

Comparison among Various Control Methods of Tomato Bacterial Spot Disease (*Xanthomonas campestris pv. vesicatoria*)

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ABSTRACT

Two different plant extracts, thyme (*Thymus vulgaris*) and eucalyptus (*Eucalyptus sp.*), two bacterial strains, *Pseudomonas fluorescens* L18 and *Bacillus subtilis* K3, one antibiotic (Streptomycin) and one bactericide (copper sulphate) were tested for their control efficacy against Tomato Bacterial Spot caused by the bacterium *Xanthomonas campestris pv. vesicatoria* under vitro and in vivo conditions.

Two different concentrations of each extract was used, under lab and greenhouse conditions. The eucalyptus extract had better effect than the thyme extract in both conditions. The inhibition zone in Petri dishes was 11.25 mm and 6.99 mm and the reduction of disease severity index in plants was 40.7% and 35.6% respectively.

The *Pseudomonas fluorescens* L18 strain had much better and was more effective than the *Bacillus subtilis* K3 strain in reduction the disease severity in plants. The disease severity index was reduced by 29.3% and 18.2% respectively.

Streptomycin used as antibiotic had a great effect on reduction of the disease severity index 83.5% and inhibition zone 22.5mm. Copper sulphate reduced the disease severity index by 87% and had 38.1mm inhibition zone and had the best effect compared with other treatments.

Keywords: *Xanthomonas campestris pv. vesicatoria*, *Bacillus subtilis*K3, Biological control, Plant extract, *Pseudomonas fluorescens*. Copper sulphate, Streptomycin

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) crop belonging to the family Solanaceae is considered the second economical important crop in all over the world after cereals. It is an economically attractive crop among farmers due to its relatively quick maturity and high yield (Rashid, 2016).

Tomato bacterial spot disease (TBS) caused by *Xanthomonas campestris pv. vesicatoria* (Xcv) is one of the most important diseases of tomato. Bacterial spot is primarily characterized by appearance of greasy-appearing, water soaked and circular lesions on leaves, stems and fruits with considerable reduction of quality and quantity of tomato yields. (Stall, 1995; Byrnea *et al.*, 2005).

Different methods such as cultural practices, application of chemical pesticides, use of plant extracts, antibiotics and biological agents have been used for controlling the disease (Kucharek, 2000; Morais *et al.*, 2002; Balestra *et al.*, 2009; Varun, 2014).

Several biological agents such as the bacteria *Pseudomonas fluorescens*, *P. putida* and *Bacillus subtilis*, have been used for the disease control under greenhouse and field conditions (El-Hendawy *et al.*, 2005, Abo-Elyousr and El-Hendawy, 2008).

The aim of this study was to evaluate the efficacy of different plant extracts, bacterial strains and antibiotics against the tomato bacterial spot disease in in vitro and in vivo conditions.

MATERIALS AND METHODS

Isolation and Purification of Xcv

Isolation of Xcv was done from different plant parts (leaves, stems, fruits and seeds). Diseased samples of tomato showing typical symptoms were collected from different locations in Erbil Province during the years (2015, 2016). Isolates were identified as *Xanthomonas campestris* pv. *vesicatoria* by standard bacteriological technique, biochemical reaction, molecular method (Polymerase chain reaction) and pathogenicity tests were carried out on tomato fruits and seedlings in order to fulfill the requirements and prove of Koch's postulates.

Pure cultures of Xcv isolated from diseased samples were maintained by subculture.

Culture of the antagonistic bacteria, *Pseudomonas fluorescens* L18 and *Bacillus subtilis* K3 *Pseudomonas fluorescens* L18 and *B. subtilis* K3 used in this study were isolated from a golf grass and oil- seed rape respectively in Sweden (Amein and Weber, 2002; Tinivella *et al.*, 2009).

