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المجلة العراقية لبحوث السوق وحماية المستهلك

EFFICACY OF *PSEUDOMONAS FLUORESCENS* AND IRON CHELATED FE-EDDHA AGAINST *FUSARIUM OXYSPORUM* F. SP. THE CAUSAL AGENT OF ROOT ROT AND WILT DISEASE ON PEPPER

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ABSTRAC

An experiment was conducted to evaluate the biological control activity of Pseudomonas fluorescens PO2 and chelated iron Fe-EDDHA (Fe) at a concentration of 0.5% and the interaction between them for inhibiting Fusarium oxysporum Fo6 on pepper. In vitro, PO2 significantly inhibited the growth of Fo6 with an inhibition rate of 83.33% compared with control, and chelated iron caused a significant decrease for Fo6 with an inhibition rate 66.67% compared with control, while was not affecting the growth of PO2. The results of the pot experiment showed that the treatment (Fo6+PO2+Fe) showed a highest significant difference in reducing the disease incidence and disease severity which amounted to 6.67% compared with control (Pathogen only) (100, 85)% respectively. Also, the treatment (PO2+Fe without the pathogen) was increased the total fresh root and vegetative weight (46.17, 136.8) g respectively and was increase in the dry root and vegetative weight as reached (4.87, 10.07) g respectively compared with control (not inoculated plants) which amounted (12.1, 75.67, 1.83, 5.33) g respectively. In greenhouse experiment the results showed the excellence of the treatment (Fo6+PO2+Fe) in reducing the incidence of infection, which amounted to 10% and the severity of injury amounted to 5.83% compared with the control which recorder to (100, 74)% respectively, as well showed a significant increase in the iron percentage due to response of plant for adding chelated iron into roots with significant differences between treatments. Depending on the results, the use of P. fluorescens with chelated iron can be recommended to control pepper wilt disease caused by F. oxysporum.

Keywords: Pseudomonas fluorescens, Fusarium oxysporum, Chelated Iron Fe-EDDHA.

تقييم كفاءة البكتريا *Pseudomonas fluorescens* والحديد المخلبي Fe-EDDHA ضد الفطر *Fusarium oxysporum* f. sp. المسبب لمرض تعفن الجذور والذبول على الفلفل

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الخلاصة

اجريت تجربة لتقييم فاعلية بكتريا المكافحة الاحيائية (PO2) *Pseudomonas fluorescens* والحديد المخلبي Fe-EDDHA بتركيز 0.5% والتداخل بينهم في تثبيط عزلة الفطر الممرض *Fusarium oxysporum* (Fo6) على نباتات الفلفل، واظهرت النتائج المختبرية ان البكتريا (PO2) اعطت قدرة تثبيطية عالية ضد نمو العزلة (Fo6) وبنسبة تثبيط بلغت 83.33% قياساً بالمقارنة التي سجلت 0%، وسبب الحديد المخلبي انخفاضاً معنوياً في نمو العزلة (Fo6) وبنسبة تثبيط بلغت 66.67%، قياساً بالمقارنة التي بلغت 0% بينما لم يؤثر في نمو المستعمرة (PO2)

* Part of M.Sc. thesis of the first author.

على الوسط الزرعي، واظهرت نتائج تجربة الاصص ان المعاملة (Fo6+PO2+Fe) اعطت اعلى فرق معنوي في خفض نسبة وشدة الاصابة بالعزلة (Fo6) اذ بلغت 6.67%، قياساً بالمقارنة بوجود المسبب فقط (Fo6) التي سجلت (100، 85)% على التتابع، كما اعطت المعاملة (PO2+Fe) بدون وجود المسبب الممرض زيادة عالية في الوزن الطري للمجموعين الجذري والخضري اذ بلغت (46.17، 136.8)غم على التتابع، وزيادة في الوزن الجاف للمجموعين الجذري والخضري اذ بلغت (4.87، 10.07)غم على التتابع، قياساً بالمقارنة (النبات بمفرده) اذ بلغت الاوزان فيها (12.1، 75.67، 1.83، 5.33)غم على التتابع، وبينت نتائج تجربة البيت البلاستيكي تفوق المعاملة (Fo6+PO2+Fe) في خفض نسبة الاصابة اذ بلغت 10% وشدة اصابة بلغت 5.83% قياساً مع معاملة المقارنة (Fo6) التي بلغت (100، 74)% على التتابع، كما واظهرت النتائج زيادة معنوية في نسبة الحديد نتيجة استجابة النبات لاضافة الحديد المخلبي الى الجذور وبفروق معنوية بين المعاملات. اعتماداً على النتائج يمكن التوصية باستعمال البكتريا *P. fluorescens* مع الحديد المخلبي للسيطرة على مرض ذبول الفلفل المتسبب عن الفطر *F. oxysporum*.

