Tikrit Journal for Agricultural Sciences (2019) 19 (3):65-71
https://doi.org/10.25130/tjas.19.3.9

IRAQI Academic Scientific Journals
العراقية المجلات الأكاديمية العلمية

ISSN:1813-1646 (Print); 2664-0597 (Online)
Tikrit Journal for Agricultural Sciences
Journal Homepage: http://tujas.tu.edu.iq

TJAS
Tikrit Journal for Agricultural Sciences

Aram N. Hussein[1]

Ramadan Y.Mohamed[2*]

Tahsein A.M. Amein[2]

[1]*Erbil Polytechnic University, Technical Institute of Khabat, Plant Protection Dept.*

2Salahaddin University/Erbil, College of Agriculture, Plant Protection Dept.

KEY WORDS:

Antagonistic bacteria, *Pseudomonas fluorescens*, *Bacillus subtilis*, Biological control, Plant extract**.**

ARTICLE HISTORY:

Received: 26/12/2018

Accepted: 11/06/2019

Available online: 10/10/2019

Tikrit Journal for Agricultural Sciences (TJAS) Tikrit Journal for Agricultural Sci.

Biological Control of Fire Blight Disease on Pear Caused by *Erwinia amylovora* in Erbil Province/Iraq

ABSTRACT

Two bacterial strains, *Pseudomonas fluorescens* L18 and *Bacillus subtilis* K3 and also five plant extracts Garlic (*Allium sativum*), Clove (*Syzygium aromaticum*), Black cumin (*Nigella sativa*), Thyme (*Thymus vulgaris*) and Sour pomegranate (*Punica granatum*) were tested for their efficacy against the fire blight disease of pear caused by the bacterium *Erwinia amylovora*. The results showed that both bacterial strains had good effect on infected shoots, but the *P. fluorescens* strain had much better and significant effect than the *B. subtilis*. This strain protected the shoots by 40% and reduced the disease severity index by 44.2 %, while *B. subtilis* protected the shoots by 20 % and reduced disease severity by 26 %. The pathogen was affected by all plant extracts but the Garlic extract showed the best effect with (34 mm) inhibition zone and Black cumin that showed the least inhibition zone (10.1 mm). It can be concluded that the investigation of different bacterial strains and herbal extracts can be concerned for control of this disease in future.

INTRODUCTION

The pear (*Pyrus communis* L.) is the fifth most widely produced fruit in the world, being produced mainly in China, Europe, and the United States (Silva *et al*., 2014). The total world pear production was more than 22 million tone based on Food and Agriculture Organization of the United Nation (FAO) statistics (2012). Fire blight caused by the bacterium *E. amylovora* (Burrill) (Winslow *et al*., 1920) was the first bacterium illustrated as a causal agent of a plant disease by Burrill in 1883. It has been known as one of the most important plant bacterial diseases worldwide and is a devastating necrotic disease affecting apples *Malus domestica*, pears *Pyrus communis*, and other rosaceous plants (Norelli *et al*., 2003). There is no any control measure for the disease that will totally eradicate it, provide an absolute cure, or fully protect an orchard **(**Mafruanescu *et al*., 2009). Many researchers have been trying to find alternative controlling pathway of the pathogen since 1980s (Vanneste, 2011). The bacterial strains belonging to the species *Bacillus subtilis* and *Pseudomonas fluorescens* have been extensively studied as potential biological control agents (Johnson and Stockwell, 2000). Also plant extract have been used complementary of chemical (Mosch *et al*., 1996). Moreover, using of plant extracts is eco-friendly and may reduce cost of cultivation. . This study was aimed to (i)

* *Corresponding author: E-mail*: ramadan.mohamed@su.edu.krd

study the effect of inoculation pear shoots with strains of *P. fluorescens* and *B. subtilis* (*in vivo*) for control of the disease and to (ii) estimate the ability of some plant extracts to inhibit the growth of *E. amylovora* isolates using well diffusion assay technique (*in vitro*).

