



# ISOLATION AND DIAGNOSIS OF *Erwinia* FROM SOILS OF DIFFERENT REGIONS OF AL- MUTHANNA GOVERNORATE

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## Abstract

Soil samples were collected from ten different regions of Al- Muthanna province which were planted with potatoes. Three isolates were obtained from *Erwinia carotovora subsp atroseptica* (E.c.a) and two isolates of *Erwinia chrysanthemi* (E.ch). The results showed the ability of all isolates to cause infection when inoculating potato slices with bacterial isolates. The results also confirmed the negative effect of these isolates on germination rate of tomato, cucumbers and mung bean seeds, all the diagnosed bacterial isolates recorded a germination rate of 0% for all plants used in the experiment.

**Keywords :** Isolation, diagnosis, *Erwinia*, soils, Al-Muthanna governorate.

## Introduction

*Erwinia* bacteria is one of the most important plant pathogens and belongs to the *Enterobacteriaceae* family within the genus of *Pectobacterium*, Such as *P. ssp. Carotovorum*, *P. carotovorum ssp atrosepticum*, and *P. chrysanthemi* (Hauben, 1998), but this nomination has not been widely accepted by plant pathologists. It is a straight bacillus dimensions  $0.2-1.0 \times 1.0 \times 3.0$  micrometer moving by peritrichous peripheral whip, and do not form spores, facultative anaerobes, gram-negative bacteria, It is characterized by its ability to produce a large amount of pectic enzymes, In addition, other enzymes on which *Erwinia* bacteria depend on the decomposition of plant cell wall (Agries, 1988). The three species *Erwinia carotovora subsp carotovora* (Ecc), *Erwinia carotovora subsp atroseptica* (Eca) and *Erwinia chrysanthemi* (E.ch) cause bacterial mildew, (Ecc) has the ability to infect a wide range of plants, while (Eca) is specialized with one host is the potato plant and this specialization may be due to environmental conditions that help to survive for the next season and consequently the infection on the same crop in temperate cold regions. While E.c.h is a pathogen of many plants that grow in tropical and subtropical areas, it can infect some crops that grow in temperate areas (corn and dahlia). *Erwinia* is an opportunistic pathogen because the disease develops

only in case of the weakness of the host resistance (Pérombelon and Kelman, 1980). The infection caused by *Erwinia* depend on temperature, E.c.a be blisters at a temperature less than 25 °C, while E.c.h be blisters at higher temperatures (Pérombelon, 1987). But recently strains of E.ch have been found in cold region causing black leg disease. E.c.c has weak pathogenicity ability, this is due to its inability to compete with E.c.a. as well as E.c.h had the ability to infect maternal tubers, But it is considered the main cause of the aerial root leg disease, competition is less intense, especially at high temperatures or when using contaminated irrigation water and over-irrigation., because of the losses caused by these bacteria, the aim of the research was to detect and identify the types of these bacteria which spread in the soil of Al-Muthanna governorate for the purpose of reducing their damage.

## Materials and Methods

### Collect soil samples

10 samples were collected from soil planted with potato crop within the geographical area of Al-Muthanna governorate within the geographical area of Muthanna governorate, a composite sample was collected by collecting samples from one field and mixing them together to reduce the error rate and homogeneity to form a representative sample of the field as much as possible.

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All samples were placed in sterile plastic bags and kept in the refrigerator until use. (Table 1) shows the numbers and source of these isolates.

**Table 1:** Bacterial isolates obtained from soil in these regions.

| Isolate source              | Sample number |
|-----------------------------|---------------|
| Samawah- Abu jwelana        | 1             |
| Rumaiitha- Alhilal          | 2             |
| Rumaiitha- Alnajmi          | 3             |
| Alkhidhir- Aljazeera region | 4             |
| Samawah- Alseweer           | 5             |
| Samawah- Eastern Albasateen | 6             |
| Samawah- Alwekaa            | 7             |
| Alkhidhir- Aldaraji         | 8             |
| Rumaiitha- Almjd            | 9             |
| Samawah- Alwekaa            | 10            |

### Isolation of bacteria

A dilution series of soil samples was prepared above by adding 10 g of each soil sample to 90 ml of sterile water in a 250 ml glass beaker and mixing well, serial dilution was carried out by transferring 1 mL of stuck soil to a test tube containing 9 mL of sterile water and repeated the process until dilution was achieved, then take 1 ml of each dilution and inoculated test tubes containing 9 ml of nutrient broth by three replicates for each dilution and then incubated tubes antenna at a temperature of 28 °C, for 48 hours, 0.1 ml of tubes that gave a positive indication were taken and spread on the surface of a petri dish containing the nutrient agar medium and the potato dextrose agar medium, the dishes were incubated at 28 °C for 48 hours, then individual colonies were elected and replanted in the same way for ascertain the purity of isolation and write down the phenotypic qualities of developing colonies. From these colonies membranes were prepared on clean glass slides, It was then stained with a gram dye and tested under a light microscope.

