Using intra-plant bacteria to control Gray Mold and promote growth in Strawberry plants

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Abstract

The laboratory study was conducted at Al Haweeja Technical Institute's Department of Medical Laboratory Techniques - Bacteriological Laboratory to isolate and diagnose bacteria within the plant. The isolates were obtained from the stems, leaves, and roots of the strawberry plant. The isolates used in the study (B1, B2, B3, B4, B5, B6), which were identified as (*Enterobacter sichuanensis*, *Pantoea agglomerans*, *Bacillus cereus*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, and *Bacillus pumilus*), were subjected to various chemical tests. The results of the laboratory experiments for the chromium dye test showed that (*Enterobacter sichuanensis, Pantoea agglomerans, Pseudomonas stutzeri, Pseudomonas fluorescens)* are negative for chromium dye, and (*Bacillus cereus, Bacillus pumilus*) are positive for the chromium dye. In the catalase test, the results showed that all isolates were capable of producing the catalase enzyme. In the oxidase test, the isolates (*Enterobacter sichuanensis, Pantoea agglomerans)* were negative, while the isolates *(Bacillus cereus, Pseudomonas stutzeri, Pseudomonas fluorescens, and Bacillus pumilus) were positive*. In the antagonism test, these isolates showed different inhibition rates, ranging from (96.3%) for *Bacillus pumilus* to (71.4%) for *Bacillus cereus*. In field scabs, the (*P. fluorescens*) treatment recorded the lowest infection rate (37.6%) and infection severity (0.42) compared to the infection treatment (77.7%) and infection severity (0.70) after two weeks of treatment, while the (*B. pumilus*) treatment recorded the lowest infection rate after four weeks (15.7%) and infection severity (0.22) With a significant difference from other treatments and the infection treatment. The results of the treatments on the dry weight and wet weight of the root system showed that the treatment with (*P. agglomerans*) had the highest effect on both dry weight (7.44 g) and wet weight (14.56 g), which is the highest among the other treatments. As for the results of the treatments for dry weight and wet weight of the vegetable group, the treatment (*B. cereus*) recorded the highest dry weight (10.89 g) and wet weight (34.8 g) with a significant difference with the infection treatment, which amounted to (1.8 g) dry weight and (8.9 g) wet weight.

Keywords: Strawberry, *Botrytis cinerea, Enterobacter sichuanensis, Pantoea agglomerans, Bacillus cereus, Pseudomonas stutzeri, Pseudomonas fluorescens, Bacillus pumilus.*

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Introduction

The strawberry plant (*Fragaria* × *ananassa* Duch) is a member of the *Rosaceae* family, comprising about 20 wild species. It is considered a highly productive plant for its size, characterised by a shallow and medium-spreading root system [1]. Strawberries rank fourth in the world in terms of production, after apples (*Malus sylvestris*), oranges (*Citrus sinensis*), and bananas (*Musa* spp.). The output of strawberry varieties depends mainly on soil fertility and water availability during the growing season, making the provision of adequate plant nutrients vital to ensure high yields and quality fruit. Strawberries are produced in 63 countries around the world, with China leading the list of producing countries with a production of 3,801,865 tones, followed by the United States of America with 1,420,570 tones [2], [3]. Strawberries are a rich source of many health benefits for consumers, including fiber and ascorbic acid, as well as an essential source of antioxidant compounds with multiple health properties, such as vitamin C and folic acid [4], [5]. Despite their numerous benefits, strawberries are susceptible to various microbial diseases, including fungi, bacteria, viruses, and nematodes. The most prominent disease, *Botrytis cinerea,* commonly known as gray mold, is one of the most serious fungal pathogens affecting strawberry crops, causing significant economic losses of up to 25% to 89%. *Botrytis cinerea* is a plant pathogen that infects more than 500 plant families, causing gray mold and resulting in significant losses to economically important fruits, ranking it second among the top ten fungal plant pathogens worldwide [6][26]. *Botrytis cinerea* produces large quantities of conidial spores, which are airborne and travel long distances, and can form stony bodies in the absence of favorable growth conditions [7]. This fungus poses a real challenge in control, especially with the increasing use of chemical pesticides, which has led to the emergence of resistant strains, as well as the environmental and health damages associated with these pesticides [8]. This prompted researchers to study alternative means, including biocontrol, as it is considered effective and safe in combating this type of pathogen without negatively affecting the environment and human health [9]. These bacteria also promote plant growth by producing phytohormones and stimulate systemic resistance in the plant [10].

