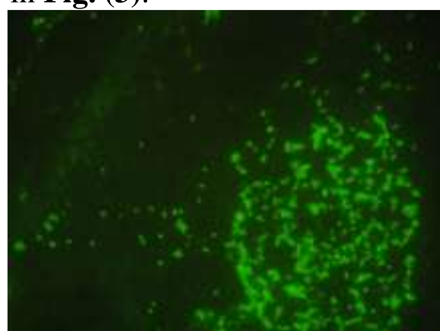

Fig. (3): Cells of *R. solanacearum* under immunofluorescent (IF) microscope (1000X).

C. Polymerase Chain Reaction (PCR):

The results of identification of the tested 12 *R. solanacearum* isolates which isolated from different habitats are shown in **Fig. (4).** In this respect, the visualized specific 718bp PCR product under UV light showed very close similarity of the isolates under investigation.

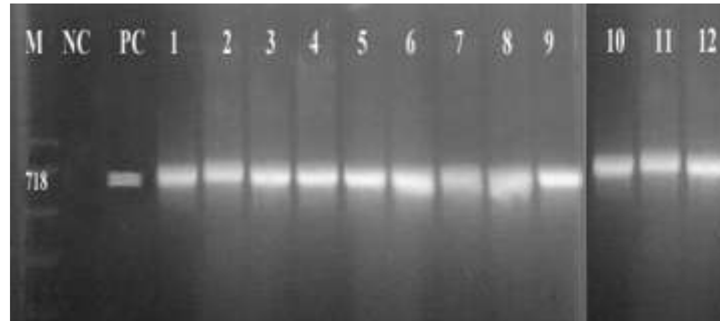

Fig. (4): A single band on the agarose gel electrophoresis at 718 bp showed no variation between the different isolates of *R. solanacearum* that represent different habitats. Where, no. 1- 12= tested *R. solanacearum* isolates, M = marker, N = negative control and P = positive control.

d. Real time PCR (Taq-Man) Assay :

The RS primers and probe are specific for detecting the race 3 biovar 2 of *R. solanacearum*. Positive results were obtained for all tested isolates, indicating that the all twelve tested isolates were *R. solanacearum* race 3 biovar 2 as clear in **Fig. (5).**

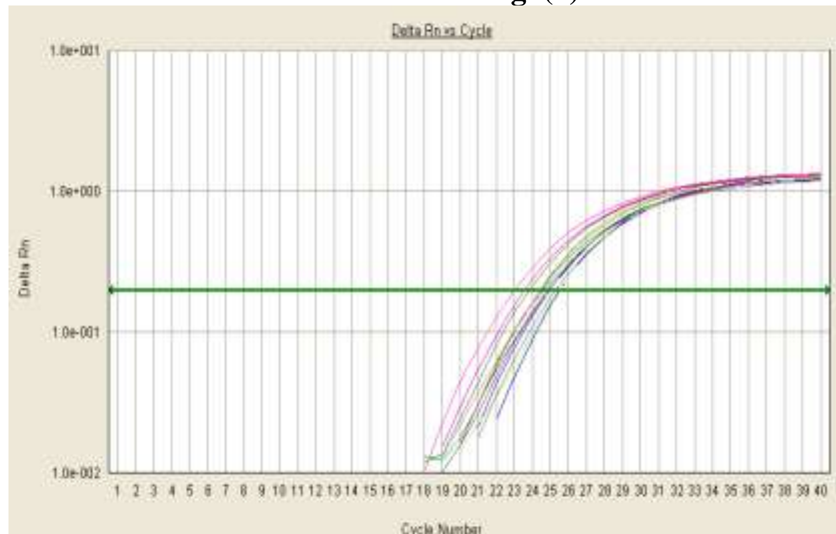

Fig. (5): Real time PCR *Taq*-Man assay of twelve isolates of *R. solanacearum* race 3 biovar 2 isolated from different habitats in Egypt.