### Detection of pathogen isolates

#### Pathogenicity test on potato slices

Potato tubers were taken and sterilized superficially by immersing them with 1% sodium hypochlorite free chlorine solution for a minute, then washed with sterile distilled water and dried on sterile filter paper.

The tubers were cut into slices of 7-8 mm thickness and superficially sterilized with 70% alcohol for a minute, then washed with sterile distilled water and dried on sterile filter paper, and then distribute slices in sterile glass dishes at a rate of 4 slices / dish, then add 10 mL per bacterial inoculum with a concentration of 105 colony forming units

/ 1 mL prepared from the bacterial isolates grown on the medium 48 hours old.

The slides in the dishes were immersed with the bacterial inoculum, the dishes were incubated at 23 °C for 7 days and the results were taken (Kado and Heskett, 1970).

#### Biochemical tests

Several tests were conducted including Catalase test, Oxidase test, Carbohydrate Fermentation (Cruckshank *et al.*, 1975), Indole test (Koneman *et al.*, 1997), Red methyl test, Voges proskauer test, and Citrate utilization test (Macrate, 1985).

#### Phosphatase test

The test medium was prepared and distributed in 5 sterile Petri dishes by drilling and leaving a dish without inoculum as a comparison. The dishes were incubated at 23 °C for 48 hr. The reaction is positive when the medium changes color to dark (Dickey, 1979).

#### Crystal Violet Pectat Medium (CVP)

This medium was used to distinguish the *Erwinia* species from the *Agrobacterium* and *Pseudomonas* species. CVP medium consisting of solutions (A) and (B) was prepared. Solution (A) was prepared by dissolving 3 ml of 10% of aqueous solution  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 10.5 ml of aqueous solution Crystal Violet 0.75%, 4.5 ml (1N) of NaOH and 5 ml of Silicon aqueous solution 0.001% in 200 ml hot distilled water, then the solution was heated with shaking with the electric mixture for five minutes and the solution was set aside. B solution was prepared by dissolving 1.0 g  $\text{NaNO}_3$ , 50 mg of extract yeast, 4.0 gm of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 15 gm of Poly galacturonic and 2 gm of agar in 300 ml of Hot distilled water. The medium Inoculated with bacterial isolates on NA medium in 24-hour old by striping method. The dishes were incubated at 23°C for 72 hours and the growth nature was recorded on this medium (Cupples and Kelman, 1974; Neill and Logan, 1975).

#### Pectin analysis test

The test medium was prepared and distributed in 5 test tubes at a rate of 5 ml / tube and sterilized the medium with the incubator and inoculated with 10 drops of bacterial culture grown on Nutrient Broth for 24 hours, the tubes were incubated at 27 °C for 5 days, it is inferred that the reaction is positive when the medium in the tubes is liquid (Kado and Heskett, 1970).

#### Effect of bacterial isolates on germination ratio

A laboratory experiment was carried out to determine the effect of diagnosed bacterial isolates on seed germination ratio (cucumber, tomato, Mung bean). The

soil was sterilized by the incubator and then placed 1 kg of soil in 1 kg plastic pots after the pot was sterilized with a medical cotton saturated with ethyl alcohol. The soil was planted with the seeds of tomatoes, cucumbers and Mung bean contaminated with bacterial isolates by three replicates and left three replicates without inoculation (comparison treatment). After one week of germination, germination ratio was calculated.