MATERIALS AND METHODS

Microorganism

a- Culture of *Erwinia amylovora*

Diseased samples of pears showing typical symptoms were collected from different locations in Erbil Province during the years (2015/ 2016). Four isolates were identified as *E. amylovora* (*Ea*1, *Ea*2, *Ea*3, and Ea4) by standard bacteriological technique (API20E) (BioMerieux/France), molecular method (Polymerase chain reaction) and pathogenicity test on pear shoots and fruits. Pure cultures of *E. amylovora* isolated from diseased samples were maintained by subculture on King's medium (KB) agar (King *et al*., 1954).

b- Culture of the antagonistic bacteria, *Pseudomonas fluorescens* L18 and *Bacillus subtilis* K3

Pseudomonas fluorescens L18 and *B. subtilis* K3 used in this study were obtained from Dr. Tahsein A.M. Amein which are isolated from a golf grass and oil- seed rape respectively in Sweden (Amein and Weber, 2002; Tinivella, *et al*., 2008).

c- Preparation of *Plant* Extracts

The plants extracts were prepared according to the methods described by Valarmathy *et al*., (2010), with slight modification. Briefly, the fresh plant materials were dried in the oven at 60 ^{o}C then powdered. Extract of each plant part used was prepared by mixing 50g of powdered material with 500 ml of 96% ethanol in Soxhlet extractor for 24 h. at 60 ^{O}C. The solution was evaporated to concentrate under reduced pressure and controlled temperature by using rotary evaporator, then dried by using oven at 70 ^{o}C. The extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) in order to prepare 100 mg/ml solution of each extract (table 1).

Table 1: The scientific, common name, plant parts and volume of the extracts used in current study

Scientific Name	Common Name	Plant Parts Used	Volume (µl)
Allium sativum	Garlic	Bulbs	50
Thymus vulgaris	Thyme	Leaves	50
Punica granatum	Sour Pomegranate	Peels	50
Syzygium aromaticum	Clove	Buds	50
Nigella sativa	Black cumin	Seeds	50

In vivo *bacterial* experiment

The procedure of Leah, (1993) with few modifications was used. Freshly pear shoots (20 cm in length) were inoculated by injection the pathogen and the antagonism through the stem mid-way between the apex and the first fully emerged leaf. Each treatment included 5 shoots (replicate). *Erwinia amylovora* and antagonistic bacteria grown overnight in Luria-Bertani Broth (LB) at 28 ^{o}C cultures were harvested by centrifugation (12000rpm) for one minute and re-suspending in sterile saline (NaCl 0.85%) to 10^{8} cfu / ml. Shoots were first inoculated with *E. amylovora* suspension and then were insert at the same place with the antagonistic agent. A gauge needle was used to deposit 50 µl of cell suspension each time. As positive control, the pathogen-alone and sterile distilled water alone as negative control were included in each assay. Pear shoots were maintained in conical flasks

filled with water for 10 days at 26 ± 2 °C and scored for infection when any of the following symptoms were seen:

1- Ooze production at/or extending from the wound site.

2- Leaf wilt and necrosis with heavy Ooze.

Percent protection was measured as follows:

(% protection = (No. uninfected pear shoots / Total no. of shoots) x 100).

Disease severity index on the inoculated shoots was estimated using a procedure of Westwood, (1978) with some modifications using a six scale grades as follow: (0= healthy shoots (Non symptoms visible), 1= stem necrosis extending from the wound site, 2= slight necrosis and oozing, 3= necrosis advancing into the petiole of leaf. 4= necrosis of leaf 5= necrosis over the whole leaf and oozing). Percent of severity of disease (Disease severity index DSI) was calculated according to the following formula (Mchinney,(1923).

$$DSI\ (\%) = \frac{\sum (\text{Class x No. of shoots in class})}{\text{Total no. of shoots x No. of grades}} \times 100$$

The experiment was carried out twice.

In vitro Screening *of* Plant Extracts

The modified well diffusion assay technique was used in *in vitro*. 10^5 cfu/mL of *E. amylovora* age 24 h. was uniformly spread on Petri dishes with nutrient agar medium using sterile cotton swab. Five mm in diameter well was punched into each agar plate with the help of sterilized Cork borer. Fifty µl of the plant extracts at concentration 10% were added to the wells by using a micropipette. The inoculated agar plates were left for one hour for proper diffusion then were incubated at 26 - 28 °C for 24 h. Dimethyl sulfoxide (DMSO) was used as negative control for each extract. Antibacterial activities were evaluated by measuring the diameters of inhibition zones in mm. Each assay was performed in triplicate and twice. Mean values were reported (Parekh and Chanda, 2007).

Data Analysis

Data were analyzed using GenStat (version 12.1.0.; Genstat VSN International Ltd 2009). Data were subjected to analysis of variance (ANOVA) and means of the treatments were compared by using least significant differences (LSD) at ($P \leq 0.05$).

