Histological and chemical Susceptibility of some Potato cultivars Infected with new Strains of *Pectobacterium carotovorum*

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Abstract

The potential losses of potato yield due to soft rot disease caused by *Pectobacterium carotovorum* constitute a major threat to this crop, as the pathogen adapts to environmental and nutritional changes resulting in genetic patterns that are more virulent in infection of plant tissue. Therefore, the current study aimed to determine the tissue and chemical sensitivity of some potato varieties infected with this bacteria. Results confirmed that the bacterial isolates belong to *P. carotovorum* according to diagnostic keys, microscopic and morphological examinations that indicated the bacteria is Gram-negative grew on the differential medium Yeast extract-dextrose-CaCO3 (YDC) as a yellow to cream colonies, convex, shiny, and circular. While on the D-3 agar medium, the colonies were white to cream, and on the Nutrient Agar medium (NA) was cream to shiny white. The outcomes of the biochemical tests indicated that *P. carotovorum* isolates gave positive results for some tests including Catalase and Hydrolysis of Gelatin. Other tests such as Oxidase, Starch hydrolysis, voges-proskauer, phynelalanen, and methyl red were negative. The isolates were molecularly diagnosed based on the 16 S rRNA gene, as new strains were obtained for the first time in the Iraq, which were deposited in GenBank (NCBI) under the accession numbers PP824981, PP824982, and PP824983. The highest percentage of infection and severity with the disease were recorded in Elmundo variety, which reached 42.10 and 22.14%, respectively. The results of the sensitivity test of some potato varieties to *P. carotovorum* showed that the highest rotted tissue was in Elmundo variety, which reached 4.98cm, compared to the control which reached 0.00cm. Based on the percentage of carbohydrates, protein and fats, Burren variety outperformed other studied varieties significantly reaching 8.44%, 1.17 mg/g and 0.15 mg/g, respectively, compared to the control treatment which reached 16.81%, 3.92 mg/g and 1.81 mg/g, respectively. While for phenols, flavonoids, nitrates and organic acids, Volare variety outperformed the other varieties, reaching 6.67 mg/g, 1.61 mg/g, 0.21% and 0.10%, respectively, compared to the control which reached 18.31 mg/g, 3.24 mg/g, 0.27% and 0.22%, respectively.

Keywords: *Pectobacterium carotovorum*, potato, biochemical tests, virulence.

Introduction

Potato (*Solanum tuberosum* L.) is belonging to the Solanaceae family and it is considered a valuable economic crop rich of nutritional elements as well as a source of energy, its fruits contain many minerals, carbohydrates, proteins, fats and vitamins. Therefore, it is the most important food crop in terms of global consumption (28). Soft rot is one of significant bacterial diseases that affect many plants including the Solanaceae family particularly potatoes and many other crops. The disease is found in tropical and temperate regions. It is one of the diseases that affect plants in the field and during storage, causing significant losses (17). The pathogen is considered one of the most important factors determining vegetable production and causes a complete loss of production if the appropriate conditions are available, as wounds constitute the main outlet for the bacteria that cause the disease. Lenticels are also one of the natural outlets for infection to occur on new tubers. The development of the disease requires high levels of moisture in the soil, and the appropriate temperature for the disease to occur in storage is (24-27) °C. Therefore, potatoes should be stored in stores with low temperatures (4-5 °C) (18).

