

Evaluation of the efficiency of some Natural and chemical Substances in inhibiting the Biofilm of *Enterobacter cloacae* and Stimulating potato plant Resistance in the field

Hawraa Razzaq Dhafer ALaboudi and Firas Ali Ahmed

Faculty of Agriculture - University of Kufa – Republic of Iraq

Corresponding author Email: hawraa1991h97h@gmail.com

DOI: <https://doi.org/10.36077/kjas/2025/v17i3.12983>

Received date: 1 /8 /2023

Accepted date: 19/ 10/2023

Abstract

Soft rot disease caused by different species of bacteria *Enterobacter cloacae*, *Pectobacterium carotovorum*, *Dickeya* is one of the most important diseases that destroy most fruits and vegetables globally. Due to the widespread use of chemical pesticides before harvesting, which led to environmental pollution which affects human health, amino acids and seaweed extract were used as natural materials and some chemicals were used to enhance plant defences against the pathogen. The results of the cell membrane revealed that the concentration of 0.5 for each of the amino acids, seaweed extract and copper 0.25 led to inhibition and biofilm formation, reaching <0.12 (0.11), <0.12 (0.10), respectively. In the field, the effect of applying the interactions A.a + Sef + cop + E.c significantly impacted the level of POD enzyme activity in plants treated in the preventive and therapeutic experiment, as it reached 33.7, 29.5 g / fresh weight. The overlap between them amounted to 31.6 g / fresh weight, compared to the control treatments, which amounted to (20.2, 18.3, 19.3), respectively. The combination A.a + Sef + cop + E.c significantly increased the activity of the PAL enzyme, as the enzyme levels reached 40.40 and 39.32 mg/fresh weight for the preventive and therapeutic methods, respectively, compared with the control treatment, which amounted to 21.25 and 20.95 mg/fresh weight. The effect of overlapping the treatment (A.a + Sef + cop) and the preventive and therapeutic experiments was 37.71 mg / fresh weight, with a significant difference from the control treatment, which amounted to 21.10 mg / fresh weight. The treatment of amino acids + E.c influenced increasing the total phenols in the plant, reaching 181.6 in the preventive experiment compared to the control treatment, which amounted to 153.4. Amino and spraying with seaweed extract led to eliminating bacterial soft rot caused by bacteria E.c and prolonging the storage process.

Keywords: *Enterobacter cloacae*, potato, Biofilm, Oligo x, Amino acid Soft rot



Introduction

Potato (*Solanum tuberosum*) is one of the strategic vegetable crops of high economic value in many countries of the world and Iraq. Potato is the fourth main food in the world's crops after rice, corn, and wheat. It is widely cultivated in various tropical and subtropical countries, with a cultivated area of more than 226 million tons (6,9). The potato crop is exposed to many pathogens, including viral, bacterial, fungal, and other pests, causing significant economic losses (15). Bacterial soft rot disease on potatoes is one of those diseases, which is one of the determinants of potato cultivation in the world, causing damage to the field and storage and a decrease in yield in quantity and quality (4). Soft rot bacteria also infect the crops of vegetables and fruits after harvest, which are severe and cause great losses, especially when appropriate conditions are available for their development, such as the occurrence of wounds and surface mechanical damage to the fruits, high temperatures and humidity, and the availability of sufficient oxygen (20).

Enterobacter cloacae is one of the most important causes of soft rot disease, which causes significant losses in crops during the growing season and during storage,

resulting in losses ranging between 30-50% in annual production (24). A plant can resist a pathogen or several pathogens through synthetic or biochemical defences that help the plant control the pathogen (17). Plant defences are activated by some biotic and abiotic factors, including using some chemical compounds that stimulate plant defences, known as Induced Systemic Resistance (ISR) (5).

Amino acids were used within the plant parts in their free form in various agricultural aspects in large aspects due to their easy permeability through the stomata and epidermal layers and their rapid absorption by the roots (23).

With the alarming burden of antibiotic resistance by some bacterial species, amino acids and seaweed extracts have been used as new alternatives to antimicrobials to treat many types of microbes, especially antibiotic-resistant bacteria, due to their antimicrobial activity and anti-biofilm activity (12). Due to the importance of soft rot disease, the study aimed to find an effective way to control soft rot disease on potatoes in the field by using some safe, environmentally friendly factors that are beneficial to plant growth.