RESULTS AND DISCUSSION

Effect of antagonistic bacteria

Application of bio-agents *P. fluorescens* L18 and *B. subtilis* K3 reduced the infection of fire blight and disease severity on pear shoots. Significant differences were observed in shoot protection and disease severity. The bacterial strain *P. fluorescens* protected the shoots by 40% and reduced disease severity by 44.2 %, while *B. subtilis* protected the shoots by 20 % and reduced disease severity by 26.5% (Figure. 1& 2), Both strains have shown good effect in controlling different crop diseases (Amein *et al.*, 2011: Amein *et al.*, 2008; Koch *et al.*, 2010; Schmitt *et al.*, 2009).

The effect of *P. fluorescens* in reduction of disease severity was better than *B. subtilis*. Schoofs *et al*., (2015), applied *B. subtilis* QST 7013 against young pear shoots under artificial inoculations conditions. They observed interesting activity of the bacterial antagonist against *E. amylovora*: a reduction in the disease progression as necrosis and a limitation of the ooze formation on the infected tissue was observed (Schoofs et *al*., 2015). Palleroni, (1984) observed that *P. fluorescens* had great potentiality to produce a broad spectrum of secondary metabolites f. ex. Hydrogen cyanide antibiotic that could be toxic to other microorganisms. Johnson and Sockwell, (2000) reported that the antagonistic bacterium had good colonizing ability in apple and pear blossoms (Stigmas) during mid-bloom. The reduction of the disease by 40–60% was obtained with applications of *P. fluorescens*. Sanna *et al*., (2012) mentioned that, the use of some strains of nonpathogenic bacteria as a biological

control against fire blight proved to be useful, cheap and safe methods to reduce shoot, blossom and immature fruit infection.

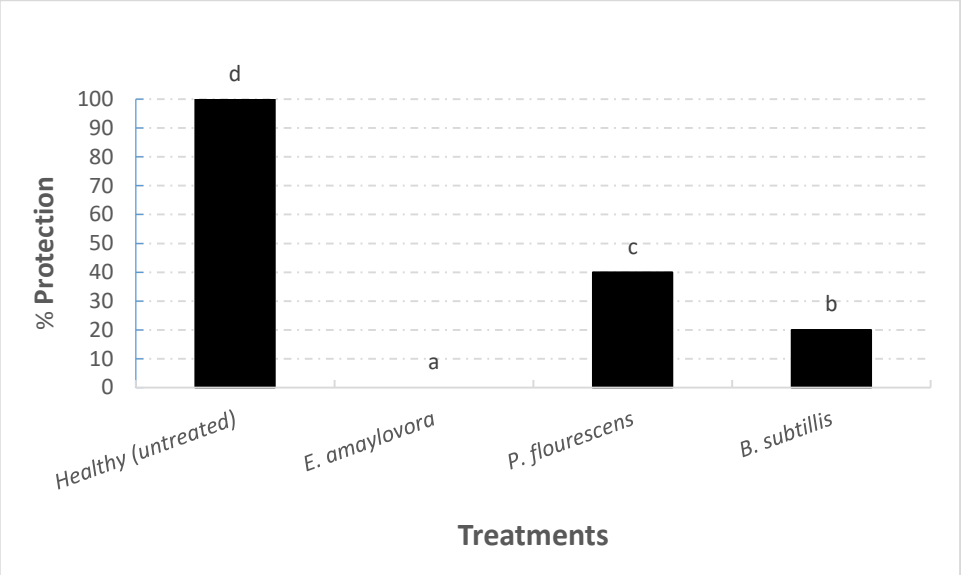

Fig. 1: Protection of Pear shoots infection caused by *E. amylovora* using two bacterial strains, *P. fluorescens* L18 and *B. subtilis* K3 in in-vivo experiment.
Column followed by different letter are significantly different at $P < 0.05$