Production losses due to bacterial soft rot caused by *P. carotovorum* can reach up to 90% (31). The bacterium has a wide host range, infecting many plants such as potatoes, carrots, celery, cucumbers, bananas, tomatoes, peppers, beans, cabbage, coffee, corn, cotton, onions, and sweet potatoes (18). The diseases on potatoes is very dangerous because the infection begins in the field and develops rapidly during transportation and storage of tubers. When infection with bacteria occurs, the severity and level of infection with these diseases often accelerates under humid environmental conditions, as the disease affects the fruits and is capable of destroying the components of plant tissues through the activity of enzymes that degrade the plant cell wall. Tubers are infected in the field and develop soft rot accompanied by an unpleasant odor (11). Soft rot caused by the bacteria *P. carotovorum* and the rot disease occurs as a result of the secretion of pectin enzymes, which dissolve the middle plate and disintegrate the cells. As the infection progresses, the rot extends inward and the infected tissues become watery and soft. Tubers surface may discolor and appear wrinkled with sticky secretions when the tuber is pressed in the infected area. The infection may spread to the main stem, causing symptoms of wilting, noting that the base of this stem becomes black in color, its tissues wrinkle, the edges of the leaflets acquire a reddish color, and the branches become erect and more solid than usual (8). Due to the importance of soft rot disease on potatoes and its widespread prevalence in some areas of Najaf, the study aimed to isolate and diagnose the bacteria causing soft rot disease and test the sensitivity of some potato varieties to the pathogen.

Material and methods

Samples collection

Potato tubers samples showing symptoms of soft rot disease were collected from potato stores in local markets within the geographical area of Najaf province (Al-Haidariya and Al-Abbasiya). Other samples of infected tubers were collected from fields planted with potatoes during the fall season 2023/2024. They were taken and placed in plastic bags with tag containing the collection of information such as the date of collection, the area from which they were collected, and the name of the cultivated variety. The number of samples taken amounted (170) healthy and infected samples. After that, the samples were transferred to the laboratory for the purpose of isolation, diagnosis, and conducting subsequent studies on them.

Estimation of the incidence and severity of disease in store

The incidence and severity of infection were estimated according to Yaganza et al. (30).

Isolation the pathogen of soft rot disease of potato

Bacteria were isolated from potato tubers that showed symptoms of soft rot disease and were brought from the mentioned areas and washed with running tap water to get rid of dust, then superficially sterilized using sodium hypochlorite NaOCl (chlorine 5%) at a concentration of 2% for 3 minutes and then washed with sterile distilled water three times to get rid of the sterile substance, and crushed using a ceramic pot after removing the outer peel of the potatoes with 10 mL of sterile saline solution. A portion of the bacterial suspension was taken using a sterile loop and inoculated with nutrient agar medium using the streak method. After that, the inoculated plates were placed in the incubator at a temperature of 28 ± 2°C for 24 hours. A portion of the growing bacteria was transferred using a sterile loop and inoculated on the NA medium using the streak method to obtain single colonies (5).

Microscopic and morphological diagnosis of *P. carotovorum*

Phenotypic and biochemical tests were performed to diagnose 20 bacterial isolates isolated from infected potato tubers, based on the bacterial diagnostic guide, taking into consideration the study of the characteristics that lead to the diagnosis of the genus and species of bacteria, according to what was mentioned by Schaad et al.,(24).

Microscopic and morphological characteristics

A portion of the pure colonies growing on the NA medium at the age of 24 hours were transferred to a clean glass slide containing a drop of (normal saline solution) and mixed well and spread on the slide and then stained with gram stain and then examined under a light microscope using a 1000X lens. Then the reaction of the cells to the dye and the shape and clustering of the cells were recorded as well as the color, shape and nature of the growth of the bacterial colonies on the NA medium (23).

1- The nature of growth on solid Nutrient Agar (NA)

The culture medium (NA) was inoculated with the bacterial isolates. The plates were then incubated at a temperature of 25±2°C for 72 hours. Growth was examined and the phenotypic characteristics were

recorded in terms of colony shape, color and texture (24).

2- The nature and color of growth on differential medium (YDC)

The YDC medium was inoculated in sterile plastic plates and incubated at 25±2°C for 72 hours, observing the yellow bacterial growth on this medium (24).

3- The nature of growth on D-3 agar medium

D-3 medium was prepared by adding 10.0 g sucrose, 10.0 g arabinose, 5.0 g casein hydrolysate, 7.0 g LiCl, 3 g glycine, 5 g lCNa, 0.3 g O2.7H4MgSO, 50 mg sodium dodecyl sulfate, 60.0 mg bromthymol blue, 100 mg acid fuchsin and 15.0 g agar to 1000 mL distilled water. The pH was adjusted to 8.2 and sterilized in an autoclave at 121°C and 15 psi for 20 min. The medium was inoculated from cultures grown on NA medium at 24 hrs by streaking. The plates were incubated for 72 hours and the presence or absence of bacterial growth was observed on the surface of the medium, which is a selective medium for the genus *Pectobacterium* sp. (24).

