First report of *Enterobacter cloacae* causing soft rot disease on potato in Iraq

Dhahir. H.R[1]*, Ahmed. F. A.[1]

[1]Plant Protection Department, Faculty of Agriculture College, University of Kufa, Kufa, Iraq.
***Corresponding author e-mail: hawraar.alaboudi@uokufa.edu.iq**

Received:
Feb. 02, 2023

Accepted:
Mar. 01, 2023

Published:
Mar. 23, 2023

Abstract

Potato is an economically important crop, considered the fourth major crop worldwide. However, it has been exposed to many pathogens that cause significant yield loss. Bacterial soft rot diseases caused by *Enterobacter cloacae* is one of the most devastating on potatoes. The pathogenicity test conducted on several bacterial isolates isolated from potato tubers showing soft rot showed that 4 of these isolates could cause disease in potatoes. The size of damaged segments of potato slices ranged between 5.50 and 7.93 cm. The isolate H.B1 caused the most significant damage of 7.93 cm of tuber slices.

Furthermore, the inoculation was carried out on 5-week-old potato plants. The bacteria were added through irrigation water at a concentration of $1x10^6$ CPU. After 20 days of infection, symptoms of wilting and yellowing were observed on the inoculated plants. The biochemical tests displayed that the bacteria are negative for Gram stain and positive for the catalase test, gelatin liquefaction, starch hydrolysis and facultative aerobic.

The nucleotide sequence analysis of 16 S marker results exhibited a similarity rate of 94%. Thus, isolate H.B1 was recorded in NCBI under registration number OP936020.

Keywords: *Enterobacter cloacae*, potato, first report

Introduction

Potato (*Solanum tuberosum* L.) is one of the important vegetable crops in many countries of the world and Iraq. Potatoes are widely cultivated worldwide, being one of the strategic vegetable crops with a high economic value [1, 2]. Potato is one of the essential crops in Iraq, which can contribute to bridging the food gap in the country [3]. Production of potatoes in Iraq during the spring and autumn seasons is estimated at 165.6 thousand tons, with an area of (24.6) thousand dunums for 2018 [4].

Potato production increased locally, especially in the past two decades, and the scope of its cultivation expanded in Iraq. However, it is still low compared to global production in countries with similar environmental conditions to Iraq [4]. The potato crop is exposed to a tremendous annual loss worldwide due to bacterial, fungal and viral diseases, as the potato crop is infected with many diseases and the bacterial soft rot disease that is caused by the. Bacteriophages *Pectobacterium carotovorum* is the most common [5], and it is one of the leading factors determining potato cultivation

around the world [6], and it is economically affecting its production [7]. The potato crop is also exposed to significant losses due to the bacterial soft rot disease caused by Enterobacter cloacae [8].

Enterobacter cloacae is one of the pathogens in different groups of plant families, as it caused soft rot on the dragon fruit (Pitaya) in Malaysia and the fruits of onions in the United States of America [9] (Schroeder and du Toit 2010) in addition to that it caused diseases on other plants such as rot ginger rootstocks in Brazil [10] and bacterial wilt of berries in China [11]. Furthermore, bacterial disease has also been observed in chilli seedlings and affects them in Mexico [12]. Given the importance of this pathogen in many countries and the lack of studies on it in Iraq, this study aimed to isolate and confirm the pathogenicity of *E. cloacae* as a significant factor causing soft rot disease in potatoes for the first time in Iraq.

Materials and Methods

Isolation and purification of bacterial strain

Samples of Elmonda variety of potato tubers showing soft rot symptoms were collected from warehouses and local markets. Other samples (infected tubers) were collected from potato fields during the fall season of 2021. The isolation sites were (Baghdad, Babylon, Karbala, Najaf, and Kirkuk). The samples were placed in polyethene bags and brought to the bacteriology laboratory at the College of Agriculture / University of Kufa. Isolation and purification of the pathogen were carried out.