5. Influence of some factors on the susceptibility of *R. solanacearum* to nanoparticles:

5.1. Influence of different exposure times on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

Exposure of *R. solanacearum* to different NPs at different incubation periods, demonstrated great effect on these bacteria. Results in **Table (3)** indicated that, the exposure of *R. solanacearum* to tested NPs for 2, 3 and 4 days demonstrates their pathogenicity efficacy. Also the results revealed that, the antimicrobial activity of NPs was found to be more when the organism was in lag phase (less than 60h.) by increasing the incubation period (84h), rate of bacterial inhibition increased with the increase of period of exposure and was influenced in Ag/CsNC, CsNPs and AgNPs. The highest effect was in Ag/CsNC, while the antimicrobial activity of treatments i.e CuO-NPs and MgO-NPs was found to be less when the organism was in phase (96h.).

Table (3): Influence of different exposure times on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

Treatment	Number of colony forming unit (CFU/mL) of *R. solanacearum*					
	24hr	48hr	60hr	72hr	84hr	96hr
Control	58×10^4	56×10^6	200×10^8	40×10^8	64×10^8	64×10^5
MgO-NPs	88×10^3	153×10^2	0	0	0	37×10
CuO-NPs	23×10^3	194×10	0	0	0	14×10
CsNPs	12×10^2	0	0	0	0	0
AgNPs	27×10^2	0	0	0	0	0
Ag/CsNC	17×10	0	0	0	0	0

5.2. Influence of different exposure temperature on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

Data recorded in **Table (4)** show that all tested NPs were superior for minimizing growth of *R. solanacearum* at 35°C. Also it was found that, Ag/CsNC was more inhibitive for *R. solanacearum* at low temperature (less than 25°C) and high temperature (45°C). Otherwise, increasing the temperature to 40°C resulted in increasing the inhibition of the tested bacterium.

Table (4): Influence of different exposure temperature on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

Treatment	Number of colony forming unit (C.F.U./mL) *of R. solanacearum*					
	15°C	25°C	30°C	35°C	40°C	45°C
Control	49×10^4	119×10^6	204×10^8	140×10^8	121×10^5	26×10^5
MgO-NPs	47×10^3	153×10^2	10×10	0	0	0
CuO-NPs	92×10^3	194×10	26×10^2	0	0	0
CsNPs	78×10^2	46×10	113×10	0	0	0
AgNPs	0	26×10^2	0	0	0	0
Ag/CsNC	0	0	0	0	0	0

5.3. Influence of different pH values on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

pH value of the medium had a role in the susceptibility of *R. solanacearum* to NPs. In this experiment, the colony forming unit of bacterium was determined after 48 hr using agar dilution method. The results in **Table (5)** and **Fig. (6)** indicated that, by adding the tested NPs, no growth of *R. solanacearum* at 5.6, 6.5 and 8 pH values. Also, the data showed that, Ag/CsNC recorded no *R. solanacearum* growth when cultivated in growth medium with pH ranging from 5.6 to 8.

Table (5): Influence of different pH values on *R. solanacearum* susceptipility to CuO-NPs, MgO-NPs, AgNPs, CsNPs and Ag/CsNC at 200 µg/mL concentration.

Treatment	Number of colony forming unit (CFU/mL) of *R. solanacearum*				
	5.6	6.5	7	7.5	8
Control	50×10^4	40×10^6	114×10^8	140×10^8	121×10^5
MgONPs	0	0	10×10	50×10	0
CuONPs	0	0	26×10^2	0	0
CsNPs	0	0	0	0	0
AgNPs	0	0	21×10	0	0
Ag/CsNC	0	0	0	0	0

