American Journal of Bioscience and Bioengineering
2017; 5(1): 41-49
http://www.sciencepublishinggroup.com/j/bio
doi: 10.11648/j.bio.20170501.17
ISSN: 2328-5885 (Print); ISSN: 2328-5893 (Online)

Management of Bacterial Spot of Pepper Caused by *Xanthomonas campestris* pv. *vesicatoria*

Eman O. Hassan[1, *], Marwa A. Zyton[2]

[1]Plant Pathol. Dept., Fac. Agric., Moshtohor, Benha Univ., Egypt

[2]Plant Pathol. Dept., Fac. Agric., Cairo Univ., Giza, Egypt

Email address:

eman_osman76@yahoo.com (E. O. Hassan), marwazyton@age.cu.edu.eg (M. A. Zyton)

[*]Corresponding author

To cite this article:

Eman O. Hassan, Marwa A. Zyton. Management of Bacterial Spot of Pepper Caused by *Xanthomonas campestris* pv. *vesicatoria*. *American Journal of Bioscience and Bioengineering.* Vol. 5, No. 1, 2017, pp. 41-49. doi: 10.11648/j.bio.20170501.17

Received: December 18, 2016; **Accepted**: February 7, 2017; **Published**: February 23, 2017

Abstract: Isolation trials from pepper leaves showing bacterial spot collected from Giza, Kalubia and Behera governorates yielded 13,12,9,6 isolates of *Bacillus subtilis*, *Pseudomonas* spp., *Xanthomonas campestris* pv. *vesicatoria* and *P. fluorescens,* respectively. The isolated strains of *X.c.* pv. *vesicatoria* were yellow Gram negative of short rods and produced Xanthomonadins and the other identification criteria proved that these isolates are *X.c*. pv. *vesicatoria.* Pathogenicity test of these isolates on Balady pepper cv. indicated that all of them were pathogenic and isolate No.3 of Kalubia governorate resulted in the highest infection. *P. putida* was most efficient bioagent in inhibiting of the pathogenic bacterium followed *by P. fluorescens*, while *B. subtilis* was the lowest efficient one. The fungicide Efdal Bakirox was the most efficient one in inhibiting growth of the causal bacterium on the medium more than the fungicides Roxil and Tango. The fungicide Tango and Roxil failed to cause any inhibition to the causal bacterium at 100 ppm. Field experiments during 2014 and 2015 growing seasons revealed that spraying pepper plants with the tested bioagents and the fungicides four times resulted in significant reduction to the severity of the natural infection by the causal bacteriaum with significant increase to the produced fruit pods compared with the control. The tested fungicides were more efficient in this regard than the bioagents. Roxil was the most efficient treatment in reducing the severity of the disease and increasing the produced pod yield. Meanwhile, the bioagent *B. subtilis* was the lowest efficient treatment and the other treatments recorded intermediate figures. Total phenols and vitamin-c content as well as the activity of chitinase, peroxidase and polyphenoloxidase in pepper leaves infected by *X.c.*pv. *vesicatoria* were greatly lowered in the infected leaves compared with the uninfected leaves and the tested bioagents and fungicides resulted in considerable increase to these chemicals compared with the infected leaves.

Keywords: Pepper, Bioagents, Fungicides, Enzymes, Management, Phenols, Vitamin-C and *Xanthomonas campestris* pv. *vesicatoria*

1. Introduction

Pepper (*Capsicum annum* L.) is one of the most important and favorable Solanaceaus cops grown in Egypt for local consumption and exportation. It is reach in vitamin-c and other mineral nutrition's, but liable to infection by bacterial, fungal, viral and viral like organisms in addition to physiological disorder and nematodes (Pernezny *et al*., 2003). However, bacterial diseases, especially bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge), Dye (*Xanthomonas axonopodis* pv. *vesicatoria*) is the most serious disease worldwide (Marco and Stall, 1983; Ward and O'Garro, 1992; Pernezny and Collins,1997; Gore and O'Garro, 1999; Pernezny *et al*., 2003; Gupta and Durga, 2011 and Li *et al*., 2015).