Biochemical tests for the diagnosis of *P. carotovorum*

The species *P. carotovorum* was diagnosed through some biochemical tests taking into consideration the study of the characteristics through several biochemical tests as follows: Catalase test, Gelatin Hydrolysis, Oxidase test, Starch hydrolysis, Methyl Red test, Voges - Proskauer test, Phenylalanine test according to (13) and Winn et al., (29).

Molecular diagnosis of *P. carotovorum* using Polymerase Chain Reaction (PCR)

Molecular identification of the bacteria causing soft rot disease on potato was performed. Three isolates were selected from 20 isolates obtained from tree varieties of infected potato (Elmundo, Volare and Burren) after confirming their microscopic, morphological and biochemical identification. The isolates were molecularly identified based on the 16S rRNA gene using the Genomic DNA Extraction Kit G-spin Total DNA prepared by Intron Company, Korea, using forward r and reverse primers (26). The PCR amplification products were subjected to electrophoresis using agarose gel at a voltage of 7V for 2 hours. The agarose gel was prepared by dissolving 1.5g in 100 mL of TBE buffer solution, then photographed under ultraviolet light. Then, the 16S rRNA gene amplification products of the forward and the reverse primers were sent to the Korean Macrogene Company for the purpose of analyzing the nitrogenous base sequence of the double nucleic acids of the bacterial isolates and to identify the isolated bacteria. The DNA sequence of each isolate was entered into the database available at the National Center for Biotechnology Information (DNA Sequence). The BLAST (Local Basic Alignment Search Tool) was used to compare the sequence results with the standard sequences available on the NCBI database.

Testing the susceptibility of some potato varieties to *P. carotovorum* and its effect on chemical properties

The susceptibility test experiment was conducted on three potato varieties grown

in Najaf, namely (Elmundo, Volare and Burren). The bacterial inoculum was prepared by inoculating NA medium with three bacterial isolates individually by the streak plate method. The plates were incubated at 25 + 2°C for 48 hours. The bacterial growth was harvested by a glass diffuser by adding 5mL of sterile physiological salt solution. A series of dilutions of 10-1 – 10-8 of the bacterial suspension of the isolated bacteria were prepared in test tubes containing sterile physiological solution. 1 ml of each of the last three dilutions was placed using a sterile pipette specific for each dilution. 20 mL of the culture medium at a temperature of 45°C was poured into each plate. The plates were moved with a gentle rotating motion to homogenize the bacterial suspension with the culture medium. Plates were incubated at a temperature of 25 + 2°C for three days. The number of colony-forming units/ml (CFU/mL) was estimated (4) according to the method of Pasco et al., (20). Five tubers were selected from each variety and each tuber was cut into two or three halves lengthwise. A hole was made in the middle with a diameter of 5 mm and a depth of 5 mm. The tubers were placed in plastic plates containing sterile and moistened filter paper. 100 microliters of bacterial suspension at a concentration of 106 was added to each hole, taking care to move the bacterial suspension to avoid cell sedimentation. Then the filter paper was moistened and the plastic plates were closed to ensure humidity then left in the incubator in a dark atmosphere for 6 days at a temperature of 28 ± 2°C. The rotting tissue was carefully removed from each half tuber using a small spoon and the cavity formed after removing the rotting tissue was filled with water using a graduated pipette. The volume of water required to fill the cavity/cm represents the rotting tissue.

Study the chemical indicators

Size of rotted tissue

The volume of decayed tissue was estimated according to Pasco et al.,(20).

2- Measuring the percentage of total soluble carbohydrates in tubers %

The percentage of total soluble carbohydrates in the tubers was estimated using a spectrophotometer, where the absorbance was read at a wavelength of 490 nm according to the method mentioned by Herbert et al., (10).