The previous method [5] was used to isolate the pathogen with minor modifications. First, the infected potato tubers were taken and washed with tap water to remove suspended dust. They were superficially sterilized with 5% sodium hypochlorite for 3 minutes and washed with sterilized distilled water several times to get rid of the effects of sterilization. Then the outer peel was removed. For potatoes it was cut into small pieces 0.5-1 cm in size using a sterile scalpel, planted in dishes containing Nutrient Agar medium, and incubated at a temperature of 28 °C for 24-48 hours on single colonies. In other ways, the bacteria were isolated according to the method described by [13] with minor modifications. Infected potato tubers were taken and washed with tap water to remove the suspended dust. They were superficially sterilized with 5% sodium hypochlorite for 3 minutes and washed with sterilized distilled water several times. Small pieces were taken by a sterile scalpel and placed in a sterilized tube containing 10 ml of autoclaved distilled water. It was closed tightly and left for 15 minutes, with the tube shaken repeatedly. The carrier loop was filled from the solution, and container dishes were laid out on the Nutrient medium. Agar plates were incubated at 28°C for 24-48 hours, and single colonies were selected for propagation.

Pathogenicity test

Pathogenicity test was carried out on potato tuber slices and 5-week-old potato plants of the cultivar from which the pathogen was isolated. Healthy potato tubers were selected free of pathological infection or visible mechanical damage. They were

superficially sterilized using a sodium hypochlorite concentration of 5% for 3 minutes. Then, they were washed with sterilized distilled water several times. Potato tubers were cut into homogeneous slices about 1 cm thick. A hole was made in the centre of each slice with a sterile cork puncher—5 mm diameter and placed in sterile plastic containers [14]. The pits were inoculated with a bacterial suspension of 100 µL, 610 CFU/ml concentration as described in [14], by three slides for each isolate and incubated at 28 °C. The development of the infection was followed daily for six days. The most virulent strains were selected based on the size of the damaged (rotting) part of the potato tuber slices / cm3, and they were multiplied on nutrient agar culture medium and kept in the refrigerator at 4 °C in 20% glycerol medium until used in subsequent experiments. The pathogenicity was also tested on potato seedlings of the mentioned age, as the bacteria were added through irrigation water at a concentration of $^{8}10$. After 20 days of infection, symptoms of wilting and yellowing were observed on the plants.

Bacterial diagnosis

Biochemical diagnosis

Several biochemical properties of bacterial strains were studied, such as (the catalase test, the starch decomposition test, the gelatin liquefaction test, and the oxidation and fermentation test. The following tests were conducted:

Molecular identification

Bacterial DNA was amplified by following the steps of the polymerase chain reaction (PCR), which were described by [15] and included:

1- Initial DNA denaturation for 5 minutes at 94 °C.
2- Final denaturation process, with 35 cycles, 30 seconds, and a temperature of 94 °C.
3- Primer annealing for 30 seconds at a temperature of 55 °C.
4- Initial extension of the PCR product for one minute at a temperature of 72 °C.
5- The final extension step stopped the PCR at 72 °C.

Gel electrophoresis

The agarose gel was prepared, and the PCR product was added to it after estimating the concentration and purity of the DNA by measuring the optical density using a Nanodrop spectrophotometer at two wavelengths (260/280 nm). A sample of DNA (3 microliters) was carried over a 1% agarose gel, according to the method of [16]. First, electrophoresis was carried out; the gel was examined by placing it on a UV transilluminator at a wavelength of 260 nm to see the DNA bands and estimate its molecular size compared with the volumetric guide. Then the PCR product was sent to Korea to sequence the nitrogenous bases.

Molecular Identification

The genetic marker16S rRNA gene was amplified utilizing the universal primer Forward 27F (5'AGA GTTTGATCMTGGCTCAAG-3') and Reverse 1492R (5'-ACCTTGTTACGACT TCAC-3'). PCR reaction consisted of 2,5 µL of template DNA; 0,4 µM of primer 27 F and 0,4 µM of primer 1492 R; 18,5 µL PCR-grade water, 2,5 µL 2x KAPA Taq extra hot-start ready mix with dye. The PCR conditions consisted of an initial denaturation at 95 ºC for 3 min and 29 cycles: denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 1 min and final

extension at 72ºC for 7 min. Identification to the species level was defined as a 16S rDNA sequence similarity with that of the type strain sequence in the gene Bank. Bioinformatics analysis of 16S rRNA sequences was performed with the gene bank using the BLAST program.