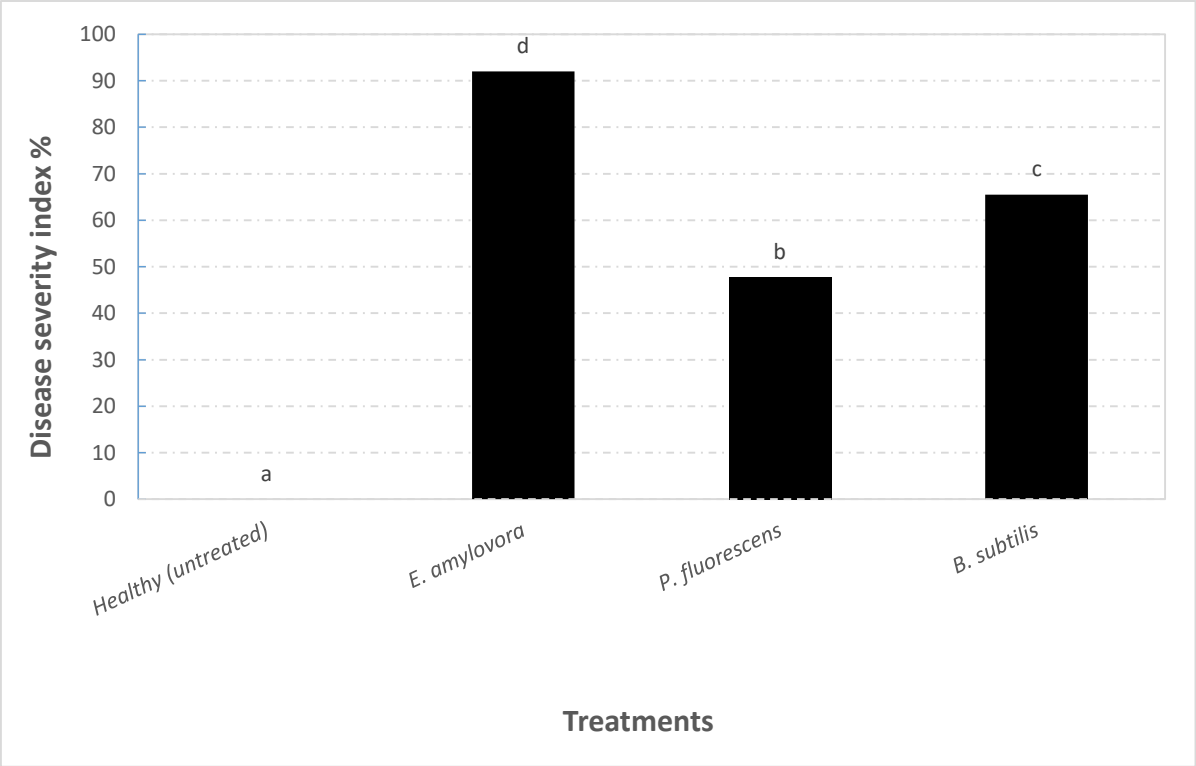

Fig. 2: Pear shoots disease severity suppression by *P. fluorescens* L18 and *B. subtilis* K3 under laboratory conditions.
Column followed by the different letter are significantly different at $P < 0.05$

Plant Extracts Sensitivity Studies

In present research, extracts of various plants are presented in table (1) investigated individually for antibacterial activity by well diffusion agar method. The results showed highly significant antibacterial activity of garlic (34mm) inhibition zone compared with all other treatments; Clove, Sour pomegranate, Thyme and Black cumin with (13.93, 12.03, 10.3 and 10.1 mm) respectively (fig. 3 & 4). There were no significant differences between *T. vulgaris* and *N. sativa*. As shown clearly in figure (4) the maximum inhibition zone was by *A. sativum* and the minimum inhibition was shown by *Nigella sativa.* Our findings are in agreement with Islam *et al*., (2014) who observed that *A. sativum* extract exhibit strong activity against *E. amylovora.*