الكلمات المفتاحية: *Pseudomonas fluorescens*، *Fusarium oxysporum*، الحديد المخلبي Fe-EDDHA.

INTRODUCTION

Pepper (*Capsicum annuum* L.) is one of the main vegetables grown all over the world, has an important nutritional and economic worth, Belongs to the Solanaceae family, the genus *Capsicum* includes more than 30 species of which five species (*C. annuum*, *C. baccatu*, *C. frutescens*, *C. chinense* and *C. pubescens*) are mainly cultivated for consumption as food and other purposes **(Parisi *et al.*, 2020)**. The consumption of pepper for being an important source of vitamins for the world's population, is increasing as a green pepper weighing 148 g contains 30 calories, 7 g carbohydrates, 2 g trophic fiber, 4 g sugar and 1 g protein in addition to vitamins A, B6, C, E, K and minerals such as Mn, P, Fe **(Kartik, 2020; Anaya-Esparza *et al.*, 2021)**, Pepper is grown in all regions of Iraq, and its cultivation has spread in greenhouses with a production of 15937 tons, while production reached 15049 tons in agricultural tunnels **(Ministry of Agriculture 2021)**. Pepper production and quality are affected by various stress factors such as environmental conditions and pathogens, which often cause economic losses of more than 70% **(Nazarov *et al.*, 2020)**, Fungal and bacterial diseases are among the determinants of pepper cultivation in greenhouse, which cause a decrease in production in quantity and quality, which is caused by a number of causes, including: *Pythium*, *Rhizoctonia*, *Fusarium*, *Phytophthora*, *Alternaria*, *Verticillium*, *Erwinia*, *Xanthomonas*, *Corynebacterium* and *Ralstonia* **(Verma *et al.*, 2020)**. With the increasing awareness in recent years towards sustainable agriculture and how to reduce the use of environmentally harmful chemicals and the trend towards using beneficial microbes and appropriate nutrients in controlling stress factors, The plant growth-promoting bacteria *Pseudomonas fluorescens* is among the organisms of great importance in the fields of medicine, environment and plant diseases **(Awda & Khalifa, 2019)**, and it was found to have the ability to control wilt disease and pepper root rot caused by *Fusarium oxysporum* **(Duc *et al.*, 2017)**. It was found that the optimal use of nutrients including chelated iron Fe-EDDHA has an important role in controlling the disease by improving the soil environment and the plant's defensive response by secreting pathogen-inhibiting compounds and secreting pathogenic-related proteins **(Aznar *et al.*, 2015)**. Therefore, the application of integrated plant nutrition is one of the basic components in sustainable agriculture and has more effectiveness in combating plant diseases as it is less costly and environmentally friendly and thus reduces disease or makes it at a controlled level with some other practices **(Newitt *et al.*, 2019)**. For reaching the best ways to control diseases and improve crop quality, the study aimed to: Evaluate the effect of the biological control agent *P. fluorescens* and chelated iron Fe-EDDHA and the overlap among themselves in inhibiting the pathogenic *F. oxysporum*.

MATERIALS AND METHODS

Preparation inoculum of the pathogenic

The isolate of the *F. oxysporum* (Fo6) was obtained from the Plant Diseases Laboratory/ College of Agricultural Engineering Sciences/ University of Baghdad deposited in the Gene bank under the accession number (OP315631), and was activated by adding it to petri dishes containing PDA media sterilized by autoclave (at a temperature of 121°C and a pressure of 1.5 kg/cm^2 for 15 min), and incubated at (25±2)°C for seven days and kept for subsequent experiments.