Results and Discussion

Isolation and purification of isolates

Samples collected from potato fields, warehouses, and local markets showed that they were infected with the bacteria that cause soft rot disease, depending on the pathological results and the biochemical and molecular diagnosis. The two methods used showed efficiency in isolating the bacteria that causes bacterial soft rot disease and obtaining pure single colonies. In addition, the method of [5, 13] saved some time and materials used in isolation and purification.

Pathogenicity test

Testing the pathogenicity of strains of bacteria that cause soft rot disease

The results of the pathogenicity test showed that ten strains of the bacteria tested on healthy potato tuber slices were able to cause disease through tissue decomposition and the emission of an unpleasant odour (Figure 1), that isolate A caused the most significant damage of 7.93 cm to the tissue of potato tuber slices and was selected for subsequent experiments.

Studies indicate the ability of pathogenic bacteria to possess virulence factors that allow them to adhere to, invade and destroy host cells. In addition, pathogenic bacteria may have the ability to overcome host immunity. These bacteria are also considered opportunistic pathogens that attack many organisms and cause them disease [17].

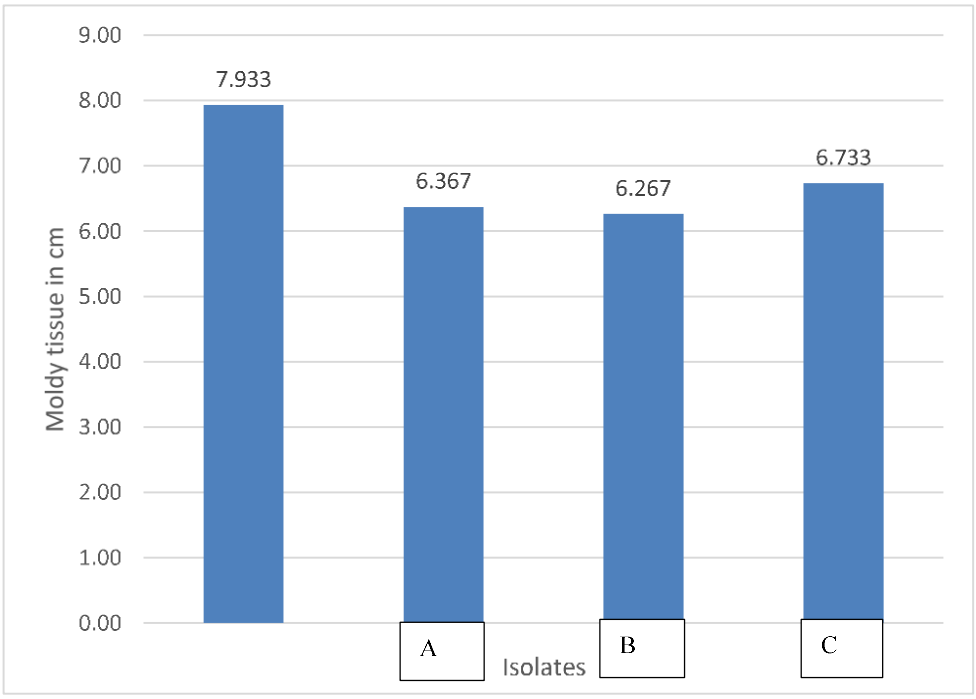

Figure (1): The pathogenicity test for strains of bacteria that cause soft rot disease on potato tuber slices

