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Isolation, diagnosis, and antibiotic resistance of the plant pathogen *Erwinia tracheiphila*

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A B S T R A C T

This study was conducted with the aim of identifying the cause of bacterial wilt disease in cucurbits, which is considered one of the most threatening diseases for melon and cucumber production because it causes great losses that can reach up to 75% of the annual production, and the study proved *that Erwinia tracheiphila* is the main cause of this disease after isolating (50) isolates of it from waterm melons and cucumbers leaves that showed symptoms of infection and performing several chemical tests to diagnose it. The effect of (6) different types of antibiotics (rifampin, cefotazima, piperacillin, ceftriaxone, tobramycin and ciprofloxacin) on it was studied by the sensitivity test method. The antibiotic ciprofloxacin gave the highest inhibition value, as indicated by the diameter of the inhibition zone around the disc, which reached (35) mm, followed by tobramycin (20) mm and piperacillin (15) mm, while other antibiotics were ineffective due to resistance. This result indicates that this bacterium is resistant to several drugs, but at the same time it can be treated with antibiotics. That they are sensitive to them, which was indicated in this study for the purpose of controlling its infection of plants, especially cucurbits. The results of this study are therefore very important in terms of promoting the overcoming of its natural resistance to many antibiotics, which limits the possibility of controlling it when resistant strains appear in a particular country.

Introduction

Bacterial wilt of cucurbits is caused by the bacteria *Erwinia tracheiphila* This is one of the most serious diseases affecting cucurbits [1]. It is the pathogen that causes significant damage to pumpkin crops, Watermelon and cucumber are among the crops most susceptible to infection, and production losses can be as high as up to 80% [2]. *E. tracheiphila* is transmitted by cucumber beetles, transmission occurs when beetles infected with the bacteria settle on fresh plant wounds, or on flower nectar, once inside the plant, the bacteria multiply in the xylem vessels to produce exogenous sugars that clog the vascular system, this causes the plant to wilt and collapse completely within 7 to 21 days [3;4]. The taxonomic status of the bacterium *Erwinia tracheiphila* is as follows [5]:

Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Erwinia*
Species:	*tracheiphila*

Some previously published studies have classified *E. tracheiphila* strains using phenotypic methods [6], relevant studies were identified Using molecular methods for *E. tracheiphila* at the strain level [7] and for the host cucurbit species from which the bacterium was isolated. On the other hand, other researchers believe that treating bacterial wilt depends on treating seeds with insecticides, but the effectiveness of these pesticides can be variable [8]. The frequent use of antibiotics to treat bacterial diseases outside the scope of plant pathogenic bacteria (PPB) is a cause for concern, particularly in *Salmonella enterica* and *Klebsiella pneumoniae*, which are important human pathogens. In addition, the transfer of resistance within the range of plant-associated bacteria needs to be further investigated, and there is a growing interest in the use of antibiotics to control major bacterial plant diseases [9].

As a result, a new wave of research began to address the problem of bacterial wilt. In this study, the potential role of using antibiotics to detect the susceptibility of bacteria associated with plant pathogenicity (*E. tracheiphila*) in the laboratory was highlighted. The research objectives were focused on achieving the following points:

1. Isolation and identification of *Erwinia* bacteria because of their pathogenic importance in economic plants.
2. To study their susceptibility to common antibiotics

Materials and methods:

Isolation and diagnosis of the pathogen:

Isolation was carried out by taking small pieces about 0.5 cm long from samples of cucumbers and watermelons infected with the disease. They were immersed in 70% ethanol for three minutes to sterilise the surface and planted in Petri dishes containing MacConkey agar medium. The growing colonies were purified by replanting until single colonies with characteristics were obtained. Appearance identical to that of *Erwinia* bacteria [10].

The growing bacteria were identified using the Gram stain test; bacterial smears were prepared from growing colonies and slides were stained with Gram's stain and examined under an oil lens at 100X magnification [11].