Materials and Methods

The effect of amino acids and seaweed extract on the inhibition of the biofilm of *Enterobacter cloacae*

Amino acids of plant and animal origin were used to test at a concentration of 0.5 ml/L, Oligo X seaweed extract at a

concentration of 0.5 ml/L, and pathogenic bacteria at a concentration of 10⁸ for each factor in the effect on bacterial cell membrane formation according to the method described by (8) with some modification. The bacterial suspension was prepared 24 hours before at a

concentration of 108 CFU; then a Microtiter Plate was taken to add N.A Nutrient broth, which had been previously prepared, with a volume of one ml for each hole of the plate. After that, the tested factors were added, each according to its concentration, by 4 replicates for each treatment, with the implementation of a comparison treatment using N.A medium without any other additions. Each hole was inoculated with 100 microliters of bacterial suspension at a concentration of 108 and incubated for 48 hours at 28 °C. After the end of the incubation period, the medium was removed from the pits, and then the pits were washed well with sterile distilled water and exposed to room temperature without a cover to dry them. Then 200 microliters of crystal violet dye (1%) were added and left to dry for 20 minutes, then washed well with sterile distilled water to remove the remaining dye. All pits and each treatment were examined under a light microscope of 40× magnification with a camera connected to a computer for imaging purposes. Then the dry layer was dissolved in each hole by adding 200 microliters of ethanol (80%) to calculate the degree of absorbance (Absorbance value) utilizing a spectrophotometer at a wavelength of 570 nm.

Effect of amino acids and Oligo X on the level and activity of peroxidase (POD) in potato plants in the field.

Leaves were collected from the center of potato plants, each according to its treatment, in polyethene bags, placed in a box containing ice, and brought to the laboratory to be washed with sterile distilled water and dried from the water using a sterile filter paper. The leaves were

cut into small pieces with sterilized scissors, and one gram of the leaves was taken from them and crushed using a sterile ceramic mortar in the presence of a phosphate buffer solution (KH_2PO_4) (10 ml) of neutral acidity and in refrigerated conditions until juice is extracted from them. The mixture was centrifuged at a speed of 1000 rpm for 20 minutes. Then add one ml of quaiacol dye (0.5%) with hydrogen peroxide (H_2O_2) at a concentration of 0.3 v/v to start the reaction.

The activity of the enzyme was measured for each treatment by adding two ml of the reaction mixture in a cell in a spectrophotometer at a wavelength of 420 nm, then adding 100 microliters of the sample and following up the change in light absorption every 30 seconds for three minutes. (21) equation was applied to estimate the change in the reading, which is proven below.

$$POD = \frac{\frac{\Delta A}{\Delta T}}{\text{fresh weight (g)}}$$

Whereas:

ΔA : change in absorbance.

ΔT : the time it takes for a change in absorption.

Effect of amino acids and Oligo X on the level and activity of phenylalanine ammonia-lyase (PAL) in potato plant.

Leaves were collected from the center of potato plants in polyethene bags and placed in a box containing ice until they arrived at the laboratory to start preparing samples by washing them with distilled water and getting rid of excess water by



drying them on sterile filter paper. One gram was taken and crushed by a ceramic mortar in the presence of 10 ml of sodium borate buffer (0.1 M, pH 7.5) and under refrigerated conditions. The extract was filtered through two layers of sterile gauze and centrifuged at a speed of 6000 r/min for 20 minutes. Transfer 0.2 ml of the filtrate to a glass tube and add one ml of 0.1 mM phenylalanine solution, pH 8.8, and 2.5 ml of 12 mM sodium borate buffer. The samples were incubated for one hour at a temperature of 38 °C, and to stop the reaction, 0.5 ml with a concentration of 1 M of trichloroacetic acid (TCA) was added. Absorbance readings were recorded using a spectrophotometer at a wavelength of 290 nm. Enzyme activity was measured based on $\mu\text{g/cinnamic acid/hour/g}$ fresh weight according to the equation described by (2).

$$Y=0.0974 X + 0.0051$$

Representing:

Y: absorbance reading.

Results and Discussion

The effect of amino acids and seaweed extract on the inhibition of the biofilm of *Enterobacter cloacae*

The test results of Table (1) showed the agents used in the experiment with the highest concentrations, the ability of bacteria to form a biofilm. The results of this study indicated the ability of the treatments Acetic acid, Streptomycin, Ampicillin and *E. cloacae* to form a high biofilm. There were no significant differences compared to the comparison treatment. In contrast, the study showed that Ceftriaxone formed the cell membrane

X: the concentration of cinnamic acid in the sample.