The antagonism test of *P. fluorescens* against *F. oxysporum* in vitro

The isolate of *P. fluorescens* (PO2) was obtained from the Plant Diseases Laboratory/ College of Agricultural Engineering Sciences/ University of Baghdad deposited in the Gene bank under accession number (ON041213.1), it was growth by preparing 250 mL glass flasks containing 100 mL of nutrient broth (NB) and sterilized, then the culture media were inoculated with the overnight culture of the PO2 and incubated at (28±2)°C for 48 h. Then the antagonism test between *P. fluorescens* against the *F. oxysporum* by taking 1 mL the bacteria inoculum and inoculated the empty petri dish then adding the sterile PDA and moving the dish. after solidify the center of the petri dishes were inoculated with a disc 5 mm of the pathogenic isolate, with four replicates, control plates were inoculated only with pathogenic fungal, after incubated, then the percent of growth inhibition after seven days was calculated, according to the equation **(da Silva Bomfim *et al*., 2015)**:

$$(\%)\ \text{Inhibition} = \frac{Average\ colony\ diameter\ in\ the\ control - Average\ colony\ diameter\ in\ the\ treatment}{Average\ colony\ diameter\ in\ the\ control} \times 100$$

In vitro, assessment of Fe-EDDHA against *F. oxysporum*

Flask of 100 mL was prepared and add to it the nutrient medium PDA and the chelated iron Fe-EDDHA at a concentration 0.5% (w/v) and shake the beaker well, then sterilize by autoclave and pour the contents of the beaker into petri dishes. Each dish was inoculated with a disc from the edge of growing pathogenic colony with four replications, control plates were inoculated with fungal only **(Dong *et al*., 2016)**. Plates were incubated at (28±2)°C for seven days. The diameters of colony were measured and the percent of growth inhibition was calculated according to the equation mentioned in the previous experiment.

In vitro assessment of Fe-EDDHA against *P. fluorescens*

Flasks with 100 mL of NB were prepared and Fe-EDDHA at a concentration of 0.5% was added and sterilized by autoclave, then the flasks were inoculated with 1 mL of bacteria suspension two day old grown on NB, for the control treatment bacteria was adding without Fe-EDDHA and incubated at (28±2)°C for 48 h, and the growth was measured by a spectrophotometer at a wavelength of 600nm **(Sharma *et al*., 2018)**.

Assessment of *P. fluorescens* and Fe-EDDHA against *F. oxysporum* in pots

Soil mixture was sterilized using autoclaved and parted in plastic pots(15 cm width × 20 cm height), then each pot was inoculated with 1% (w/w) inoculum of isolate FO6 that was grow on local millet seeds (*Panicum miliaceum*). After two days pepper seedlings were planted in pots, and all pots were irrigated with water. Three days later, inoculum of *P. fluorescens* at 1% (v/w) and chelated iron Fe-EDDHA at 0.5% were added. The treatments were carried out with three replications for each treatment as follows:

1. Pathogen only (Fo6).
2. Fo6 + *P. fluorescens* (Fo6+PO2).

3. Fo6 + Fe-EDDHA (Fo6+Fe).
4. Fo6 + *P. fluorescens* + Fe-EDDHA (Fo6+PO2+Fe).
5. *P. fluorescens* only (PO2).
6. Fe-EDDHA only (Fe).
7. *P. fluorescens* + Fe-EDDHA (PO2+Fe).
8. Seedlings only (C).

After 30 days, the infection rate was calculated and symptoms evaluation was calculated using the following equation: Disease severity % =∑ [scale no. × Plants of infected/ highest scale × total no. of plants] ×100, it was made based on scale of symptoms according to **Sun *et al*. (2015)** and as follows: 0= no symptoms on roots, 1= less than 25% of the roots are brown, 2= 25-50% of the roots are brown, 3= 50 -75% of the roots are brown, 4= 75-100% of the roots are brown and plant dead. Also, wet and dry root and vegetative weight were measured.