As well as the sources for the diagnosis of *Erwinia* bacteria are the following biochemical tests:

- Catalase test; this was done by transferring a portion of a fresh, pure colony for each isolate using a loop culture loop to a clean glass slide to which 2 drops of hydrogen peroxide (H_2O_2) had been added. It was observed that O_2 bubbles rose from the hydrogen peroxide substance [12].
- Oxidase test; the test was performed by plating a portion of the pure colony of each bacterial isolate on the for 24 hours on the surface of a filter paper saturated with reagent (diamine dihydrochloride tetra-methyl-phenylene) and observing the change in colour. The appearance of a purple colour in 10 seconds indicates the ability of the bacteria to produce the oxidase enzyme and has a positive effect on the test [13].
- 36°C growth test; a load of bacterial growth is transferred to the surface of the nutrient agar medium, inoculated using a plating method and the dishes are incubated at 36°C for a period of 48-72 hours. The appearance of bacterial growth on the surface of the inoculated dishes is a positive result [14].
- Levin production test; the purpose of this test is to detect the enzyme sucrase. A load of test bacteria is transferred to the surface of a nutrient agar medium containing 5% sucrose. A positive result is the formation of colonies from a convex fold [15].
- Growth test on medium containing 2% sodium chloride; a load of bacterial growth was transferred from the surface of nutrient agar containing 2% sodium chloride and inoculated using the plating method. Plates were incubated at 28°C for 24-48 hours. The appearance of

bacterial growth on this medium is a positive result [16].

- Agglutination test; the strip prepared by Neogen (UK), consisting of the test solution, positive control solution and negative control solution, was used. The test was carried out according to the manufacturer's recommendations by placing a drop of the bacterial suspension of the isolate to be tested in the centre of the strip prepared by the company and adding a drop of the test solution. The test was performed in the same way. Test with positive and negative reference solutions and record the result.

Testing the effect of antibiotics on bacterial growth in the laboratory:

The disc diffusion method was used by placing ready-made antibiotic discs from the Turkish company Bio-analyse (Table 1) on the prepared agar-Muller-Hinton medium from the company Hi-media, which was inoculated by spreading the bacteria on its surface. The dishes were incubated at 28°C for 24-48 hours, then the diameters of the growth-free areas around each disc were measured in mm and the results recorded [17].

Table (1): Antibiotics used, their concentrations and abbreviations

No.	Antibiotics	Conc. µg/disc	Code
1	Rifampin	5	RA
2	Ceftriaxone	10	CRO
3	Cefotaxime	10	CTX
4	Tobramycin	10	TOB
5	Piperacillin	30	PRL
6	Ciprofloxacin	10	CTP

Results and discussion:

Isolating the pathogen:

Isolation results from infected leaves of watermelon and cucumber plants showed bacterial growth around the infected parts grown on MacConkey medium, as shown in Figure (1).

Figure (1): Colony of *Erwinia* bacteria on MacConkey agar medium.

Purification of bacterial colonies:

The purification results showed large colonies (2-5 mm in diameter), convex, shiny, opaque and light pink in color.

Diagnosis of the bacteria :

The results of staining the bacteria with Gram's stain and examining them under the microscope at 100× magnification (Figure, 2) showed that the bacterial cells, Bacillus brevis, were Gram-negative and did not form spores. This is identical to what was indicated by Bal, [18], and the results of the tests mentioned in Table 2, by diagnosing the bacteria isolated from the infected samples, the isolate belongs to the species *Erwinia tradheiphila*, and these results are consistent with what was stated in the diagnostic systems approved by AL-Dahmashi, [19], the bacteria were positive for the catalase test because bubbles appeared when the colony was immersed in the 3% H_2O_2 reagent. It was also negative for the oxidase enzyme, as it did not turn a dark purple colour when the oxidase reagent was added. No bacterial growth was observed when the inoculated dishes were incubated at 36°C and the bacteria gave a positive result on the fibroin production medium, indicating the formation of large colonies of a sticky, slimy nature. The results of the bacterial growth test on a salt medium (2% sodium chloride) showed that the bacteria were able to tolerate and grow on the salt medium.

Figure (2): *Erwinia* bacterial cells under a light microscope at 100× magnification, stained with Gram's stain.