Plant Extracts

Preparation of Plant Extracts

Plant extracts of thyme and eucalyptus were prepared as follow: Fresh leaves of each plant were washed with sterile distilled water and were dried in the oven at 60 °C then powdered. Extract of leaves was prepared by mixing 50g of powdered materials with 500 ml of 96% ethanol in Soxhlet extractor for 24 h. at 60 °C. The solution was evaporated to concentrate under reduced pressure and controlled temperature by using rotary evaporator, then dried by using oven at 70 °C. The extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) in order to prepare 100mg/ml stock solution of each extract (Valarmathy *et al.*, 2010).

1. In Vitro Studies

Screening of Plant Extracts

The antimicrobial activity of thyme and eucalyptus extracts were tested in *in vitro* (Bacterial growth inhibition test). Three different concentrations of extracts *i.e.*, (5, 10 and 20%) were prepared. (0.1 ml) of Xcv bacterial suspension (1×10^8 / ml, 48 h old culture) were spread onto NA plate medium with saturated and sterilized cotton buds. Six mm in diameter well was punched in each agar plate with the help of sterilized Cork borer. 50,100 and 200 μ l of each plant extracts at each concentration (5, 10, 20%) were added to the wells individually by using a micropipette. The inoculated agar plates were left for one hour for proper diffusion then plates were incubated at 26-28 °C for 72 h. Dimethyl sulfoxide (DMSO) was used as negative control for each extract. Antibacterial activities of each concentrate were measured in form of inhibition zones in mm surrounding the punchers. Each assay was performed in five replicates and mean values were reported (Morais *et al.*, 2002 and Parekh and Chanda, 2007).

Biological Agents

Two bacterial strains (*P. fluorescence* and *B. subtilis*) were used in this study. The method used was as above except that instead of plant extracts, bacterial suspension (1×10^8 cfu/ ml, 48h old culture) were used. Diameter of inhibition zone was measured as described by (Boudyach *et al.*, 2001).

Antibiotics

The antibiotics used in this study were streptomycin, with concentration (0.3mg/ml) and Copper based compounds (copper sulphate) with concentration of (19%). The method used was as above for plant extracts. Effect of tested antibiotics were measured in form of inhibition zone surrounding the punchers according to (Loo *et al.*, 1945).

2. In vivo Study

This experiment was conducted to evaluate the efficacy of different compounds (Thyme and eucalyptus extracts, *P. fluorescens* and *B.subtillus* strains, streptomycin and copper sulphate) in reduction of the bacterial spot disease in tomato caused by Xcv.

In all *in vivo* studies, pots (13cm diameter and 14cm deep) containing one kg sterilized commercial peat mixture / pot, were planted with one seedling/pot (4 week old) of 122-321 oval

Cocktail tomato cultivar. Each treatment had nine replicates. The same amount of plants (pots) not treated (healthy) were used as control.

Symptoms and disease severity were recorded after 12 -14 days from inoculation. Inoculated plants were kept on a greenhouse bench and rated for foliar bacterial spot disease using the following scale: 1 = symptomless, 2 = a few necrotic spots on a few leaflets, 3 = a few necrotic spots on many leaflets, 4 = many spots with coalescence on few leaflets, 5 = many spots with coalescence on many leaflets, 6 = severe disease and leaf defoliation, and 7 = plant dead (Abbasii *et al.*, 2002).

A disease severity index (DSI) was calculated using the following formula:

$$\text{DSI (\%)} = \frac{\sum (\text{class x no. of plants in class})}{\text{Total no. of plants x (no. of classes - 1)}} \times 100$$

Five ml of each Thyme and eucalyptus plant extract at 20% concentration, the two bacterial strains *P. fluorescence* and *B. subtilis*, streptomycin (0.3mg/ml) and (copper sulphate) 23% concentration were sprayed individually on plant leaves one day after tomato seedlings inoculation with the *Xcv* bacterial suspension and maintenance in a humid chamber for 24 h. Disease incidence and severity were recorded, after 12 - 14 days. Pots spread with *Xcv* only were served as a positive control and only spread with water served as negative control.