Effectiveness measurement of total phenols

The presence of total phenols was measured in the leaves of potato plants 10 days after spraying the plants with seaweed extract at a concentration of 0.5 ml/L. The method of (18) was followed by crushing one gram of leaves in a ceramic mortar with 10 ml of methanol (80%), then transferring them to tubes, which were later placed in a water bath at a temperature of 70 °C for 15 minutes, with continuous stirring during the incubation period. Transfer one ml of the filtrate with 5 ml of sterile distilled water and 250 microliters of Folin reagent into a sterile glass tube and incubate at 25 °C for 30 minutes. The amount of phenols was measured on the basis of milligrams of phenols per gram of soft plant tissue by a spectrophotometer at a wavelength of 725 nm.

(Biofilm) moderately compared to the comparison treatment and other treatments. The rate the absorption is 0.16 nm.

It was also shown from the tables show that the factors Copper and x Oligo were the best among the other factors, as they gave the lowest rate of absorption values, 0.10 and 0.11, respectively, indicating that the cell membrane was not formed at all. Thus it was significantly superior to the treatments Acetic acid, Streptomycin, Ampicillin and *E. cloacae* in terms of their effect on the formation of the biofilm.



Table 1. The effect of amino acids and seaweed extract on the inhibition of the biofilm of *Enterobacter cloacae*

Treatment	Rang 570nm	Standard range of OD	Biofilm	Adherence
Copper	0.10	0.12 >	Non	Non
Acetic acid	0.11			
x Oligo	0.11			
Control	0.00			
Ceftriaxone	0.16	0.12-0.24	Moderately	Moderately
Streptomycin	0.27	0.24 <	High	High

The results of the test with the lowest concentrations are in Table No. (2) showed the ability of the factors used in the experiment to form a biofilm. None of the studied factors was able to form a biofilm and not stick to the plate used in the experiment within the lowest range of the standard rate used in the experiment (19), While the cell membrane formation appeared moderately in each of Oligo,

Copper, and Ceftriaxone, as the absorption values were (0.12, 0.12, 0.24), respectively, the results also showed that the treatments (Acetic acid, Streptomycin, Ampicillin, and *E. cloacae*) were the highest in cell membrane formation among the other treatments, as the absorption values were (0.30, 0.36, 0.32, 0.72), respectively.

Table 2. Testing the ability of *Enterobacter cloacae* to form biofilm and the ability of the studied agents to prevent the formation of bacterial cell membranes at low concentrations.

Treatment	Rang 570nm	Standard rang of OD	Biofilm	Adherence
Control	0	0.12 >	Non	Non
Oligo	0.12	0.12-0.24	Moderately	Moderately
Acetic acid	0.13			
Copper	0.12			
Ceftriaxone	0.24			
Streptomycin	0.36	0.24 <	High	High
Ampicillin	0.32			
<i>E. cloacae</i>	0.72			

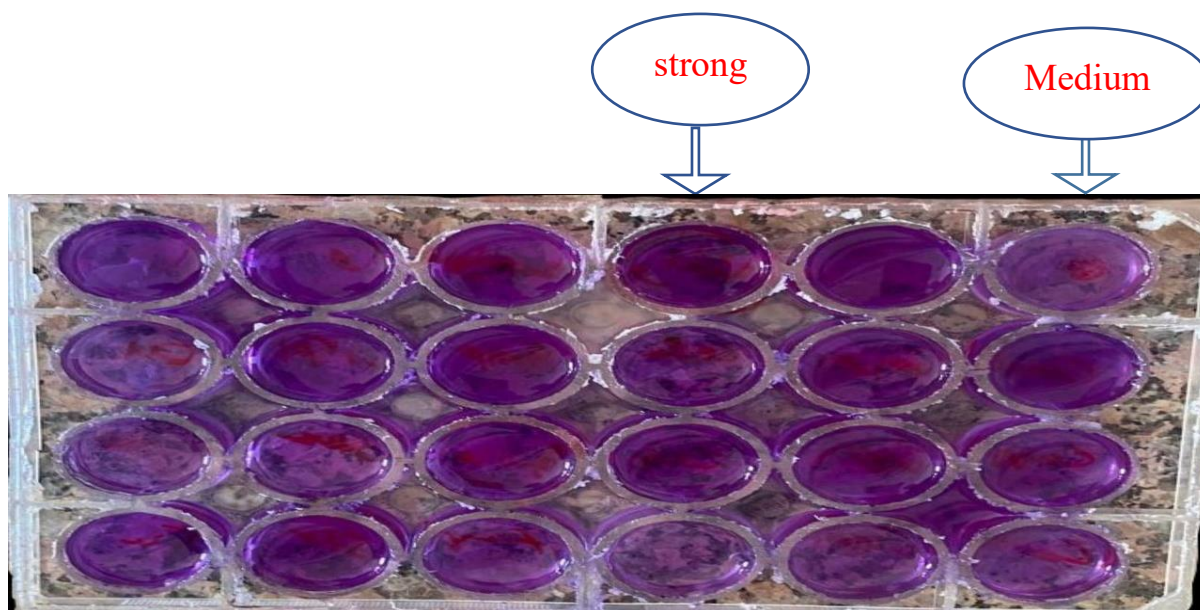


Figure 1. Biofilm formation of *E. cloacae* isolate by microplate method (MTP)