This study aims to evaluate the role of endophytic bacteria in reducing the rate and severity of infection, as well as to investigate the effect of bacteria on vegetative and root growth characteristics, and their ability to inhibit fungal growth.

Materials and Methods

Isolation of bacteria:

This Strawberry plant samples were washed thoroughly with sterile distilled water, then sterilised with sodium hypochlorite solution (NaOCl) for 5 minutes, and to remove the residual sodium hypochlorite solution, the samples

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were washed with sterile distilled water in three stages, for 1 minute each, then the samples were dried using sterile filter paper, and then cut into small parts of 1 cm, each of the stem, root and leaves. The plant parts were planted on different culture media, namely: Blood Agar, Nutrient Agar, and MacConkey Agar. Five plant parts were placed in each petri dish, with three replicates of each plant part. The dishes were placed in the incubator. Other parts of the same plant, of the same size, were taken and placed in the neutral buffer solution. Then, 1 ml of the solution was taken and spread evenly over the entire surface of the dish.

The dishes were placed in the incubator for 24 to 48 hours. Petri dishes containing N.A. medium were prepared and inoculated using a sterile swab taken from the growing bacteria with a sterile needle. The dishes were then placed in the incubator for 24 hours. Symbols were written on the dishes to indicate the isolated plant parts after the incubation period. The bacterial isolates were divided based on their phenotypic characteristics, including size, color, and edge morphology of the bacterial colonies, as well as other tests. Finally, the isolates were divided into six different groups [11], [12].

Identification of Bacteria

Phenotypic examination

After the growth of bacterial colonies on culture media (Blood Agar, Nutrient Agar, MacConkey Agar), the shape, color, and size of the colony were observed, and this is considered a preliminary diagnosis.

Figure 1. Phenotypic form of bacteria

Microscopic examination

A drop of neutral saline solution was placed on a clean glass slide. Smears were taken from pure colonies using a sterile carrier (Lop), then placed and spread on a glass slide, and then passed over a flame three times for fixation. The smears were stained with Cram's dye and dried. The smears were examined by light microscopy under an oil lens to observe the shapes of the cells, their arrangement, and their reaction to Cram's dye.

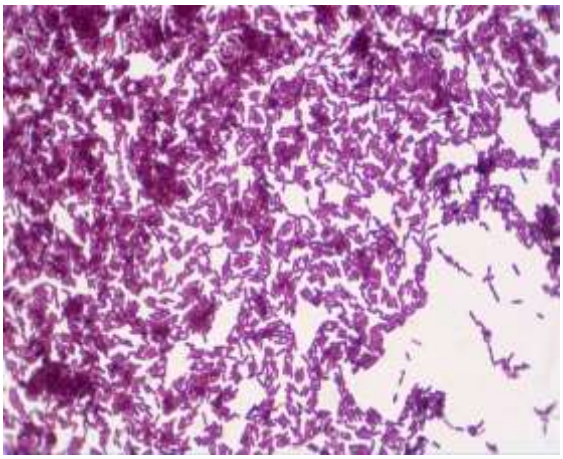

Figure 2. Microscopic form of bacteria

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Chemical tests:

Catalase test

A swab is taken with a sterile carrier (Lop) and then placed on a glass slide. A drop of catalase reagent (hydrogen peroxide) is added. When bubbles appear, this indicates a positive test [13].

Oxidase test

1 g of (Tetra methyl 1 _p_phenylene diamine dihydrochloeide) was dissolved in 100 ml of distilled water and used to determine the ability of bacteria to produce the oxidase enzyme [14].