Testing the effect of *P. fluorescens* with Fe-EDDHA against *F. oxysporum* in a greenhouse

The field experiment was conducted for the season (2021-2022), the field was prepared, and the experiment was carried out according to the RCBD design with three replications then pepper seedlings was planted and followed the recommended agricultural except procedures for the plant protection, then the roots were artificially contaminated by adding the pathogen inoculum at 1% (w/w), after two days *P. fluorescens* was added at 2% (v/w), and chelated iron Fe-EDDHA at a concentration 0.5% to the area surrounding the roots. Treatments were carried out in the previous experiment. After two months, the disease incidence & severity were calculated. The ratio of iron was measured before and after treated the plant for each treatment separately using Atomic Absorption Spectrophotometer AA-7000.

RESULTS AND DISCUSSION

The antagonism test of P. fluorescens against F. oxysporum in vitro

The results of the antagonism test showed (Table 1 and Figure 1) that P. fluorescens has a high inhibition against F. oxysporum, the growth rate of the colony treated with bacteria was 1.5 cm with an inhibition rate of 83.33% and a highly significant difference from the control (Pathogenic only) which had a colony rate of 9 cm and an inhibition rate of 0%. This result was consistent with Razi et al. (2019) who stated that the reason for the inhibition is due to that the bacteria P. fluorescens produces antifungal receptors such as Protease and hydrogen cyanide (HCN), as well as the ability to produce the enzyme β-1,3-glucanase that inhibits a number of bacteria. Also noted Hua et al. (2020) that areas of inhibition were evident around the colony of F. oxysporum in all the dishes treated with bacteria with a decrease in the diameter of the colony, which indicates its ability to synthesize antibiotics Anti-fungal.

Table (1): Pathogenicity of P. fluorescens against F. oxysporum*.

Treatment	Colony growth rate (cm)	Inhibition percentage (%)
F. oxysporum + P. fluorescens	1.5 **b**	83.33 **a**
Control	9 **a**	0.0 **b**

*** Each number of four replicates.**

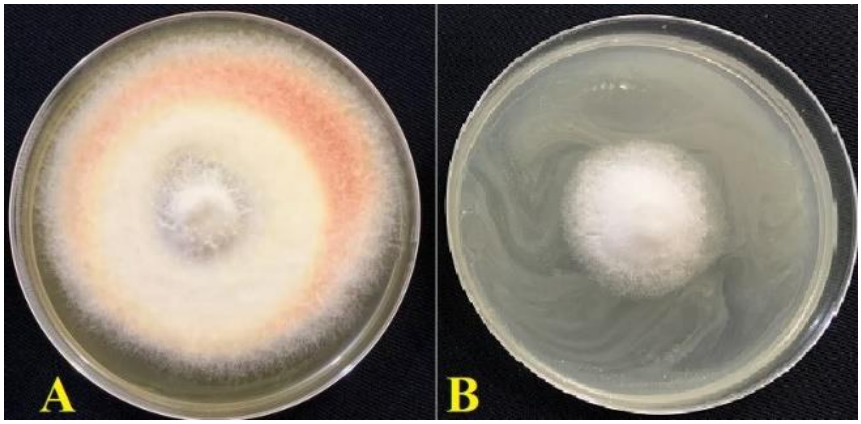


Figure (1): Antagonism test. A: *F. oxysporum*, B: *F. oxysporum* Vs *P. fluorescens.*

In Vitro assessment of Fe-EDDHA against *F. oxysporum*

The results of the test (Figure 2) showed that chelated iron Fe-EDDHA at a concentration of 0.5% caused a significant reduction in the growth rate of colony *F. oxysporum*, reaching 3 cm and an inhibition rate of 66.67%, compared to the control (Pathogenic only) in which the growth rate of the colony reached 9 cm and an inhibition rate 0%. Our results are in agreement with **Sulochana *et al.* (2014)** who observed that the radial growth of *Fusarium* decreased with the addition of chelated iron to the culture media.

Also in another study conducted by **Gajewska *et al.* (2022)** indicated that the presence of chelated iron in the culture media may affect the growth of the fungal hyphae due to its toxicity that affects the growth of pathogenic fungi, and that the conidia of the *F. oxysporum* was very sensitive even to low amounts of iron, as iron treatment reduced the germination rate of conidia by about 40%, moreover, it contributed to the reduction in the production of Fusaric acid from the fungus in vitro **(Dong *et al*., 2016)**.