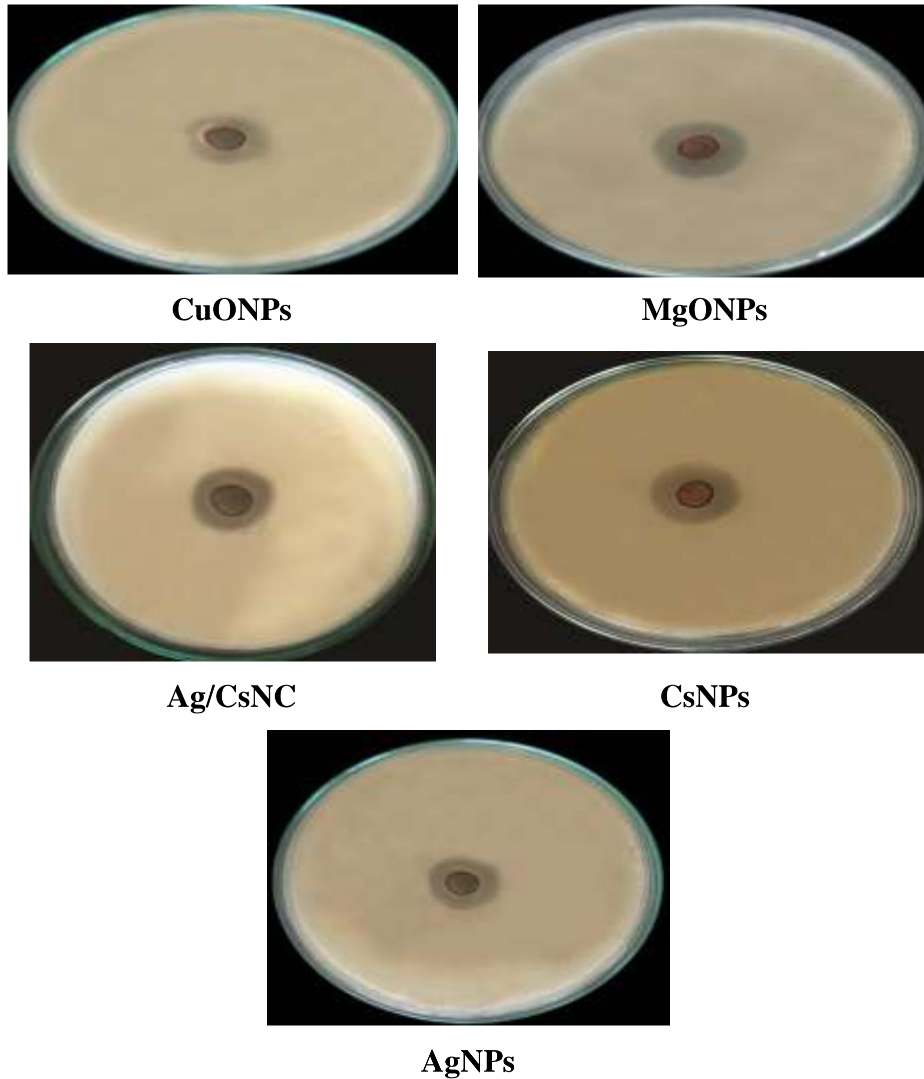

Fig. (6): Agar plates seeded with *R. solanacearum* strain treated with NPs at optimum inhibition conditions (pH8, 60 hr and 35°C).

DISCUSSION

Potato (*Solanum tuberosum* L.) is an important popular vegetable crop throughout the world after wheat, rice and maize and it is economically important in Egypt for local consumption and exportation. Egypt is ranked the first African potato producer. Exportation of potato crops, especially to the European markets affected by potato brown rot disease caused by *R. solanacearum.* European and Mediterranean Plant Protection Organization (EPPO) has listed *R. solanacearum* as an A2

quarantine pest (**Lee** ***et al*****., 2012**). *Ralstonia* (*Pseudomonas*) *solanacearum* was reported for the first time in Egypt at El-Gemmeiza farm, Gharbia governorate based on symptomatology only by **Briton-Jones (1925)**, there after many researches were carried out in Egypt by **Balabel, (2006); Saad, (2011); Abdel-Aal, (2015) and Messiha** ***et al.*** **(2019)**.