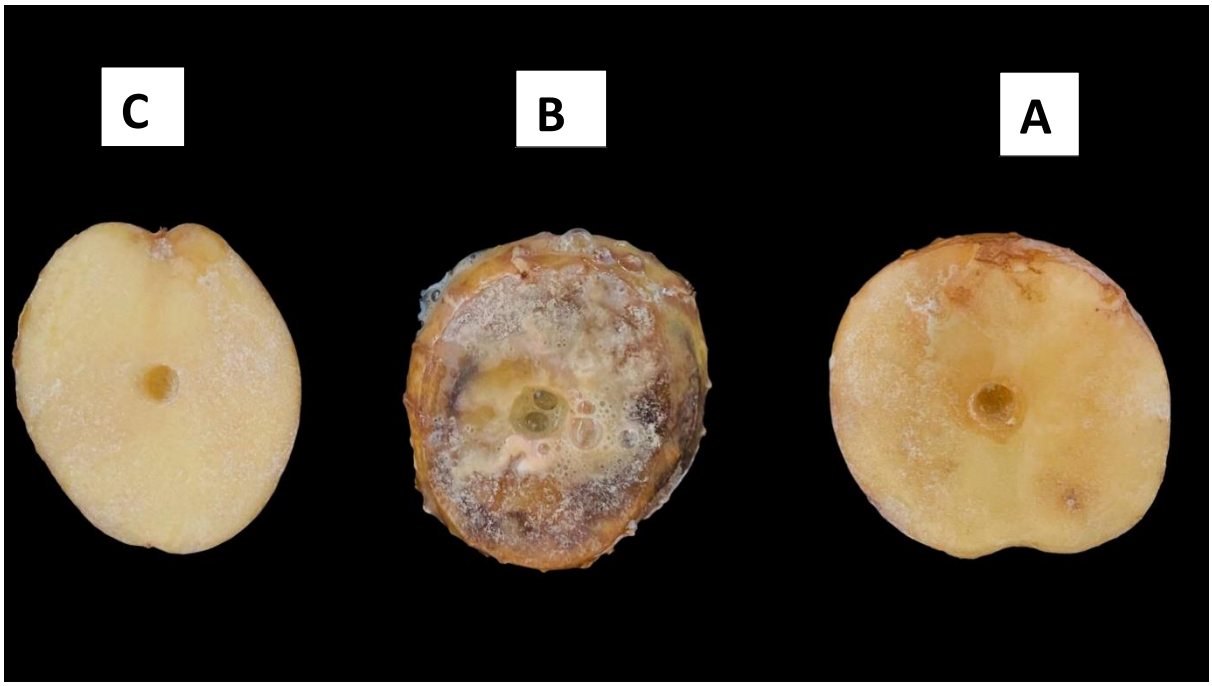

Figure (2): The pathogenicity of the severe bacterial isolate on potato tuber slices: (A) Pathogenicity after three days, (B) Pathogenicity after six days, (C) Control treatment (sterile distilled water only)

Figure (3): Pathogenicity of the severe bacterial isolate on potato seedlings in anvils after 20 days of infection

Diagnosis of the bacteria causing soft rot disease

Biochemical diagnosis

The biochemical diagnosis of the *Enterobacter cloacae* isolate showed that it was positive for a group of tests conducted. The results indicate that the bacterial strains (18 and 21) belong to the Enterobacteriaceae (Table 1), where these results coincided with the findings of previous works [12, 8].

Table (1): Biochemical tests for bacterial strains isolated from infected potato tubers. The sign (+) means a positive test result. A sign (-) indicates a negative test result

Bacteria	Test	KOH	Catalase	Dilute gelatin	Starch	Oxidative fermentation
E. cloacae		+	+	+	+	+

Molecular Diagnostics

It was possible to obtain an amount of 30 microliters of DNA per 1.5 mL of bacterial suspension, with a purity of 1.8 ± 2, depending on the absorption of the ultraviolet spectrum by the Nanodrop device at the two wavelengths of 260 and 280 nm, as mentioned previously [14] by transferring three microliters of DNA on a 1% agarose gel. The results showed a sample band, as shown in Figure (3).

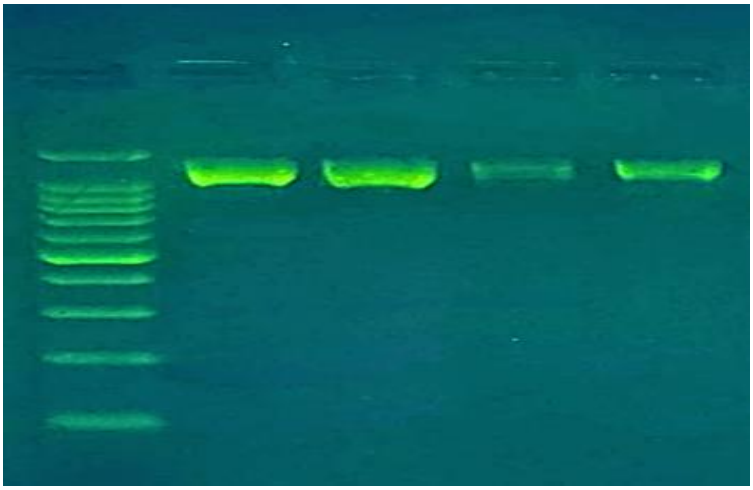

Figure (4): Electrophoresis on a 1% agarose gel of DNA samples extracted from bacterial strains