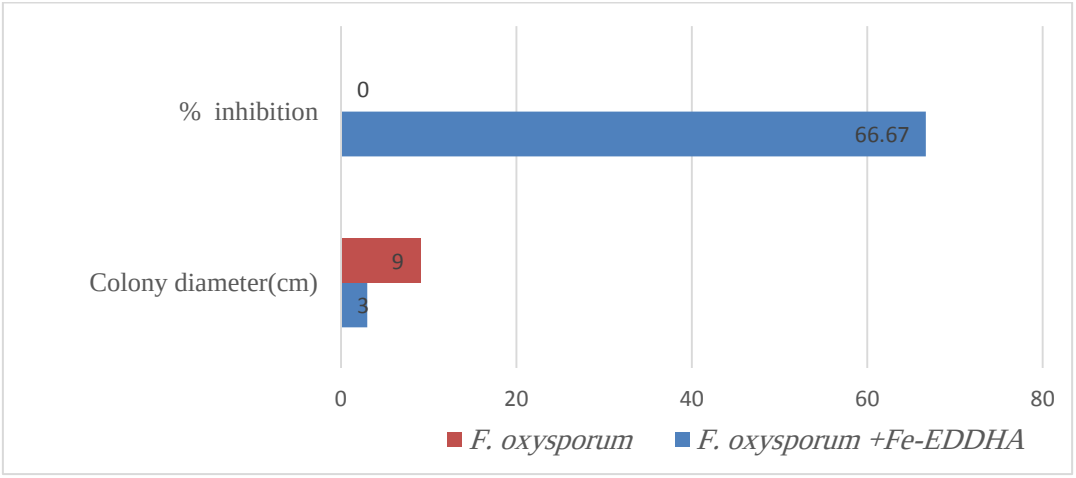


Figure (2): The effect of Fe-EDDHA against *F. oxysporum.*

In Vitro assessment of Fe-EDDHA against *P. fluorescens*

The results test of the effect chelated iron Fe-EDDHA 0.5% on the growth of *P. fluorescens* (Table 2) showed that the growth of the isolate was not affected by the addition of

iron, as the average absorption was 0.994 and without a significant difference from the control treatment, which amounted 0.997. These results agreed with what was mentioned **Gyawali & Ibrahim (2014)**, who found that the addition of iron does not negatively affect the growth of bacteria and on the contrary, may enhance growth in the nutrient medium, Also mentioned **Bubici *et al.* (2019)** that the addition of Fe- EDDHA induces *P. fluorescens* to produce antibiotics that have a toxic effect on pathogens, thus increasing their biological control effectiveness.

Table (2): The effect of Fe-EDDHA on *P. fluorescens**.

Treatment	Average absorption
F. oxysporum + Fe-EDDHA	0.994 a
Control	0.997 b

*** Each number of four replicates.**

Assessment of *P. fluorescens* and Fe-EDDHA against *F. oxysporum* in pots

The results showed (Table 3) significant differences in reducing the rate and severity of infection and differences in the total fresh and dry of root and vegetative weights of pepper plants infected in pots, compared with the control with the presence and absence of the pathogenic, as the treatment of adding *P. fluorescens* and iron was achieved in the presence of the pathogen (Fo6+PO2+Fe) had the highest significant difference in reducing disease incidence and severity of infection, which was recorded at 6.67%, and achieved the tow treatments (Fo6+PO2),(Fo6+Fe) reduction in the rate of injury amounted (26.67 and 33.33)% respectively, and the severity of injury was recorded (11.67 and 28.33)% respectively, compared with control (Pathogenic alone), in which the percentage and severity of infection reached (100 and 85)%, respectively.

These results indicate that the use of more than one agent gives promising results in combating the pathogen as a result of the compatibility between the factors, and that the addition of iron with the biological control bacteria *P. fluorescens* stimulated the plant to increase the resistance of the pathogen by activating the defense enzymes in the host, and the effect of the action of bacteria comes It produces a wide range of biologically active metabolites such as Antibiotics, Volatiles, Siderophores, Bacteriocins and Phytotoxins that stimulate systemic resistance in the plant and make it act as a biological control agent. It also has the ability to inhibit some soil-borne pathogens and prevent the growth of pathogenic microorganisms **(Garrido-Sanz *et al.*, 2017; David *et al.*, 2018)**,

Iron also has effect on increasing plant tolerance to disease through its role in activating hormone signals that stimulate cellular responses, leading to the strengthening of cells against pathogens **(Karapetyan & Dong, 2018)**, Our results are in agreement with what was mentioned **Nejad & Chorom (2016)** Which concluded that the addition of Fe-EDDHA to the soil led to a reduction in germination and disease severity with the *F. oxysporum* by a third to a half.