Antagonistic ability test of bacteria with the pathogenic fungus:

Add 1 ml of N.A. medium for bacterial growth to a petri dish containing N.A. and distribute the bacterial inoculum homogeneously by moving the dish in a circular motion to ensure that the bacteria are distributed throughout the dish, then place the disc of the pathogenic fungus with a diameter of 0.5 cm^2 in the centre of the dish. The plates were incubated for 5 days at 37 °C, and the growth of the fungus was measured using a ruler [24][15],[16].

Inhibition Percentage Formula:

Inhibition ratio = control colony diameter - treatment colony diameter/control colony diameter× 100%

Table 1. Treatments

Treatments	Treatment Code
Enterobacter sichuanensis	B1
Pantoea agglomerans	B2
Bacillus cereus	B3
Pseudomonas stutzeri	B4
Pseudomonas fluorescens	B5
Bacillus pumilus	B6

Results and Discussion

Results of the antagonistic ability of the bacteria against the pathogenic fungus

The laboratory results showed the percentage of antagonism between the pathogenic fungus and the bacteria, the bacterial isolate *Bacillus pumilus* recorded the highest effectiveness in inhibiting the pathogenic fungus with an inhibition rate of 96.3%, followed by *Pseudomonas flurescens* isolate with an inhibition rate of

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90.3%, which is the highest in the inhibition rate, indicating its high efficiency in inhibiting the fungus. The isolate *Pseudomonas stutzeri* exhibited an inhibition rate of 87.41%, ranking third among the inhibition rates. The isolates *Enterobacter sichuanensis* and *Pantoea agglomerans* had an inhibition rate of 79.26%, while *Bacillus cereus* was the least effective isolate with an inhibition rate of 71.48%. This indicates a relative weakness in its ability to inhibit the growth of the fungus compared to the other isolates. The antagonistic ability can be attributed to the different bacterial isolates' genetic structure and their production of various biomaterials, as some of them produce antifungals, such as phenazine and pyoluterotin, that inhibit fungal growth, or produce the enzyme catalase, which degrades the fungal cell wall [15], [16].

Effect of treatments on incidence and severity of infection two weeks after treatment

The results showed that all bacterial treatments achieved a reduction in the percentage and severity of infection, with *P. fluorescens* (37.9%) and severity of infection (0.42), followed by *B. cereus* (41.6%) and severity of infection (0.34), and *P. agglomerans* showed the highest infection rate (42.2%) and severity (0.39), and *P. stutzeri* recorded an infection rate (44.2%) and severity (0.33), these results are medium and close in reduction rate, while *B. pumilus* recorded an infection rate (52.5%) and severity (0.47), which is the highest infection rate. This difference in the percentage of inhibition is attributed to the ability of these bacteria to produce some inhibitory compounds and pathogen cytolytic enzymes, as well as stimulate the systemic resistance of the plant [17] [25], and producing organic acids such as lactic acid and succinic acid, which work to increase plant growth and reduce fungal diseases [18].

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Table 2. Shows the effect of treatments on the incidence and severity of infection two weeks after the onset of infection.

Treatments	Percentage of infection	Severity of infection
B1	48.4	0.32
B2	42.2	0.387
B3	41.6	0.34
B4	44.2	0.327
B5	37.6	0.42
B6	52.5	0.467
with disease	77.7	0.70
without disease	0	0.00
L.S.D	15.51	0.1754

Effect of treatments on the incidence and severity of infection after four weeks

The results showed that all treatments showed a significant effect in reducing the percentage and severity of infection after four weeks, as the treatment (*B. pumilus*) recorded a percentage of infection (15.7%) and severity of infection (0.21), indicating the cumulative action of this bacterium and its efficiency in reducing the infection rate through multiple mechanisms including the production of lipopeptides, lipolytic enzymes (chitinase, cellulase and glucanase), induction of systemic resistance in the plant and the production of microbial volatile organic compounds [19]. Followed by (*P. agglomerans*) with a 22.8% infection rate and infection severity (0.15), as it achieved a balance between infection rate and severity, indicating its ability to reduce the spread of the pathogen within the plant, while the treatments recorded (*E. sichuanensis*) (24.9%) (0.20), and (*B. cereus*) (26.1%) (0.20) and (*P. fluorescens*) (25.9%) (0.19), these results are close to the percentage and severity of infection and this variation is related to the ability of these bacteria to produce effective compounds against pathogens [20]

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Table 3 Shows the effect of treatments on the percentage and severity of infection four weeks after the onset of infection.