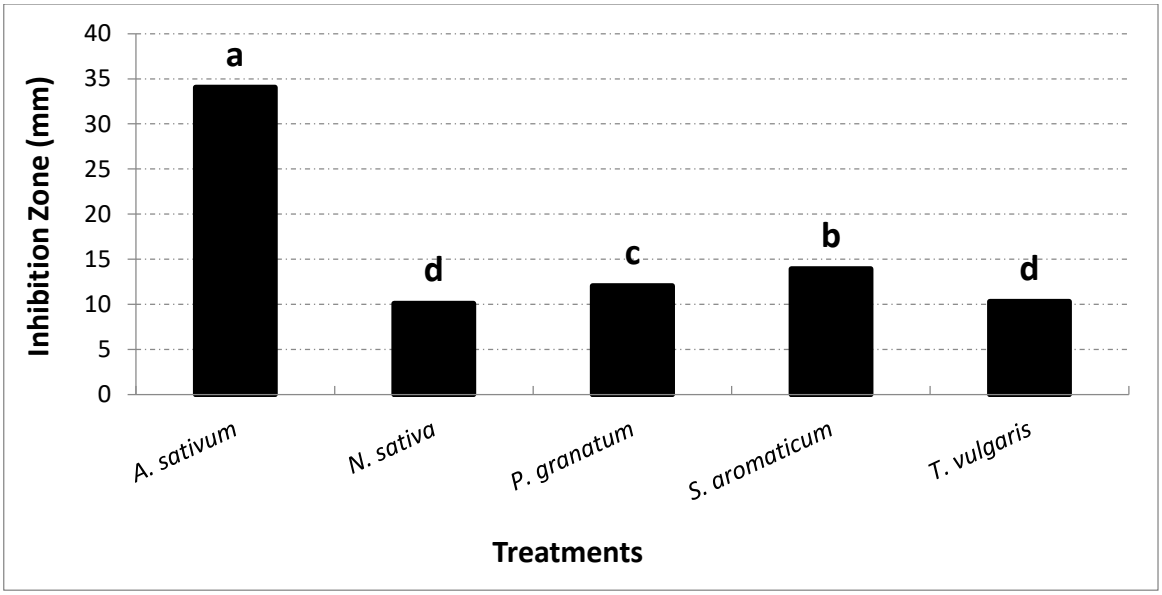

Fig. 3: Effect of different plant extracts on growth (mm) of *E. amylovora* in NA Petri dishes experiment.(Column followed by the same letter are significantly not different at *P* 0.05.)

Similar result was also found by Ali other researcher, who observed garlic and thyme water extracts were more effective on suppressive the growth of *E. amylovora*. All concentrations of thymol (purified from garden thyme) were appeared to inhibit growth of the pathogen . As well as, all the concentrations of sulfur compound from garlic were appeared to inhibit the growth of *E. amylovora* (Ali,2010). Curtis *et al*., (2004) concluded that antimicrobial activity of allicin from *A. sativum* exhibit great activity against *E. carotovora*. Although the exact active components of the extracts that showed these effects were not identified, but the antibacterial activity of garlic and thyme may be due to its contents of active component, sulfur compound in garlic, thymol in thyme (Ali, 2010), phenolic compound in pomegranate peel (Reddy *et al*., 2007), eugenol in clove and alkaloid compound in black cumin which may interact with cysteinyl residue of protein and other active groups leading to inhibiting of bacterial growth, disrupting of cell membrane and cell collapse (Saad *et al*., 2005).

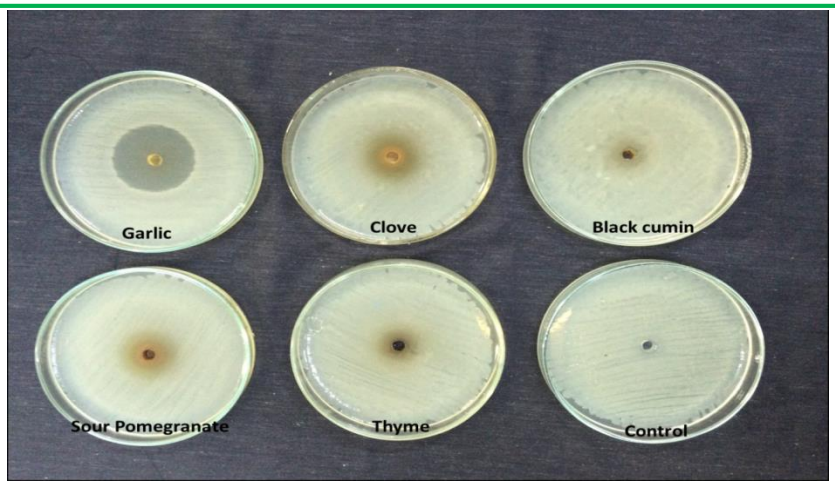

Fig. 4: Antibacterial activity of some plant extracts against *E. amylovora* in nutrient agar media.

CONCLUSIONS

Applications of *P. fluorescens* and *B. subtilis* as bio-agents proved their efficacy in reducing fire blight disease severity and control the pathogen on young pear shoots and *P. fluorescens* had better effect than *B. subtilis* in disease control under laboratory conditions. *A. sativum* extract showed significant antibacterial activity against *E. amylovora in vitro*.