Also the result show significant differences in the total fresh and dry of root and vegetative weights in comparison with the presence or absence of the pathogen (Table 3 and Figure 3), The treatment (Fo6 + PO2 + Fe) recorded a highly significant difference in the increase in the fresh of root and vegetative weight which amounted (42.13 and 105.57)g respectively, and an increase in the dry for the root and vegetative weight, which amounted (4.2 and 6.47)g compared with control (Fo6) whose weights reached (4.00, 27.73, 0.77 and 1.73)g respectively. The treatment (PO2+Fe) without the pathogen also recorded a significant

difference in the weight gain which amounted (46.17, 136.8, 4.87 and 10.07)g in comparison with control (Plant alone) which recorded (12.1, 75.67, 1.83 and 5.33)g on the arrangement.

These differences in weights come to the role of these additive factors and their direct and indirect effect on increasing growth, as one study indicated that the addition of chelated iron at different concentrations gave an increase in growth rates through an increase in the activity of antioxidant enzymes **(Torbat, 2014)**, It was also found that *P. fluorescens* bacteria have the ability to produce extracellular enzymes such as Amylase, Protease, Chitinase, Cellulase and Gelatinase which bind the iron ion leading to the formation of Indole Acetic Acid (IAA), which helps in increasing growth and number for root, These enzymes assist in the absorption of nutrients and the production of Siderphore, which have the ability to attract the iron ion from the soil and thus prevent the pathogen from obtaining it **(Bhatti *et al.*, 2017)**.

Table (3): Percentage and severity of infection, fresh, dry weight for root and vegetative*.

No.	Treatment	Rate of infection (%)	Severity of infection (%)	Fresh weight of root (g)	Fresh weight of vegetative (g)	Dry weight of root (g)	Dry weight of vegetative (g)
1	**Fo6**	100.00	85.00	4.00	27.73	0.77	1.73
2	**Fo6 + Po2**	26.67	11.67	16.53	66.0	1.3	5.43
3	**Fo6 + Fe**	33.33	28.33	35.23	93.13	3.73	6.0
4	**Fo6 + PO2 + Fe**	6.67	6.67	42.13	105.57	4.2	6.47
5	**PO2**	0.00	0.00	35.33	119.37	2.4	8.83
6	**Fe**	0.00	0.00	30.53	116.37	2.4	8.37
7	**PO2 + Fe**	0.00	0.00	46.17	136.8	4.87	10.07
8	**Control**	0.00	0.00	12.1	75.67	1.83	5.33
L.S.D 0.05		**12.96**	**16.16**	**2.25**	**3.51**	**0.67**	**0.32**

*** Each number of three replicates.**

Figure (3): A: *F. oxysporum* (Fo6), B: *P. fluorescens* + Fe-EDDHA (PO2+Fe).

Testing the effect of *P. fluorescens*, Fe-EDDHA against *F. oxysporum* in greenhouse

In greenhouse the results showed (Table 4) the decrease in the rate and severity of infection after adding factors to plants infected with the pathogen *F. oxysporum*, the treatment (Fo6+PO2+Fe) recorded a rate and severity of injury that amounted to (10 and 5.83)% respectively, with a highly significant difference from the comparison treatment (Fo6) which recorded (100 and 74)% respectively, followed by the treatment (Fo6+PO2) which recorded the rate and severity of infection amounted (16.67 and 10.17)% respectively, then the treatment (Fo6+Fe) which recorded (30 and 22.5)% respectively. These results were near to a number of studies, As it was found that the optimal use of nutrients is one of the important strategies in improving foods **(Mahdi, 2016),**