Statistical Analysis

For interpretation of data, analysis of variance (ANOVA) was used, with sources and amounts of variation compared using an *Xcv* ratio test. To compare treatment means, Duncan's test at $\leq p$ 0.05 were calculated. Means followed by different letters demonstrated a significant difference.

RESULTS AND DISCUSION

In vitro

Plant extract

The test results showed that eucalyptus leaves extract was more effective than thyme leaves in inhibiting the bacterial growth in laboratory condition. Also results revealed that, all tested concentrations of extracts had good inhibitory effect on growth of the *Xcv* bacteria compared with the control Fig. (1). Increasing the concentration increased gradually the inhibition zone. The best effect with 11.25 mm inhibition zone had the 20% concentration of eucalyptus extract. 10% of eucalyptus extract had (8.74 mm) better and significantly affect than 20% thyme extract (6.99 mm). Both extracts in all concentrations inhibited the pathogen growth in Petri dishes.

Santos and Waterman (2001a); Santos and Waterman (2001b); De vincenzi *et al.*, (2002); Mustafa and Yeşim, (2005) and Rosato *et al.*, (2007) reported that eucalyptus and thyme antibacterial extracts have a great effect on reducing the disease severity. The obtained results are also in harmony with those reported by Morais *et al.*, (2002) who tested the antimicrobial activity of 45 extracts of medicinal plants against *X. c. pv. vesicatoria*. Besides, the obtained findings of Basim *et al.*, (2006) confirmed the antimicrobial effect of plant extracts against *Xcv* where they screened the effect of aqueous extracts from leaves of 30 higher plants, *in vitro* for their antibacterial activity against different pathovars of *Xcv* bacterium. Eight plant species showed antibacterial activity, based on the inhibition zone in a diffusion assay. This concept is currently more flexible, and various compounds that are considered resistance inducers also exert direct action on the pathogens. Active compounds of the extract act directly on the pathogens or induce host resistance through the production of phytoalexins, increased PRP activity, synthesis of structural compounds, and biochemical plant defense (Mustafa and Yeşim, 2005).

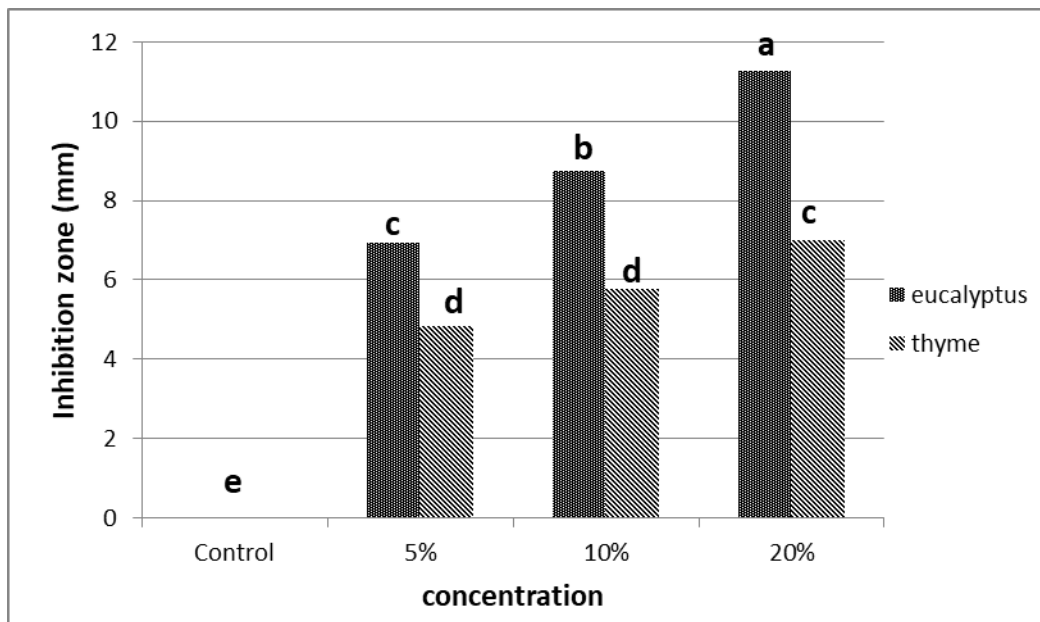


Fig. 1: Effects of eucalyptus and thyme plant extracts on *Xcv* inhibition zone based on Duncan's multiple.