## Results and Discussion

Phenotypic and microscopic characteristics of the pathogen. Some colonies growing on the NB and PDA media showed phenotypic and microscopic characteristics identical to *Erwinia* sp. The colonies took smooth, convex circular shapes of yellow or cream with regular or irregular edges 48 hours after incubation (Ruissen and Janse, 1988; Palacio-Bielsa *et al.*, 2006). The colonies on CVP medium were white, inclined, convex and opaque. When bacterial cells were smeared and stained with gram dye and tested under a light microscope, negative staining, a short, rounded bacillus with a single and mostly double end, appeared negative (Janse and Spit, 1989; Palacio-Bielsa *et al.*, 2006).

### Detection of pathogen isolates on potato slices

Test results of the pathogenicity ability on the potato slices have proven showed that five isolates showed phenotypic and microscopic congruence with *Erwinia*

sp isolated from soil cultivated with potato crop which was capable of causing disease. These isolates are 2, 3, 6, 8 and 10 and showed symptoms of bacterial soft rot on the potatoes in the dishes with an unpleasant odor when the dish was opened after three days of inoculation. The results are consistent with the results of (Kelment and Rudolph, 1990; Schaad, 1980). So, on this basis, the five isolates were selected for the purpose of studies.

### Biochemical tests

The results of biochemical tests in table 2 showed that all bacterial isolates are not able to produce Cytochrom Oxidase, while the results of all isolates showed their ability to produce Catalase enzyme through exhalation of oxygen bubbles resulting from the decomposition of hydrogen peroxide  $H_2O_2$  when treated by isolates of bacteria. The results of the IMVIC tests, which include the test of indole, red methyl and Voges proskauer, which are used to distinguish between the two strains of bacteria *Erwinia carotovora subsp carotovora* (Ec.c) and *Erwinia carotovora subsp atroseptica* (Ec.a). The isolates that gave negative test results for the test of indole, red methyl and Voges proskauer were isolates (E.c.a), while the isolates that showed positive results for these tests were the strains of bacteria (E.c.c) and *Erwinia chrysanthemi* (E.c.h) (Koneman *et al.*, 1997). To distinguish bacterial isolates, citrate testing was carried out, the isolates belonging to (E.c.a) and (E.c.c) given positive results for citrate test,

while isolates belonging to (E.ch) gave a negative result. Regarding the fermentation test of sugars (lactose, maltose, sucrose)

The isolates that gave a positive result by converting the color of the medium from red to yellow are bacterial (E.c.a). (E.c.c) (Kleman and Dickey, 1988). The results showed that all isolates were able to analyze pectin. From the results of the table it was found that bacterial isolates 2, 3 and 10 belong to *Erwinia carotovora subsp atroseptica* (E.c.a), while isolates 6 and 8 are due to *E. chrysanthemi*.

### Effect of bacterial isolates on germination ratio

This test showed the negative effect of all isolates on the seed germination ratio used in the experiment, all isolates recorded a

**Table 2:** Biochemical tests.

| Results |   |   |   |   | Tests                           | Isolate no.        |
|---------|---|---|---|---|---------------------------------|--------------------|
| 10      | 8 | 6 | 3 | 2 |                                 |                    |
| -       | - | - | - | - | Response of cells to Gram stain | 1                  |
| +       | + | + | + | + | Motility test                   | 2                  |
| -       | + | + | + | + | Gelatinase test                 | 3                  |
| +       | - | - | + | + | Citrate utilization test        | 4                  |
| -       | - | - | - | - | Oxidase test                    | 5                  |
| +       | + | + | + | + | Catalase test                   | 6                  |
| -       | + | + | - | + | Methyl Red test                 | 7                  |
| -       | + | + | - | + | Indole test                     | 8                  |
| -       | + | + | - | + | Voges proskauer test            | 9                  |
| +       | - | + | + | + | Gelatinase test                 | 10                 |
| +       | + | + | + | + | Urease test                     | 11                 |
| -       | - | - | - | - | Starch hydrolysis test          | 12                 |
| -       | + | + | - | + | Growth at 37 ° C                | 13                 |
| +       | - | - | + | + | Growth in 5% of NaCl            | 14                 |
| -       | + | + | - | - | Phosphatase test                | 15                 |
| +       | + | + | + | + | Pectate degradation             | 16                 |
| +       | - | - | + | + | Lactose                         | Fermentation<br>17 |
| +       | - | - | + | - | Maltose                         |                    |
| +       | - | - | + | - | Sucrose                         |                    |

germination rate of 0% for all types of plants, while the germination ratio of comparison treatment was 90%, 100% and 100% for Mung bean, tomato and cucumber plants respectively.

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