Treatments	Percentage of infection	Severity of infection
B1	24.9	0.20
B2	22.8	0.1533
B3	26.1	0.20
B4	30.4	0.1733
B5	25.9	0.1933
B6	15.7	0.2067
with disease	88	0.76
without disease	0	0.00
L.S. D	17.82	0.13771

Effect of treatments on wet and dry weight of the vegetable group

The results showed that all isolates positively affected the wet and dry weight of the vegetative population, with (*B. cereus*) (10.89 g) dry weight and (34.8 g) wet weight, followed by (*P. stutzeri*) (9.33 g) dry weight and (24.4 g) wet weight, which outperformed all treatments and had a significant effect with the infection treatment, which recorded (1.8 g) dry weight and (8.9 g) wet weight. This effect is attributed to the ability of these isolates to stimulate growth hormones, such as auxins and cytokinins, in addition to increasing the uptake of elements, such as phosphate [21]. (*P. fluorescens*) treatment was (9.11 g) dry weight and (28.6 g) wet weight, and the treatment (*E. sichuanensis*) (8.44 g) dry weight and (26.9 g) wet weight, and (*B. pumilus*) (6.89 g) dry weight and (20.8 g) wet weight, which is the lowest and this limited effect is attributed to the fact that this isolation affects the induction of resistance against diseases rather than growth.[23]

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Table 4. Shows the effect of treatments on the dry and wet weight of the vegetable group.

The treatments	Dry weight (g)	Wet weight (g)
B1	8.44 g	26.9 g
B2	7.67 g	31.3 g
B3	10.89 g	34.8 g
B4	9.33 g	24.4 g
B5	9.11 g	28.6 g
B6	6.89 g	20.8 g
with disease	1.8 g	8.9 g
without disease	4.01 g	9.3 g
L.S.D	4.487 g	18.23 g

Effect of coefficients on dry weight and wet weight of rootstock

The results showed that all treatments had an effect on improving root growth, as (*P. agglomerans*) showed (7.44 g) dry weight and (14.56 g) wet weight, followed by (*P. fluorescens*) with (7.0 g) dry weight and (13.78 g) where it outperformed all the treatments and its impact was significant with the treatment of the infection that was recorded (1.9 g) dry weight and (2.4 g) wet weight, while (*B. cereus*),(*E. sichuanensis*) and (*B. pumilus*) treatments recorded (6.33 g) (13.11 g), (6.22 g) (12.0 g), and (6.33 g) (13.67 g) dry and wet weight, respectively, these isolates affected the increase in dry and wet weight, which indicates the effect of the treatments compared to the infected witness, as the effect is attributed to improving the absorption of nutrients and stimulating the production of growth hormones such as auxins and cytokinins that stimulate root hair growth, dissolve phosphate, potassium and calcium, and produce siderophores that facilitate iron and elements necessary for root growth [22].

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Table 5. Shows the effect of treatments on the dry and wet weight of the root system.

Treatments	Dry weight (g)	Wet weight (g)
B1	6.22 g	12 g[i]
B2	7.44 g	14.56 g
B3	6.33 g	13.11 g
B4	5.89 g	12.22 g
B5	7 g	13.78 g
B6	6.33 g	13.67 g
with disease	1.9 g	2.4 g
without disease	4.8 g	7.76 g
L.S.D	3.189 g	7.888 g

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Conclusion

The results of this study demonstrated the effectiveness of the bacteria used in controlling *Botrytis cinerea*, also known as gray mold, and its impact on plant growth. Treatment (*B. pumilus*) showed the highest inhibition rate and high efficiency in reducing the incidence and severity of infection after four weeks. The treatments (*P. agglomerans*) and (*P. fluorescens*) also enhanced plant growth and vegetative and root growth characteristics. This study demonstrated the potential of these bacteria for use in biological control as an alternative to chemical pesticides and for enhancing plant growth.

Figure 4. Symptoms of gray mold disease

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