3- Quantitative estimation of protein

The percentage of protein in potato tubers was measured according to C.A.O.A. et al. (1).

4- Quantitative measurement of fats

Fats were estimated according to the method of (9).

5- Quantitative estimation of phenols

The method described by Meena et al., (15) was followed for the quantitative determination of phenolics in potato tubers.

6- Quantitative estimation of flavonoids

Flavonoids were estimated according to Mbaebie et al., (14).

7- Quantitative estimation of nitrates

The nitrate content in tubers was measured according to Cataldo et al., (3).

8- Quantitative estimation of organic acids

Organic acids were estimated according to Palikiva, (19).

Results and Discussion

Isolation soft rot pathogen on potatoes

Three bacterial isolates were isolated from potatoes that showed symptoms of soft rot disease from all samples collected from potato stores in different areas of Najaf province. Bacterial growth appeared after 12 hours on the NA culture medium. Individual colonies were distinguished after 24 hours of incubation. The colonies appeared in a creamy to white color, shiny, circular, with regular and complete edges and a convex surface with 0.5-1mm diameter. Most studies that addressed this topic indicate that these characteristics are identical to the phenotypic characteristics of *P. carotovorum* (33).

Testing the incidence and severity of infection percentage in the storage

Results of estimating the incidence and severity of soft rot in storage of potato tubers as shown in Figure (1) and indicated that all stored potato varieties are susceptible to the disease. The incidence and severity of infection varied according to storage conditions and the type of stored tubers. The results of the statistical analysis showed that the highest incidence of the disease was in Elmundo and Volare varieties, which amounted 42.10 and 33.01%, respectively, and the lowest incidence was in Burren variety, which amounted 20.21%. Figure (2) showed that the highest severity was recorded in Elmundo and Volare varieties, which reached 22.14 and 18.25%, respectively, and the lowest severity of infection was in the Burren variety with 15.43%. This percentage cannot be ignored as it causes economic losses to the plant. The reason for the variation of varieties in their sensitivity to infection with the disease in the same storage conditions is due to the high rate of infection and its severity with the disease in the store, which was due to the presence of suitable conditions to provide a suitable temperature for the development of the latent infection present in the tubers and infected in the field with the availability of suitable humidity in the form of a water layer on the surface of the tuber, which leads to the creation of anaerobic conditions that increase the activity and virulence of the bacteria causing the disease (8). The genetic factor also affects the nature and quality of tubers and their possession of positive resistance qualities such as the thickness and strength of the tuber peel, the cohesion of the plant tissue, and the presence of phytolexins, which work to reduce the effectiveness of the pectin-degrading enzyme Pectinase secreted by bacteria. The availability of a suitable temperature for the development of the latent infection present in tubers and infected in the field, with the availability of suitable moisture in the form of a water layer on the surface of the tuber (12). The reason for the appearance of distinctive symptoms, the most important of which is tissue decomposition and the emission of unpleasant odors, is due to the activity of bacteria and its ability to produce enzymes capable of dissolving the middle plate containing pectin materials and the cell wall containing protopectin, and pectic acid are decomposed by a pectin enzyme such as Pectin Methyl Esterase to Polygalacturonic acid, which is converted to Monogalactouronic acid or to oligosaccharides by the enzymes

Depolymerase and Polygalactouronase or converted to Oligouronides by the enzyme Pectinlyase (32).