REFERENCES

Ali, H.A. (2010). Studies on the efficacy of some chemicals and plant extracts in the control of plant pathogenic bacteria. M.sc thesis, Department of Plant Pathology, Faculty of Agriculture, Cairo University Egypt: 93pp.

Amein, T. & Weber, Z. (2002). Seed treatment with strains of *Pseudomonas fluorescens* as potential bio control agents of wheat take-all. *Journal of Plant Diseases and Protection*, 109: 655- 661.

Amein, T**.** S. A. I. Wright, M. Vikström, E. Koch, A. Schmitt, D. Stephan, F. *et al.*, Groot.(2011). Evaluation of non-chemical seed treatment methods for control of *Alternaria brassicicola* on cabbage seeds. Journal of Plant Diseases and Protection, 118 (6), 214–221).

Amein, T**.** Z. Omer and C. Welch (2008). Application and evaluation of

Curtis, H.; Noll, U.; Stormann, J. & Slusarenko, A. (2004). Broad-spectrum activity of the volatile phyto anti cipinallicin in extracts of garlic (L.) against plant pathogenic bacteria, fungi and Oomycetes. *Physiol. Mol. Plant Pathol*. 65: 79- 89.

Eckhard Koch, Annegret Schmitt, Dietrich Stephan, Carola Kromphardt, Marga Jahn, *et al.*, (2010). Evaluation of non-chemical seed treatment methods for the control of *Alternaria dauci* and *A. radicina* on carrot seeds. European J. of Plant Pathology. 127: 99 – 112.

Food And Agricultural Organization of the United Nation FAO. (2012). Available from: http://faostat.fao.org/site/339/default.aspx (Accessed 14/9/ 2016).

Islam, M.A.; Alam, M.D.; Urmee, S.A.; Rahaman, M.H. & Razu, M.H. *et al*. (2014). Isolation, Identification, In Vitro Antibiotic Resistance and Plant Extract Sensitivity of Fire Blight Causing *Erwinia amylovora*. J. *Plant Pathol. Microb*., 5: 233.

Johnson, K.B. & Stockwell, V.O. (2000). Biological control of fire blight. In: Fire blight, the disease and its causative agent, *Erwinia amylovora*. Ed. J. Vanneste. CABI Publ., Wallingford, UK: 319-338.

King, E.O.; Ward, M.K. & Raney, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med., 44: 301-307.

Leah K. (1993). Biological control of *Erwinia amylovora* by *Erwinia herbicola*. Ph. D. Thesis, Coll. Molecular Biology, Plant and Microbial Sci. Dep., Univ. Canterbury: 191pp.

Mafruanescu, L.; Saviuc, C.; Oprea, E.; Savu, B. & Bucur, M. *et al*. (2009). In vitro susceptibility of *Erwinia amylovora* (Burrill) Winslow *et al*. to Citrus maxima essential oil. Roum. *Arch. Microbiol. Immunol*., 68: 223-227.

Mchinney,H.H(1923).Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*.J.Agric.Res.26,195-217.

Mosch, J.; Zeller, W.; Rieck, M. & Ullrich, W. (1996). Further studies on plant extracts with a resistance induction effect against *Erwinia amylovora*. *Acta Horticulturae*, 411: 361-366.

Norelli, J.L.; Jones, A.L. & Aldwinckle, H.S. (2003). Fire blight management in the twenty-first century: using new technologies that enhance host resistance in apple. *Plant Disease*, 87: 756-765.

Palleroni, N.J. (1984). Family I. Pseudomonacea. Wilson, Broadhurst, Buchanan, Krumwide, Rogers and Smith 1917.pp.143-213 In: Bergey's manual of systematic Bacteriology, vol. I (Krieg, N.R. and Holt, K.G.,eds), Wiliams and Wilkins, Baltimore, M.D.

Parekh, J. & Chanda, S. (2007). In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants. *Turkish Journal of Biology*, 31(1): 53-58.

Pseudomonas strains for biocontrol of wheat seedling blight. Crop Protection 27, 532–536.

Reddy, M.; Gupta, S.; Jacob, M.; Khan, S. & Ferreir, D. (2007). Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Med*., 73: 461-467.

Saad, B.; Azaizeh, H. & Said, O. (2005). Tradition and perspectives of Arab herbal medicine: A review. Evidence-based complementary and alternative medicine, 2(4): 475-479.