The present investigation was conducted during (2019-2021). In this study isolation of the pathogen was made from infected potato tubers, irrigation water, soil and weed collected from El- Sharqia, El-Ismailia El- Dakahlia and El-Behera Governorates on the SMSA medium, selective for the pathogen. In this pecularity, **Tohamy** ***et al.*** **(2007)** isolated 24 *P. solanacearum* from soil, water and tuber. Fluidal, irregular, white and/or white with pink centers colonies, typical for *R. solanacearum* virulent form, were picked up in the current study and twelve isolates showed an agreement with *R. solanacearum* race 3 biovar 2 characteristics using serological test as Immunofluorescence antibody staining (IFAS) and conventional PCR techniques with specific primers. The PCR showed the specific bp PCR product visualized under UV light revealed very close similarity of the twelve isolates selected from the aforementioned sources. These results agree with those reported by **Balabel (2006)** and **Abdel-Aal, (2015)**. **Mahdy** ***et al.*** **(2012) and Serag** ***et al.*** **(2020)** applied the PCR technique to identify *R. solanacearum* strains**.** Taq-Man (the flourogenic PCR), being the most recent advances in this regard for detection of all biovars confirmed the identification of the pathogen (**Weller** ***et al*****., 2000**). Twelve isolates in this study, indicated by the Taq-Man assay didn't show any variations revealed that the isolates were all *R. solanacearum* biovar 2 race 3. **Balabel, (2006)** discussed that positive results were obtained in Taq-Man assays with all isolates from different sources, indicating that the tested isolates were *R. solanacearum* biovar 2 race 3. Similarly, **Shehata, (2001)**; **Balabel, (2006)**; **and Stulberg** ***et al.*** **(2016)** reported that results noticed in Taq-Man PCR bioassays revealed non specific differences between isolates. Pathogenicity tests of the selected twelve isolates were carried out in the green house on potato seedlings. Pathogenicity was confirmed by the development of wilt symptoms on tested plants followed by reisolation and identification of the causal organism from diseased plants (**Elphinstone** ***et a1.,*** **1998**). Results of the current study revealed variable degree in wilting noticed among isolates as shown from the disease severity group and the wilt symptoms of the inoculated seedlings. The disease severity was higher in Sponta and Kara representing 87.1 and 83.8% respectively than Mundial cultivar representing 60%. Results of the current study proved that potato plants inoculated with the isolate obtained from Behira Governorate, Nubaria district coded R9 were the

highest virulent and showed the highest severity of disease incidence and were highly pathogenic to all of the studied five potato cultivars of Spunta, Kara, Draga, famosa and Mundial. Transmission of *R. solanacearum* from one area to another mainly occurs through infected seed and farm implements infested soil and surface water, including irrigation water, are the primary sources of inoculums, the pathogen infects roots of susceptible plants, usually through wounds. In this respect several workers confirmed these results (**Pradhanang *et al.*, 2005, Swanson *et al.*, 2005 and Abdel-Aal, 2015**).

The effect of various factors like pH, reactive time and temperature on the antibacterial activity of NPs was studied *In Vitro* to demonstrate the susceptibility of *R. solanacearum* to NPs. The growth of NPs treated cells decreased along with reactive time indicating the decrease in number of viable cell with increased reaction time. *R.solanacearum* were more susceptible in the logarithmic growth phase at 30ºC ,This may be due to the massively produced young bacteria at this stage are the most sensitive. Similarly, researchers recently found that the susceptibility of Gram-negative *E. coli* and *Pseudomonas aeruginosa* to graphene oxide is the highest in the exponential growth phase, in which the bacteria exhibit physiological changes, and bacteria are quite resistant during the stationary phase, in which the cells do not grow (**Das *et al.*, 2011**). There was slightly change in CuONPs and MgONPs treated cells through 24 hr treatment period. These results clearly demonstrated that the application of CuO-NPs and MgO-NPs could effectively exert antibacterial activity against *R. solanacearum* by disturbing after a longer incubation time. Growth inhibition in cells of *R. solanacearum* treated with NPs and grown at 15ºC and 25ºC as almost the same as in those grown at 45ºC. However, the growth of cells incubated at 30ºC was slightly lower than that of cells incubated at 25ºC, and the growth of cells in the control group incubated at 40ºC was slightly lower than that of cells incubated at 30ºC.The results are in accordance with previous results of **Kim *et al.* (2011)** who discussed the extent of growth inhibition in cells of *S. aureus* and *E. coli* treated with Ag-NPs and grown at 17ºC and 25ºC as almost the same as in those grown at 37ºC.