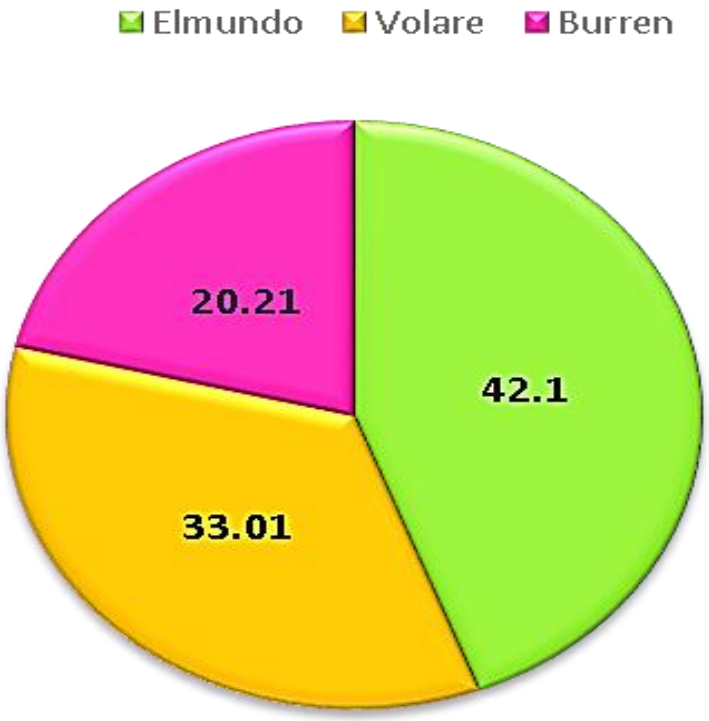

Figure 1. The percentage of incidence of soft rot on potato in the storage.

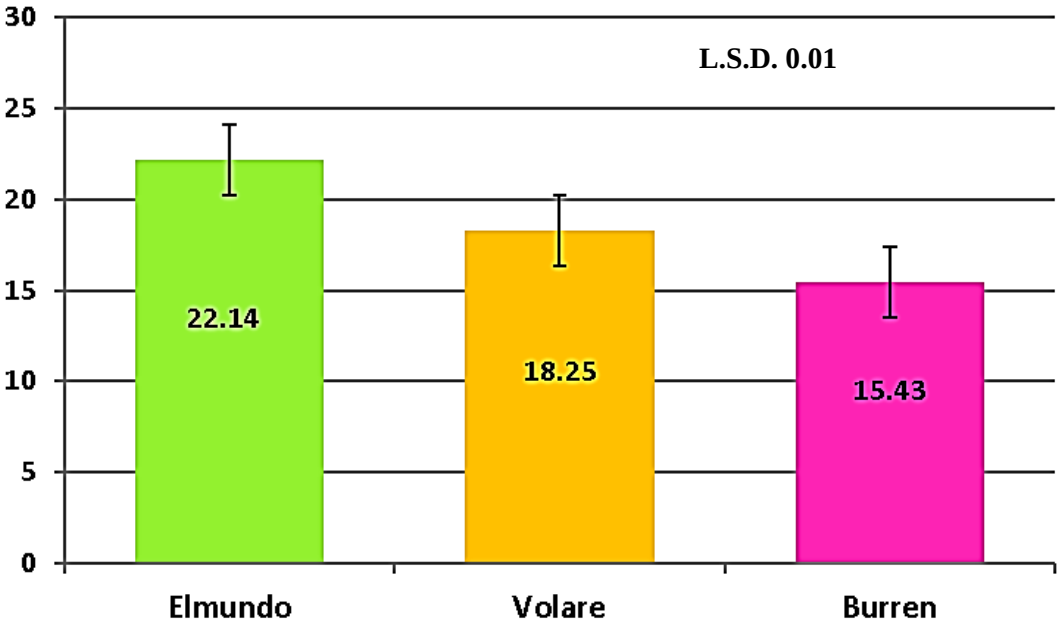

Figure 2. The severity of soft rot disease on potato in the storage.

The microscopic and morphological characteristics of *P. carotovorum*

The microscopic and morphological characteristics of 20 bacterial isolates were studied. Three bacterial isolates were obtained and confirmed microscopically and morphologically that they belong to the genus *Pectobacterium*. The results of the microscopic diagnosis indicated the appearance of short, Gram-negative bacilli-shaped bacteria. The cell aggregation showed most of them were single and some were clustered. Results of the morphological examination after 24 hours for the bacteria growing on the YDC differential medium, showed that the colonies appeared as yellow to creamy, convex, shiny, circular with a complete edge and on the D-3 Agar medium, the colonies were white to creamy, convex, circular. While, on the NA medium, the colonies were creamy to shiny white, circular, with regular and complete edges, convex and with a diameter of 0.5-1 mm (27 and 22).