*Column followed by the different letter are significantly different at $P < 0.05$

Effect of Bioagents:

Pseudomonas fluorescens and *B. subtilis* strains were tested for their abilities to inhibit the growth of *Xcv* on nutrient agar (NA) medium. None of the two bacterial strains had positive effect on the growth of the pathogen and the inhibition zone was zero mm. The results disagree with Sahin *et al.*, (2000). They reported the good antagonistic effect of these strains against *Xcv*. Treatments had significant effect in reduction the disease. Different strains of *P. fluorescens* and many other *Xanthomonas* pathovars had clear inhibitory effects on *Xcv* under *in vitro* conditions (Campbell, *et al.*, 1998).

Antibiotics:

The data results in Fig. (2) indicate that both tested antibiotics had an inhibitory effect on the growth of the pathogenic bacteria compared with the control. Copper sulphate had better inhibition zone (29.7 mm) than the streptomycin with (26 mm) but this difference was not significant. These two treatments were better than all other treatments in laboratory condition. Similar results were obtained by Carrillo-Fasio *et al.*, (2001); Roberto *et al.*, (2002); Martin and Hamilton, (2004) and Shukla and Gupta, (2004). They all reported inhibition zones and reduction in diameter of bacterial growth when treated with streptomycin and copper based bactericides. Also, Carrillo-Fasio *et al.*, (2001) tested thirty nine *X.cv* strains for their sensitivity to several copper formulations and combinations of copper + mancozeb, copper + antibiotics, in laboratory and field experiments.