R. solanacearum treated with CsNPs and Ag/CsNC was more susceptible at pH 7.5 and the rate of growth inhibition in cells treated with AgNPs and grown at pH 5.6 and 8.0 were almost the same as that in cells grown at pH 7.0. Conditions at pH 8 did not affect the growth of CuO-NPs treated cells. **Kim *et al.* (2011)** reported that the growth curves of AgNPs treated *S. aureus* and *E. coli* cells incubated at pH 5.6 and 8.2 did not differ from those of cells grown at pH 7.2. The growth rate of *S. aureus* cells in the control group held at pH 5.6 was slightly lower than that of cells incubated at pH 7.2.

Investigations have also showed that different environmental conditions cause significant differences in antimicrobial activity. For example, **Liu *et al.* (2010&2011)** announced that the efficiency of graphene for inactivating *E. coli* and *B. subtilis* bacteria increased with increasing shaking speed, time, and concentration. In this respect **Chen *et al.* (2016)** concluded that the antibacterial activity of the MgO-NPs and bulk MgO occurred in a dose- and time-dependent manner. Also, the temperature of the environment has a potent influence on antibacterial activity due to its effect on the ROS generation rate. When nanoparticles are stimulated by temperature, electrons are captured at the active sites. Afterward, the electrons interact with oxygen (O_2) to produce ROS, thereby enhancing the antimicrobial effectiveness of nanoparticles **(Wang *et al.*, 2017)**. The pH of the environment influences *In Vitro* antimicrobial activity, a decrease in the pH increases the dissolution rate of ZnO nanoparticles, which resulted in greater antimicrobial properties **(Wang *et al.*, 2017)**. Moreover, **Chen, *et al.* (2019)** mentioned that the zeta potential of the CuO-NPs decreased with increasing pH. The zeta potential had a positive value below pH 8 due to the high level of hydrogen ions, indicating that the nanoparticles were stable in the bacterial culture medium (pH 6.5–7.0). The dispersion possessed a negative charge when the pH was greater than 8.0, which was possibly associated with the adsorption of carboxy groups on the surface.

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"تأثير بعض العوامل على حساسية ميكروب رالستونيا سولاناسيرم الحاد مرضياً للجزيئات النانومترية"

أميرة جمال الدين ربيع محمود[1] ، أبتسام نعيم حسينى[2] ، نجلاء موسى بلابل[1] ، محمد سليمان حنفى[1] ، غديرالدسوقى السيد ديغم[2]

1–قسم الأمراض البكتيرية–معهد بحوث أمراض النباتات–مركز البحوث الزراعية– الجيزة

2– كلية العلوم فرع البنات – جامعة الازهر

تم أجراء بعض التجارب المعملية لدراسة القدرة المرضية لبكتيريا ***R. solanacearum*** المسببة للذبول البكتيرى وبالأخص العفن البنى للبطاطس فضلا عن تقييم قدرة خمسة جزيئات نانوية مختلفة CuO–NPs, MgO–NPs, AgNPs, CsNPs and Ag/CsNC بتركيز 200 ميكروجرام \مل لكل منهم تحت تأثير بعض العوامل للحد من القدره الإمراضيه لبكتيريا ***R. solanacearum*** .