The formation of the biofilm in liquid media may be due to the action of the flagella present in the bacteria, which helps them to swim and move freely to form biofilms. This was confirmed by (13) in a study in which he showed biofilm formation in gram-negative bacteria using flagella-mediated movement until attachment occurs in a specific site and its attachment to the form of elastic tissue at first.

Our study agreed with the study conducted by (3) on the ability of *E. cloacae* isolates to form the highest biofilm. (10) indicated that the virulence of plant pathogens is attributed to their ability to form the cell membrane.

The inhibition of biofilm formation of *Enterobacter cloacae* is due to the fact that the inhibitor affects the flagella present in the bacteria, which in turn affects their motility and affects the production of

The bacteria's resistance to antibiotics and inhibitors is due to encapsulating itself inside special capsules to protect itself from external influences. This is consistent with what was confirmed by (1) in his study, in which he showed that microorganisms encapsulate themselves inside biofilms within a matrix of polymeric materials outside the cell that can act as a barrier and be resistant to various hostile conditions such as sterilizers, antibiotics, and others.

Adenosine triphosphate (ATP) is critical in bacterial membrane adhesion and biofilm formation through cell lysis and extracellular DNA (16) release.

polysaccharides, in addition to analyzing the association between the formed biofilms as well as reducing the thickness of the biofilms (14).

Effectiveness of application of agents on potato plants in affecting the level and activity of the peroxidase enzyme (POD).

The results shown in Figure 5 showed that the treatment of adding the triple mixture alone (A.a. + Sef + cop) and the triple mixture with the pathogenic bacteria was significantly superior in achieving the highest rate of peroxidase enzyme in the plant, which amounted to 32.7 and 33.7 minutes/gm fresh weight, respectively, compared to the control treatment, which amounted to 20.2 minutes/gm fresh weight, after (10) days of adding seaweed extract at a concentration of 0.5 ml/liter. As for the treatment method, the two treatments of adding amino acids and the triple mixture with pathogenic bacteria (A.a + Sef + cop + E.c) excelled without a

significant difference between them, reaching 29.5 minutes/gm fresh weight for both treatments, with a significant difference from the comparison treatment, which gave 18.3 minutes/gm fresh weight.

As for the preventive and therapeutic methods, it was evident from the same table that the preventive method of adding treatments significantly differed from the therapeutic method (24.2 minutes/gm fresh weight). It also showed the overlap between the treatments of the two therapeutic and preventive methods. The treatment of the triple mixture with pathogenic bacteria was significantly superior (31.6 minutes/gm fresh weight) to the comparison treatment, which averaged 31.6 minutes/gm fresh weight.

Table 3. The effectiveness of applying agents to potato plants in affecting the level and activity of the peroxidase enzyme (POD)

	Treatments	Protective (min/g fresh weight)	Therapeutic (min/g fresh weight)	Treatments effect
1	Distilled water	20.2	18.3	19.3
2	Pathogenic bacteria E.c	22.9	18.9	20.9
3	Amino acids A.a	21.2	29.5	25.3
4	ceftriaxone Sef	17.6	14.6	16.1
5	Copper Cop	20.2	18.5	19.3
6	A.a Sef + cop	32.7	26.2	29.4
7	Ec + A.a.	28.0	26.1	27.1
8	Ec. Sef	24.9	23.2	24.1
9	Ec + Cop	20.3	18.3	19.3
10	A.a + Sef + cop + E.c	33.7	29.5	31.6
Method Average		24.2	22.3	
Treatment L.S.D. 0.05		1.54		
Method L.S.D. 0.05		0.48		
Interaction L.S.D. 0.05		2.17		

The effectiveness of applying agents on potato plants in affecting the level and activity of the enzyme phenylalanine ammonia-lyase (PAL). The results of the effect of treatments by the preventive method showed that the treatment of potato tubers with a mixture of (amino acids + ceftriaxone + copper + pathogenic bacteria) (A.a + Sef + cop + E.c) was significantly superior to the rest of the treatments after 10 days of spraying with seaweed extract at a concentration of 0.5 ml/liter of water, followed by the treatment of amino acids, which did not give a significant difference in increasing the activity of PAL enzyme in potato plants, which reached 40.40 and 38.74 mg/g fresh weight, respectively compared to the control treatment, which amounted to 21.25 mg/g fresh weight (Table 5).