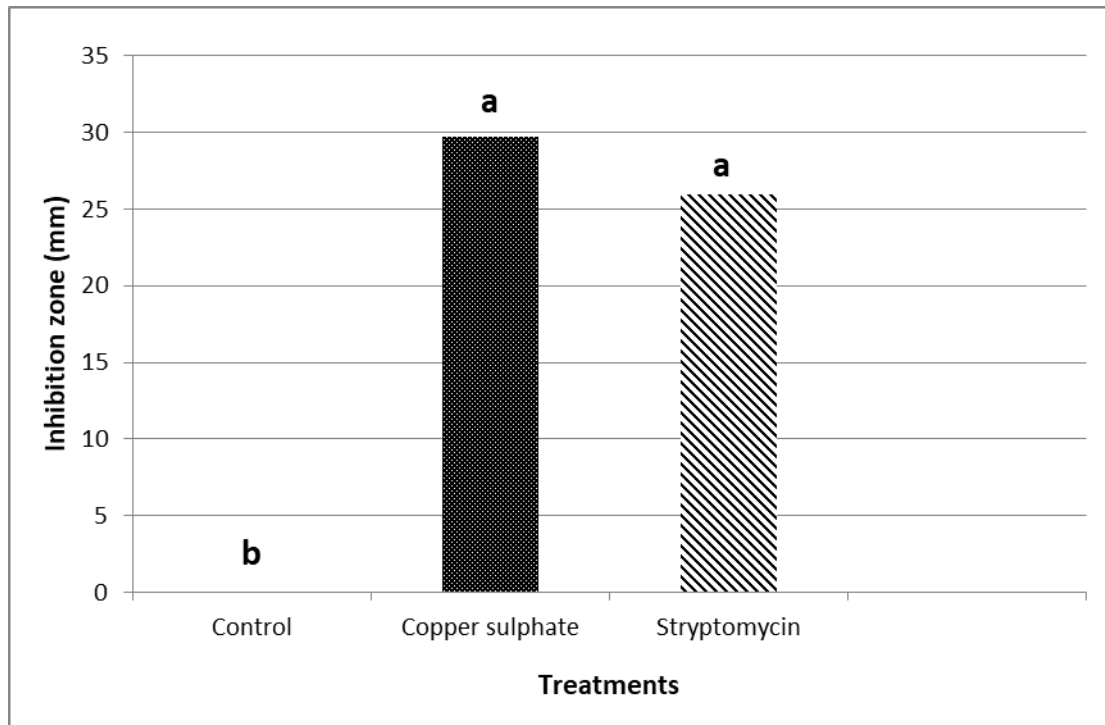


Fig.2: Effects of Streptomycin and the Copper sulphate on the growth of *Xcv* on NA medium based on Duncan's multiple.

Column followed by the different letter are significantly different at $P < 0.05$

Greenhouse Experiment:

The results presented in Fig. (3) Shows that all treatments (plant extracts, bioagents, antibiotics and bactericide) were effective in controlling bacterial spot disease on tomato leaves. All treatments significantly reduced the disease severity of foliar disease in greenhouse conditions. The bactericide and the antibiotic were the most effective in reducing the disease severity with 83.5 % and 79.4% respectively compared with the untreated control. The Eucalyptus extract reduced disease severity by 40.7% and the Thyme extract by 35.6%. Lucas *et al.*, (2012) mentioned that plant extracts and essential oils have significant role in reducing the disease.

The *Pseudomonas* strain reduced the disease severity by 29.3% and the *Bacillus* strain only by 18.2%. The results by Moustaine *et al.*, 2019, showed that the bacterial strain *Pantoea agglomerans* reduced 70% of the onset of disease symptoms, bacterial canker of tomato incited by *Clavibacter michiganensis* spp *michiganensis* *Serratia proteamaculans* 45% and *Bacillus cereus* 75%. Significant variations in reduction of disease incidence, enhancement in plant growth and yield were observed in tomato plants treated with these PGPR strains as compared to the control. *Aureobasidium pullulans* and *Pantoea agglomerans* the two strains also were able to inhibit, *in vitro*, the growth of *C. michiganensis* subsp. *Michiganensis* and their antagonistic effects were confirmed in greenhouse conditions (El kinany *et al.*, 2017).

Copper and antibiotic results are in agreement with Jones *et al.*, (1991a, b); Stall, (1995) who stated that copper formulations proved there effect in reduction bacterial spot development. The chemical control has been extensively used for controlling bacterial spot diseases because copper based formulations are capable of inhibiting or delaying bacterial cell multiplications and could reduce losses. But, chemical sprays often are not very effective in long time, because their extensive use led to the development of copper resistant strains (Kousik and Ritchie, 1996; Teixeira *et al.*, 2008; Nisa *et al.*, 2010). Disease management practices, based primarily on fixed copper bactericides, do not give consistent, effective protection (Abbasi *et al.*, 2002). They reported that foliar applications of ammonium lignosulfonate (ALS), and the fertilizer potassium phosphate (KP)

tested for their ability to control this disease under both greenhouse and field conditions do not significantly increased total tomato or pepper yield.

Behki and Khan, (2001) and Aguiar *et al.*, (2003) mentioned that resistance is widespread in *Xcv* in several geographical regions, where copper was not effective for disease control. Loo *et al.*, (1945); Roberts *et al.*, (2008) and Kizheva *et al.*, (2013) stated that streptomycin is also effective against the tomato bacteria spot pathogen.

Hasan *et al.*, (2005) stated that plant extracts have played significant role in reducing the incidence of seed-borne pathogens and in the improvement of seed quality and emergence of plant seeds in the field. Both bacterial strains had the less effect in controlling the disease.

Our results are in agreement with Tzeng *et al.*, (1994); Byrne *et al.*, 2005; Abo-Elyousr and El-Hendawy, (2008) and Walters, (2009). All of them mentioned that biological control provided good protection against the disease in small-scale plots. For example *P. fluorescens* gave promising inhibitory effects, while their results are disagree with Ali, (2011) who stated that biological control is not comprehensively practiced.