و يمكن تلخيص النتائج في الآتي:

1- تم جمع عينات من مصادر مختلفة من محافظات ومناطق مختلفة من مصر من محافظات الشرقية و الدقهلية و البحيرة والإسماعيلية. و سجلت النتائج أن أعلى نسبة إصابة بكتيرية (87.2%) كانت العينات التي تم الحصول عليها من محافظة البحيرة بينما سجلت العينات التى تم الحصول عليها من محافظة الإسماعيلية أدنى عدد للخلايا البكتيرية (26.7%) وقد تم عزل إثنى عشرة عزلة بكتيرية إيجابية وفعالة وخضعت هذه العزلات للدراسات التالية.

2- أجرى اختبار إحداث القدره الإمراضيه علي شتلات نباتات البطاطس وأظهرت النتائج أن جميع العزلات المختبرة كانت ممرضة لنباتات البطاطس، وقد تم تحديد شدة الذبول عن طريق حساب نسبة الاوراق المصابه في كل نبات، ودلت النتائج أن العزلة (**R9**) كانت الأكثر فاعلية لحدوث المرض طبقا لدرجة شراسة العزلة.

3- جميع أصناف البطاطس الخمسة المختبرة (سبونتا و كارا و دراجا و فاموزا و مونديال) كانت عرضة للإصابة من قبل الممرض بنسب مرضية مختلفة مع العزلة المختبرة (**R9**).

4- تم تعريف العزلات من مصادر مختلفة تبعا لصبغة الفلورسنت للأجسام المضادة وأكدت الدراسة أن العزلات البكتيريه هي بالفعل لبكتيريا ***R. solanacearum*** و التى ظهرت على شكل اسطوانى قصير باللون الخضر اللامع و أظهرت أيضا النتائج عدم وجود اختلاف واضح بين العزلات البكتيرية.

5- أظهرت نتائج تقنية تفاعل البلمره المتسلسل **PCR** لتعريف الإثنى عشرة عزلة المختبرة من بكتيريا ***R.solanacearum*** و التي أعطت أعلي شدة إصابة وجود تصوير محدد من **DNA pb 718** و ذلك تحت ضوء الأشعة فوق البنفسجية مما يدل علي وجود تشابه وثيق بين العزلات تحت الدراسة.

6- أظهرت نتائج إختبار **(Taq-Man)** أنة لا يوجد إختلاف بين العزلات الإثنى عشرة المختبرة وأكد أن هذه العزلات تنتمى إلى الجنس 3 والطراز البيولوجى 2 حيث يعتبر إختبار **Taq-Man** أكثر تطوراً تحديداً ويستخدم للكشف عن السلالة والطراز البيولوجى ل ***R. solanacearum*** بإستخدام بكتيرية مرجعية.

7-تم إجراء مزيد من الدراسات علي النشاط التثبيطي لجزيئات النانو بتركيز 200 ميكروجرام/ مل ضد نمو بكتيريا الذبول تحت عوامل مختلفة للحصول على أقصي قدر من النشاط المضاد للبكتيريا الممرضة. تم الحصول على الأنشطة القصوى بعد **4 ,3 ,2** أيام مع جميع **NPs** و كانت جميعها متفوقه فى تقليل نمو ***R. solanacearum*** ,سجلت النتائج حدا أقصى للنشاط عند درجة حرارة 35 درجة مئوية ، دلت النتائج أن: **Ag/CsNC** كان أكثر تثبيطاً لنمو الكائن عند درجة حرارة منخفضة (أقل من 25 درجة سليزية) و درجة حرارة عالية (45 درجة سليزية) وبين درجة الحموضة 5.6 – 8 ضد سلالة ***R.solanacearum*** وذلك عند مقارنة أعداد الخلايا البكتيرية ***R. solanacearum*** **(CFU/mL)** المعاملة بجزيئات النانو بمجموعة المقارنة (الكنترول).