The molecular analysis of the 16S rRNA sequence amplified in this study successfully identified the isolated bacteria at the species level. The pairwise analysis (Figure 5) revealed that the bacterium was *Enterobacter cloacae* by showing the highest percentage of nucleotide sequence similarity, 89%, with different global isolates and strains of the same bacterium. Thus, its sequence was deposited into the GenBank database under accession number OP936020.1. As well as, The phylogenetic analysis grouped the isolated *E. cloacae* (OP936020.1) with other strains, particularly the strain *E. cloacae* AR2019-6 with accession number MN150528.1 in the same clade (Figure 6).

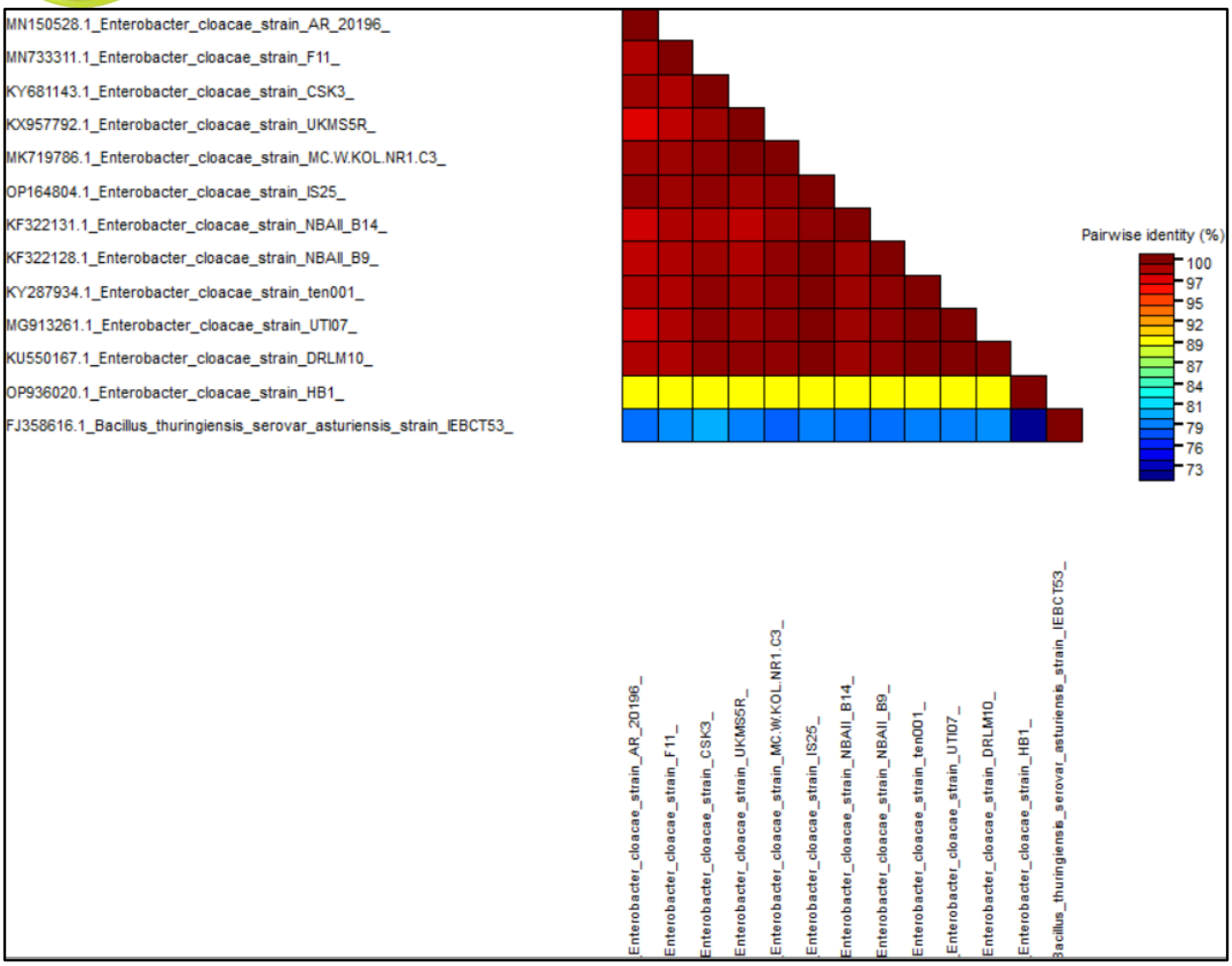

Figure (5): The pairwise analysis of the isolated *Enterobacter cloacae* with other global strains. The diagram was built based on the 16S rRNA sequences similarity using Sequence Demarcation Tool v.1.2 software [18].