As for the effect of treatments by the therapeutic method, the two treatments of the mixture of factors (A.a + Sef + cop + E.c) and (A.a. + Sef + cop) were superior to the rest of the treatments by giving them rates of 39.32 and 37.80 mg/g fresh weight compared to the comparison treatment, which had an average of 20.95 mg/g fresh weight. It did not differ from the treatments A.a. + Sef + cop, amino acids, and amino acids + E.c, which averaged 36.27, 36.36, and 36.86 mg/gm fresh weight. Concerning the therapeutic and preventive methods, the results showed that there were no significant differences between the therapeutic and preventive methods after spraying with seaweed extract at a concentration of 0.5 ml/liter of water, as this treatment gave rates of 32.56 and 32.42 mg/g fresh weight compared to the control treatment, which averaged 21.25 and 20.95 mg/g fresh weight.

Table 4. The effectiveness of applying agents on potato plants in affecting the level and activity of the enzyme Phenylalanine ammonia-lyase (PAL).

	Treatments	Protective (min/g fresh weight)	Therapeutic (min/g fresh weight)	Treatments effect
1	Distilled water	21.25	20.95	21.10
2	Pathogenic bacteria E.c	31.69	34.46	33.08
3	Amino acids A.a	38.74	33.98	36.36
4	ceftriaxone Sef	28.84	30.73	29.79
5	Copper Cop	34.50	32.42	33.46
6	A.a Sef + cop	37.63	37.80	37.71
7	Ec + A.a.	34.81	37.74	36.27
8	Ec. Sef	29.66	28.30	28.98
9	Ec + Cop	28.13	28.55	28.34
10	A.a + Sef +cop + E.c	40.40	39.32	36.86
Method Average		24.2	32.56	
Treatment L.S.D. 0.05		1.28		
Method L.S.D. 0.05		0.40		

Interaction L.S.D. 0.05**1.81**

Evaluation of the effectiveness of total phenols

The results of the use of pathogenic bacteria with copper and pathogenic bacteria with amino acids and a mixture of A.a. + Sef + cop in the treatment of potato tubers immersion by the preventive method and seaweed extract at a concentration of 0.5 ml/liter by spraying on the plants gave an increase in the rate of accumulation of phenols. It was significantly superior in the rate of accumulation of phenols after 10 days of spraying seaweed extract for all other treatments, with rates reaching 181.6, 181.5, and 181.0 mg/g fresh weight, compared with the control treatment, which amounted to (153.4) mg/g fresh weight (Table 7).

As for the therapeutic method, the treatment of amino acids and pathogenic bacteria with copper was significantly superior to the rest of the treatments, with rates of 181.1 and 178.3 mg/gm fresh weight, respectively, compared to the control treatment, which averaged 147.2 mg/gm fresh weight. The results also showed that the preventive treatment method was significantly superior to the therapeutic method in increasing the accumulation rate of phenols, reaching 170.3 mg/g fresh weight. When comparing the effect of the treatments among them, it was found that the treatment with pathogenic bacteria with amino acids and the interaction A.a. + Sef + cop had a significant superiority over all other treatments. They were 178.4 and 178.2 mg/gm fresh weight.

Table 5. Estimation of the effectiveness of total phenols

	Treatments	Protective (min/g fresh weight)	Therapeutic (min/g fresh weight)	Treatments effect
1	Distilled water	153.4	147.2	150.3
2	Pathogenic bacteria E.c	170.3	158.1	164.2
3	Amino acids A.a	165.6	181.1	173.3
4	ceftriaxone Sef	161.9	136.9	149.4
5	Copper Cop	156.7	153.1	154.9
6	A.a Sef + cop	181.0	175.5	178.2
7	Ec + A.a.	181.6	175.3	178.4
8	Ec. Sef	174.0	154.4	164.2
9	Ec + Cop	181.5	178.3	179.9
10	A.a + Sef + cop + E.c	177.0	165.7	171.3
Method Average		170.3	162.5	
Treatment L.S.D. 0.05		7.91		
Method L.S.D. 0.05		2.50		
Interaction L.S.D. 0.05		11.18		

Conclusion

Amino acids and spraying with seaweed extract have an inhibitory effect on the growth of pathogenic bacteria. It has an inhibitory effect on preventing the formation of the cell membrane Biofilm that pathogenic bacteria form in the laboratory. In the field, they significantly

increase the level of enzymes POD, PAL and phenols related to enhancing plant resistance.

Conflict of interest

The author has no conflict of interest.

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