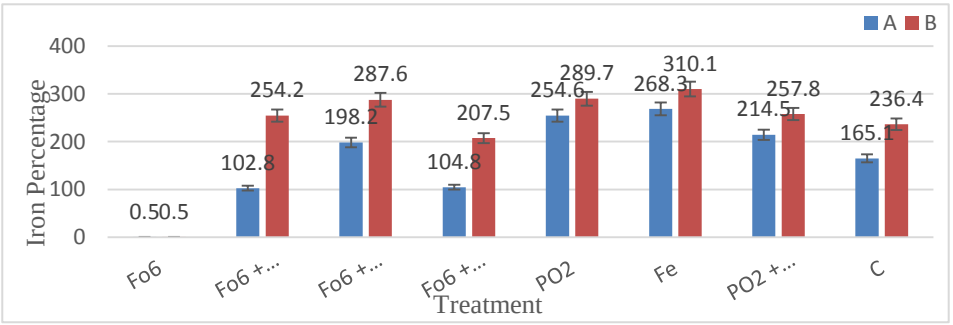
Also it was found that when chelated iron Fe-EDDHA was added to soil containing *Pseudomonas* bacteria, the soil became repressive to Fusarium wilt pathogens, due to competition for iron, and that the availability of iron in the infection area could stimulate the suppression of pathogens Fusarium wilt **(Dong *et al.*, 2016)**. and also affected of defense hormones such as Salicylic acid, Jasmonic acid and Ethylene for iron uptake response in plant roots, and components of the plant root iron uptake signaling pathway are essential for the initiation of induced resistance (ISR) **(Aznar *et al.*, 2015)**. It was found that the use of micronutrients with biological control agents gives better protection from pathogens in addition to improving growth parameters **(Liu *et al.*, 2021)**.

As the results indicate shows differences in the percentage of iron after adding the factors compared with control (Fo6), this indicates the response of the plant to the addition of chelated iron (Figure 4), and these results are consistent with many studies **(Ghasemi *et al.*, 2014; Roosta *et al.*, 2015)**. Who concluded that the iron content in the leaves was improved when chelated iron was added, the iron exposure enhanced the activities of the enzymes Catalase Ascorbate, Peroxidase, and There are three reasons for iron absorption are either competition for uptake by transporters in root cells, disturbances in the removal of heavy metals from roots or competition for entry into xylem cells. The source of iron for plants is the soil, which is available in the form of iron sulfate and is subject to oxidation and reduction, thus forming Fe^{+2} and Fe^{+3}, both of which have limited solubility, and this indicates that iron is not readily available to plants and is stored in chloroplasts as iron protein complexes known as Phytoferritin. The chemical properties of iron are also responsible for its limited accumulation in plants **(Thakur *et al.*, 2016)**.

Table (4): Percentage and severity of infection after adding factors in the greenhouse*.

No.	Treatment	Rate of infection (%)	Severity of infection (%)
1	**Fo6**	100.00	74.00
2	**Fo6 + PO2**	16.67	10.17
3	**Fo6 + Fe**	30.00	22.50
4	**Fo6 + PO2 + Fe**	10.00	5.83
5	**PO2**	0.00	0.00
6	**Fe**	0.00	0.00
7	**PO2 + Fe**	0.00	0.00
8	**Control**	0.00	0.00
L.S.D 0.05		**10.02**	**8.36**

*** Each number of three replicates.**



L.S.D 0.05 A= 26.74, B= 32.35

Figure (4): Iron percentage before and after adding it to plants. A: before adding, B: after adding.

CONCLUSION

Pepper is an economically important crop due to its high nutritional value, it is exposed to a number of pathogens the most important of which are wilt and root rot caused by the fungus *Fusarium oxysporum*. For the biological control of this disease were selected the biological control bacteria *Pseudomonas fluorescens* and chelated iron Fe-EDDHA. The results showed in vitro, pots and greenhouse that the addition of *Pseudomonas flueresence* and Fe-EDDHA at concentration 0.5%, each individually, inhibited the pathogenic, and overlap together gave the best significant result in reducing the rate and severity of infection with pathogenic, and the addition of these factors led to a significant and increase in some growth parameters, including the total fresh and dry of root and vegetative weights, Also led to an increase in the iron content in plants, which gives a high nutritional value in the crop. However, more studies are needed to confirm these results on different crops and in different conditions.

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