Table (2): Biochemical tests used to identify bacteria *Erwinia tradheiphila*

No.	Biochemical tests	Results
1	Gram stain test	-
2	Catalase test	+
3	Oxidase test	-
4	36°C growth test	-
5	Levin production test	+
6	Medium containing 2% sodium chloride growth test	+

Agglutination test results:

The results of the agglutination test (Figure 3), using standard sera prepared by Neogen, confirmed that all the isolates tested were positive for the agglutination test. These results, on the other hand, agreed with the results of Alwakil [20], which indicated that the isolated bacteria belonged to the *Erwinia tradheiphila* species. The researchers emphasised the importance of diagnosing the cause of bacterial wilt in cucurbits based on diagnostic methods that include the use of selective media,

pathogenicity tests, as well as molecular and immunological methods [21], because the symptoms of this disease are similar to other diseases in terms of pathological symptoms in the early stages of infection. As with blossom blast disease, which is caused by the bacterium *Pseudomonas*, the absence of bacterial ooze in blossom blast disease is one of the symptoms that is useful in distinguishing between the two diseases [22]. As well as the disease Nectria twig blight, caused by the fungus *Nectria*, which can be distinguished from bacterial wilt disease in cucurbits by the presence of bright orange fruiting bodies on infected surfaces [23]. It is necessary to use specialised and sensitive methods to detect the bacterium *Erwinia tracheiphila* in seedlings in order to prevent the disease from entering areas free of it and to determine the spread of new bacterial strains in areas where the disease is already present [24].

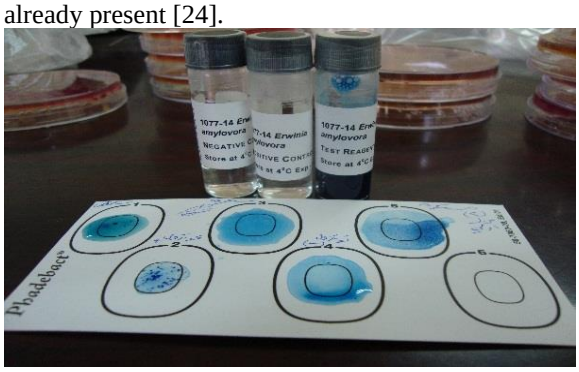

Figure 3: Agglutination test results

Laboratory test of the effect of antibiotics on bacterial growth:

Table (3) and Figure (4) show that the antibiotic ciprofloxacin has the greatest effect on laboratory bacterial growth as the diameter of the growth inhibition zone reached (35) mm, followed by the antibiotic tobramycin (20) mm and then the antibiotic piperacillin (15) mm, while other antibiotics were ineffective, and this may be due to the *Erwinia* bacteria, which belong to the Enterobacteriaceae family, whose members are characterised by a high ability to resist many antibiotics, both natural and acquired [25]. Note that antibiotics are organic compounds produced by some microorganisms that have a selective inhibitory effect on the growth of microorganisms, such as nucleic acid metabolism, or affect specific enzymes or the cell membrane or cell wall of the target cell [26].

Table (3): The effect of antibiotics on laboratory growth of bacteria

No.	Antibiotics	Inhibition zone(mm)	Result
1	Rifampin	----	R
2	Ceftriaxone	----	R
3	Cefotaxime	----	R
4	Tobramycin	20	S
5	Piperacillin	15	S
6	Ciprofloxacin	35	S

--- :with effect, R : Resistance, S :Sensitive

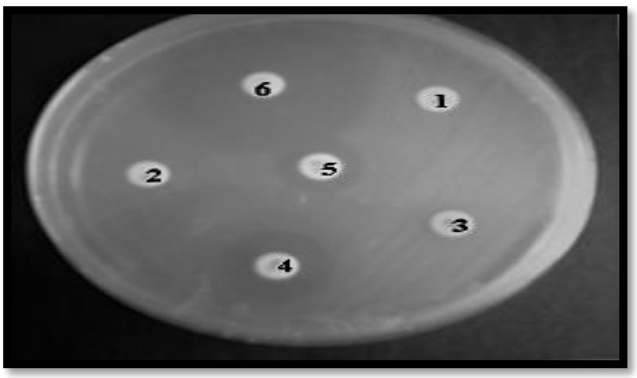

Figure (4): The effect of antibiotics on laboratory-isolated bacteria

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