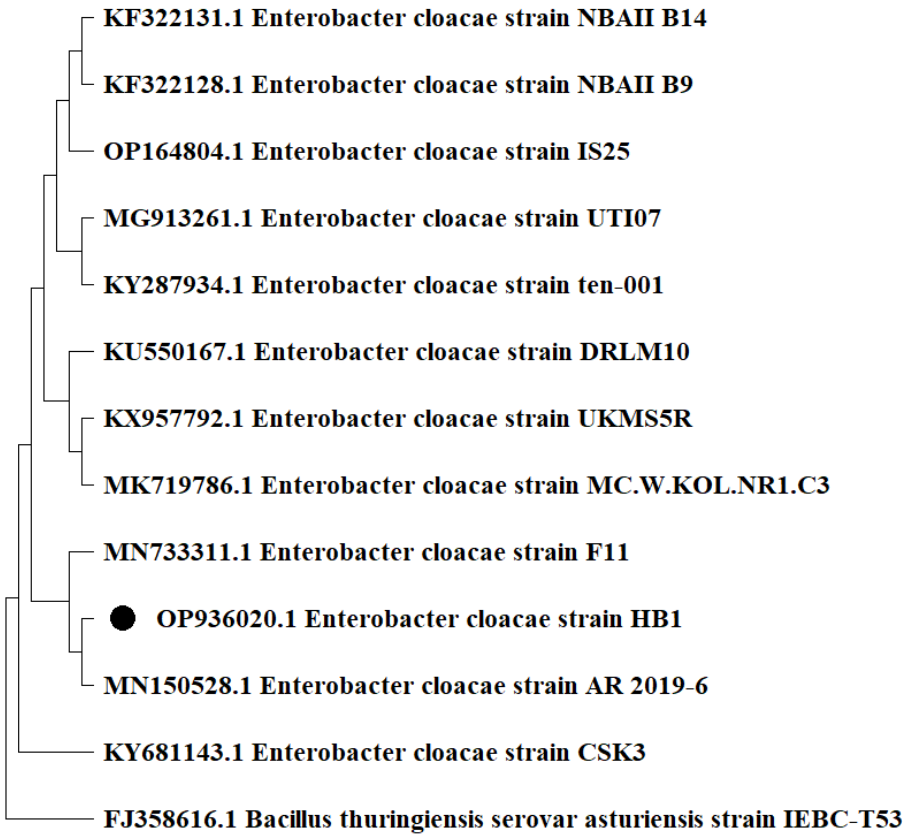

Figure (6): The phylogenetic analysis of the isolated *Enterobacter cloacae* with other global strains. The Phylogenetic tree was built based on the 16S rRNA sequences using MEGA v.11 software [19]

To the best of our knowledge, this is the first record of *E. cloacae* causing soft rot disease on potatoes in Iraq.

Acknowledgements
The first author would like to thank admission of the Dept. of Plant Protection / Faculty of Agriculture/ Kufa University for allowing using the department facilities.

References

1) **A**frous, A.; M. Pour and Ghanad, S.2014.Water Use Efficiency (WUE) is an Index of optimum water use in Agriculture (Case Study: Southwest of Iran, Dezful). ABR.,5(2):201-202.
2) Binod, P.L.; B. K. Bhim,; C. Duryodhan,; P. P. Bishnu,; J. S. Sung,; H. On-Sook, and Yul, R.K.2015.Growth and yield characters of potato genotypes grown in drought and irrigated conditions of Nepal. Int. J. Appl. Sci. Biotechnol.,3:513–519.
3) Al-Mashhadani, Abdullah Muhammad. 2005. Financial calendar for potato crop production in Iraq ((Al-Mashada village in Yusufiyah / Baghdad as a study model))

for 2002. Department of Agricultural Economics / College of the Agriculture / University of Baghdad. Iraqi Agricultural Sciences Journal. 36 (3): 151–156.