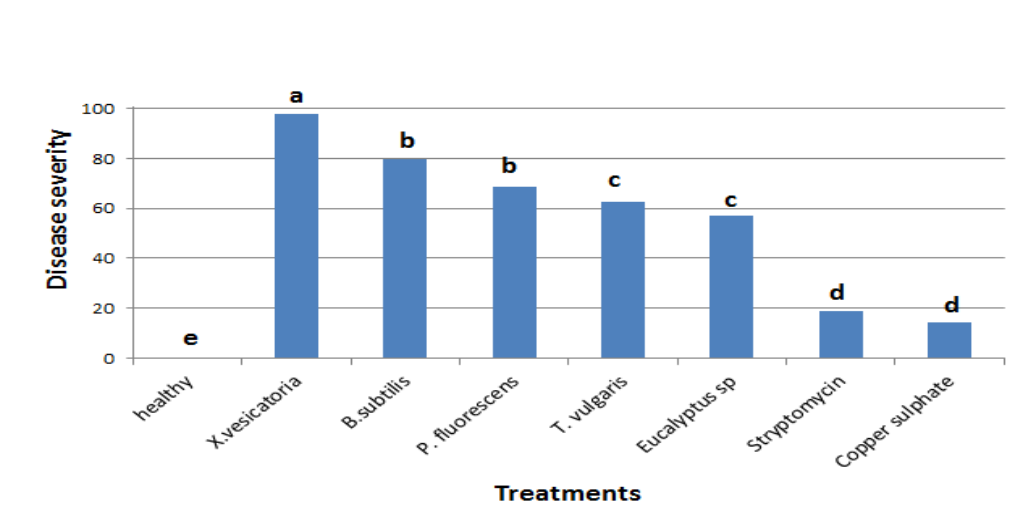


Fig 3: Effects of plant extracts, bacterial strains, antibiotic and bactericide on tomato bacteria spot disease reduction under greenhouse condition based on Duncan's multiple.

* Column followed by the different letter is significantly different at $P < 0.05$

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طرائق مكافحة مرض التبقع البكتيري للطماعة المتسبب عن البكتيريا *Xanthomonas campestris pv.vesicatoria*.

المخلص

تم استخدام مستخلصين نباتيين، اوراق الزعتر (*Thymus vulgaris*) واليوكالبتوس (*Eucalyptus sp.*) و سلالتين بكتيرية، *Bacillus subtilis K3* و *Pseudomonas fluorescens L18* (ستريتومايسين)، والمبيد بكتيري (كبريتات النحاس). تم اختبار تأثيرها ضد التبقع البكتيري للطماعة التي تسببها بكتيريا *Xanthomonas campestris pv.vesicatoria* في المختبر والحقل.

تم استخدام تركيزين مختلفين لكل مستخلص تحت ظروف المختبر والحقل. كان لمستخلص اليوكالبتوس تأثير أفضل من مستخلص الزعتر في كلتا الحالتين. كان منطقة التثبيط 11.25 ملم و 6.99 ملم و انخفاض مؤشر شدة المرض كان 40.7 % و 35.6 % على التوالي.

كانت سلالة *P. fluorescens L18* أفضل بكثير وكانت أكثر نشاطاً من سلالة *B. subtilis K3* في تقليل شدة المرض. خفضت هذه السلالات مؤشر شدة المرض بنسبة 29.3 % و 18.2 % على التوالي.

كان للستريتومايسين المستخدم كمضاد حيوي تأثير كبير في انخفاض من مؤشر شدة المرض 83.5 % ومنطقة تثبيط 22.5 ملم. كبريتات النحاس المستخدمة تسبب بانخفاض شدة المرض بنسبة 87 % وكان منطقة التثبيط 38.1 ملم وكان لها أفضل تأثير مقارنة مع الطرق الأخرى.