Diagnosis of *P. carotovorum* based on biochemical tests

Biochemical tests were used to diagnose the type of 20 bacterial isolates and showed that 3 isolates of these isolates belonging to the type *P. carotovorum* as shown in Table (1). These isolates gave positive results for some tests, including Catalase test, Hydrolysis of Gelatin, while other tests such as Oxidase test, Starch hydrolysis, Voges-Proskauer, Phynelalanen, and Methyl red were negative, which indicates that the isolates carry the characteristics and features of the type *P. carotovorum*, and this is consistent with what was indicated by previous studies (7).

Table 1. Biochemical tests for *P. carotovorum*

Biochemical test	Result
Gram stain	-
Catalase test	+
Oxidase test	-
Hydrolysis of Gelatin	+
Voges-Proskauer	-
Phynelalanen	-
Methyl red	-
Starch hydrolysis	-

Molecular diagnosis of *P. carotovorum* subsp. *Carotovorum* using PCR technique

Polymerase chain reaction (PCR) technique was used to confirm the molecular diagnosis of *P. carotovorum* bacteria. Three bacterial isolates from Elmundo, Volare and Burren potato varieties were diagnosed after confirming their microscopic, morphological and biochemical characteristics, which were among the most pathogenic isolates on

potatoes that were isolated from infected potato tubers from Al-Haidariya and Al-Abbasiya areas in Najaf province, which were diagnosed based on the 16 S rRNA gene. The results of electrophoresis on agarose gel for the amplification products of the gene showed the appearance of a band with a molecular size of 375 base pairs. The results of the nucleotide sequence analysis of the bacterial double-stranded DNA bands, which were compared with the data available at the National Center for Biotechnology Information (NCBI), indicating that the isolates belongs to *P. carotovorum* as a new strain of *P. carotovorum* which was deposited at NCBI under the accession Number: PP824981, PP824982, and PP824983, as showed in Table (2).

Table 2. Shows *P. carotovorum* strains registered in NCBI.

Strain	Accession number	Potato variety
Pectobacterium carotovorum strain Sa-Elmundo	PP824981	Elmundo
Pectobacterium carotovorum strain Sa-Volare	PP824982	Volare
Pectobacterium carotovorum strain Sa-Burren	PP824983	Burren

Testing the susceptibility of some potato varieties to *P. carotovorum* on chemical properties

Results of the statistical analysis as shown in Table (3) for testing the sensitivity of potato varieties to the bacteria *P. carotovorum*, which causes soft rot disease on potato tubers, indicated that the bacteria are capable of causing the disease through tissue decomposition, as the highest rotted tissue was in Elmundo variety, reaching 4.98 cm, and the least rotted tissue was in Burren variety, reaching 4.19cm, compared to the Control, which reached 0.00cm. The reason for the occurrence of infection and tissue decomposition on potato tuber is due to enzymes capable of breaking down the plant cell wall, including pectinase, cellulase, and proteinase. This make the tissue to become soft and secrete sticky materials, which consist of bacterial cells, plant cell decomposition products, and some gases with an unpleasant smell (6).

Bacteria is multiplying in the spaces between plant tissues, as the degrading enzymes work to dismantle the cells, causing the tissue to lose its natural systems and mechanical strength, and increasing osmotic pressure, which causes cell sap exit, softening of tissue, secretions, and smell of rot (21). Through the current study and the results that appeared in the experiment, there are significant differences Table (4). The superiority of the Burren-infected variety in the percentage of carbohydrates, protein and fats over the other varieties significantly, as it reached 8.44%, 1.17 mg/g and 0.15 mg/g, respectively, compared to Burren-uninfected treatment, as it reached 16.81%, 3.92 mg/g and 1.81 mg/g, respectively.