4) Central Statistical Organization. 2019. Production of cotton, yellow corn and potatoes for the year 2018. Ministry of Planning and Development Cooperation. The Republic of Iraq. http://cosit.gov.iq/ar/agri-stat/veg-prod

5) Des Essarts, , Y. R.; J. Cigna; A. Quêtu-Laurent; A. Caron; E. Munier; A. Doolotkeldieva, T.; S. Bobusheva and Suleymankisi, A. 2016. Biological Control of *Erwinia carotovora* and *Carotovora* ssp. by *Streptomyces* Species. Advances in Microbiology. 6, 104-114.

6) Kapsa, J.; M. Koodzeijczki and Ochrona, R. 2005. The most important disease of potato during vegetative growth, Tribology Int., 50: 27-31.

7) Nykyri, J. 2013. Virulence of Soft-Rot Enterobacteria Affecting Potato. The University of Helsinki, Department of Agricultural Sciences, Doctoral Thesis in Plant Pathology. Finland. 45p.

8) Abd-Elhafeez, E., AlKhazindar, M., & Sayed, E. (2018). Isolation and characterization of *Enterobacter* strains causing potato soft rot disease in Egypt. *Minia Science Bulletin, Botany Section*, *29*(1), 1-13.

9) Schroeder, B. K., Waters, T. D. and du Toit, L. J. 2010. Evaluation of onion cultivars for resistance to *Enterobacter cloacae* in storage. *Plant Dis.* 94:236-243.

10) Moreira, S.I., Dutra, D.D.C., Rodrigues, A.C., Oliveira, J.R.D., Dhingra, O.D. and Pereira, O.L., 2013. Fungi and bacteria associated with post-harvest rot of ginger rhizomes in Espírito Santo, Brazil. *Tropical Plant Pathology*, *38*, pp.218-226.

11) Wang, G.F., Praphat, K., Xie, G.L., Zhu, B., Li, B., Liu, B. and Zhou, Q., 2008. Bacterial wilt of mulberry (*Morus alba*) caused by *Enterobacter cloacae* in China. *Plant Disease*, *92*(3), pp.483-483.

12) Garcia-González, T., Sáenz-Hidalgo, H. K., Silva-Rojas, H. V., Morales-Nieto, C., Vancheva, T., Koebnik, R., & Ávila-Quezada, G. D. (2018). *Enterobacter cloacae*, an emerging plant-pathogenic bacterium affecting chili pepper seedlings. *The plant pathology journal*, *34*(1), 1.

13) Goszczynska, T. And J. J. Serfontein, 1998. Milk Tween Agar, a semiselective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *Phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*. Journal of Microbiological Methods, 32(1):65-72.

14) Kamysz, W.; A. Krolicka; K. Bogucka; T. Ossowski; J. Lukasiak and Lojkowska, E. 2005. Antibacterial activity of synthetic peptides against plant pathogenic *Pectobacterium* species. Journal of Phytopathology, 153:313-317.

15) Wang, F., Jiang, H., Shi, K., Ren, Y.I., Zhang, P.A.N. and Cheng, S., 2012. Gut bacterial translocation is associated with microinflammation in end-stage renal disease patients. *Nephrology*, *17*(8), pp.733-738.

16) Clark MS (1997). In: Plant Molecular Biology - A laboratory manual; Springer-Verlog Berlin Heidelberg, New York. pp. 305-328.

17) Fernández-Bravo, A., & Figueras, M. J. (2020). An update on the genus Aeromonas: taxonomy, epidemiology, and pathogenicity. Microorganisms, 8(1), 129.

18) Lahuf, A. A. (2019). *Alternaria alternata* causes leaf blight of rosy periwinkle (*Catharanthus roseus*) in Iraq. Australasian Plant Disease Notes, 14(4):1-4.

19) Lahuf, A. A.; Kareem, A. A.; AL-Sweedi, T. M. and Alfarttoosi, H. A. (2019). Evaluation the potential of indigenous biocontrol agent *Trichoderma harzianum* and its interactive effect with nanosized ZnO particles against the sunflower damping-off pathogen, Rhizoctonia solani. IOP Conf. Series: Earth and Environmental Science 365: 012033.