Sanaa, S. A. K.; El-Sheikh, M. H.; Elsabagh, A. S. & Aboulenein, A. M. (2012). Performance of "Le Conte" Pear Trees in Response to Biological control of Fire Blight Disease. *Journal of Applied Sciences Research*. 8(8): 4511-4524.

Schmitt, A.; E. Koch, D. Stephan, C. Kromphardt, M. Jahn, H.-J. Krauthausen, G. Forsberg, S. Werner, T. Amein *et al.,* (2009). Evaluation of non-chemical seed treatment methods for the control of seed-borne infections by *Phoma valerianellae* on lamb`s lettuce. Journal of Plant Diseases and Protection, 116, 200 - 207.

Schoofs, H.; Verjans, W.; Deckers, T.; De Maeyer, L. & Bylemans, D. (2015). The use of the bacterial antagonist bacillus subtilis and the plant defense enhancer fosetyl-al against fire blight on pear. *Acta*. *Horticulture*, 1094: 485-492.

Tinivella, F.; Hirata, L. M.; Celan, A.; Wright, S. A. I.; Amein, T. & Schmitt, A. *et al*. (2009). Control of seed-borne pathogens on legumes by microbial and other alternative seed treatments. *European Journal of Plant Pathology*, 123: 139-151.

Valarmathy, K.; Gokulakrishnan, M.; Kausar, M. S. & Paul, K. (2010). A study of antimicrobial activity of ethanolic extracts of various plant leaves against selected microbial species. *International Journal of Pharma Sciences and Research*, 1(8): 293-295.

Vanneste, J.L. (2011). Biological control agents of fire blight: Successes and challenges. *Acta Horticulturae*, 896: 409-416.

Westwood, M. N. (1978). Fruit and nut species. In: Temperate-Zone Pomology, M. N. Westwood (ed) Chapt. 3, W. H. Freeman and Co., San Francisco CA: 653pp.

Winslow, C.E.A.; Broadhurst J.; Buchanan R.E.; Krumwiede C.; Rogers, L.A. & Smith, G.H. (1920). The families and genera of the bacteria. Final report of the Committee of the Society of American Bacteriologists on characterization and classification of bacterial types. *Journal of Bacteriology*, 5: 191-229.

المكافحة الحيوية لمرض اللفحة النارية على الكمثرى المتسبب عن *Erwinia amylovora* في محافظة اربيل– العراق

ارام نجم الدين حسين ورمضان يوسف محمد وتحسين عبدالعزيز محمد امين

المستخلص

اختبرت فعالية سلالتين من البكتيريا وهي *Pseudomonas fluorescens* L18 و *Bacillus subtilis* K3 بالاضافة الى خمسة انواع من المستخلصات نباتية وهي الثوم Garlic (*Allium sativum*) والقرنفل Clove (*Syzygium aromaticum*) و الكمون الأسود Black cumin (*Nigella sativa*) والزعتر Thyme (*Thymus vulgaris*) والرمان الحامض (*Punica granatum*) لمكافحة مرض اللفحة النارية على العرموط (الكمثرى) المتسببة عن البكتيريا *Erwinia amylovora*. أظهرت النتائج بأن السلالتين كانت لهما تأثير جيد على الافرع المصابة، ولكن سلالة البكتريا *P. fluorescens* كانت لها تأثير أفضل وقد تباين تأثيرها وبتفوق معنوي على السلالة *B. subtilis*. اذ حققت خفض في شدة المرض بنسبة 44.2 ٪ و 26 ٪ على التوالي وكذلك حافظت السلالة *P. fluorescens* على البراعم بنسبة 40 ٪ ، في حين أن *B. subtilis* حافظت على البراعم بنسبة 20 ٪. المسبب المرضي كان متأثرا مع جميع المستخلصات النباتية وبتفوق معنوي لمستخلص نبات الثوم Garlic (*Allium sativum*) وكانت قطر منطقة التثبيط (34 مم) اما الكمون الأسود أظهر أقل تأثيرا وكانت قطر منطقة التثبيط (10.1 مم). لهذا يمكن التوصية بان السلالات البكتيرية المختلفة والمستخلصات النباتية يمكن الاستفادة منها لمكافحة مرض اللفحة النارية في المستقبل.

الكلمات المفتاحية: البكتريا العدائية *Pseudomonas fluorescens* وBacillus subtilis, المكافحة الحيوية، المستخلصات النباتية.