Elmundo-infected was the lowest variety in the percentage of carbohydrates, protein and fats, as it reached 5.66%, 0.12 mg/g and 0.10 mg/g, respectively, compared to Elmundo-uninfected treatment that reached 12.61%, 2.01 mg/g and 1.11 mg/g, respectively. The results of Table (4) showed that Volare-infected variety was superior in phenols, flavonoids, nitrates and organic acids over the other varieties, as it reached 6.67 mg/g, 1.61 mg/g, 0.21% and 0.10% respectively, compared to Volare-uninfected treatment, as it reached 18.31 mg/g, 3.24 mg/g, 0.27% and 0.22% respectively. The Elmundo-infected was the least variety in phenols, flavonoids, nitrates and organic acids, as it reached 5.43 mg/g, 0.32 mg/g, 0.03% and 0.05% respectively, compared to Elmundo-uninfected treatment, which amounted 10.77 mg/g, 2.18 mg/g, 0.19% and 0.14% respectively. The results obtained show that there is a relationship between the content of varieties in the percentage of carbohydrates, protein, fats, phenols, flavonoids, nitrates, and organic acids and their sensitivity to dry soft rot disease. This relationship may have an impact on the sensitivity of varieties due to the fact that each variety contains different genetic factors that affect resistance to dry soft rot disease, including membrane permeability, low sugar levels (2). The high percentage of carbohydrates and proteins related to the cell wall in the tuber makes it less susceptible to infection, as carbohydrates and proteins accumulate in potato tubers and occupy a larger area of the tuber size, which gives the tuber hardness, resistance, and high quality to protect itself from stresses and pathogens. The decrease in the percentage of carbohydrates indicates an obstruction to the vital activity of the potato plant, in addition, proteins are also a product of photosynthesis, which is important in all functions. The increase in the percentage of nitrates in tubers exceeding the permissible limit have a major impact on human health, so the bacteria can affect the nitrate content in potatoes, and appropriate storage conditions must be provided (25). The bacteria have an effect on the amount of fats as it affects the biological composition of the potato plant by inhibiting the action of amylopectin and thus affecting chlorophyll. Storage conditions can also play a major role in preserving tubers as they cause soft rot, weight loss, solid content, carbohydrates and organic acids, and thus cause thinning of the cell wall, which leads to the tuber being damaged, since phenolic compounds play an effective role in protecting the plant in addition to their role as defensive toxic substances against pathogens such as bacteria. Flavonoids, along with phenols, play an important role as antioxidants, especially in cases of water stress and infection with pathogens, however, the infection with bacteria that cause rot reduces the percentage of phenols and flavonoids in potato tubers (16).

Table 3. The effect of *P. carotovorum* on rotten tissue size, carbohydrates, proteins, and lipids of potato varieties

Variety	Rotten	Carbohydrates %	Proteins	Lipids

	tissue size (cm)		mg/g	mg/g
Elmundo- infected	4.98	5.66	0.12	0.10
Uninfected- Elmundo	0.00	12.61	2.01	1.11
Volare - infected	4.38	6.54	1.01	0.14
Uninfected - Volare	0.00	14.91	2.51	1.41
Burren - infected	4.19	8.44	1.17	0.15
Uninfected - Burren	0.00	16.81	3.92	1.81
L.S.D	0.01	0.02	0.01	0.02

Table 4. The effect of *P. carotovorum* on phenols, flavonoids, nitrates and organic acids of potato varieties

Variety	Phenols mg/g	Flavonoids mg/g	Nitrates %	Organic acids %
Elmundo- infected	5.43	0.32	0.03	0.05
Uninfected- Elmundo	10.77	2.18	0.19	0.14
Volare - infected	6.67	1.61	0.21	0.10
Uninfected - Volare	18.31	3.24	0.27	0.22
Burren - infected	6.01	1.32	0.11	0.03
Uninfected - Burren	21.33	2.11	0.25	0.18
L.S.D	0.02	0.01	0.02	0.33

Conclusion

The current study determined the reasons that led to the increased spread of soft rot disease on potatoes, which causes great losses in Najaf province, as a result of the lack of suitable storage conditions of potato crop. Three new bacterial isolates were recorded and deposited in NCBI, which were isolated from tubers of three potato varieties. The outcomes expand the information base for the management of bacterial diseases using modern methods of control.

Conflict of interest

The authors have no conflict of interest.

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