Symptoms of leaf spot of pepper caused by *X.c. pv. vesicatoria* appear as water-soaked spots that change from green to dark-brown as infection progresses. The spots or lesions are often surrounded by yellow zones called halos. The size of lesions is fairly variable. On some cultivars, leaves may display several small lesions (0.25 to 0.5 cm) covering over 80% of leaf area, whereas on others fewer large lesions (larger than 0.5 cm) may be visible. In some

cases a combination of small and large lesions may be found on the leaves. Fruit can also be infected by bacteria and show symptoms similar to those exhibited by leaves. The appearance of the symptoms on leaves and fruit is influenced by several factors, including the cultivars, strain of the causal bacterium and environmental conditions. As a result of severe infection on the plants defoliation can occur. The disease affects stems, leaves and fruits and causes significant losses when environmental conditions are suitable for the pathogen (Diab *et al.,* 1982 and Pernezny *et al*., 2003).

The disease may be distributed in different localities in Egypt, due to cultivation of different exotic hybrids and cultivars of pepper and tomato from foreign countries, where the disease is well known to be seed borne and causes epidemic infection on both plants. High relative humidity and warm temperature are favorable for the development of the disease, especially when these plants are grown under plastic tunnels or houses (Jones *et al*., 1991 and Abolmaaty and Abd El-Ghafar, 2010).

Bashan *et al.* (1982) mentioned that *X. c*. pv. *vesicatoria* developed as endophytes in the leaves and rhizosphere of apparently symptomless plants grown under mist, but not under dry conditions. The causal bacterium enters plant tissue through wounds and natural openings such as stomata on leaves. In warm weather and under moisture conditions such as those provided by prolonged light rainfall, high relative humidity (higher than 85%), or overhead irrigation, populations of the bacterium can reach high levels and cause severe infection. As a result of the severe infection plant defoliation can occur with high yield loss (Pernezny *et al*., 2003).

Resistance to plant disease is supposed to be a dynamic and multifactorial process. It is assumed that plant defense response can be activated by specific recognition of some microorganisms by the plant. There may be whole organisms or products secreted by microorganisms under the influence of which plants initiate defense response (Albersheim *et al.,* 1978 and Akram *et al*., 2013].

The use of *Bacillus* and *Pseudomonas* spp. as bioagents increase the activity of some enzymes such as chitinase, peroxidase and polyphenol oxidase, which play an important role in plant defense mechanisms against plant pathogens (Li *et al*., 2009 and 2015).

2. Materials and Methods

2.1. Isolation, Purification and Identification of the Associated Bacteria

The infected leaves collected from Giza, Kalubia and Behera governorates were thoroughly washed with tap water to remove soil debris and then air dried. Bacterial lesions were then cut and surface disinfected by first dipping in 95% ethanol for 5 s, then sodium hypochlorite solution 1.25% for 20 s followed by two successive rinses in sterile water. Each lesion was homogenized in 0.2 ml sterilized distilled water using a sterile Hun and pestle. The homogenate was then streaked onto nutrient medium. Five colonies from each lesion were randomly selected and retained. The associated bacterial colonies were purified and identified using the description of Parry et *al*. (1983) and Holt and Krieg (1984). Also, the identification of *X. campestris* pv. *vesicatoria* was confirmed by testing for Xanthomonad determinative characteristics (Table, 3) including (using of standard bacteriological methods (Schaad *et al.,*2001). Tests were conducted twice on a random sample of the isolates of the pathogen. Identity of the pathogen was confirmed by testing the sample for pathogenicity on pepper (cv. Balady) as previously described by O'Garro and Tudor (1994).

2.2. Pathogenicity Test of the Tested Isolates of X. c. pv. vesicatoria

Pepper seedlings (cv. Balady), of one month old, apparently free from any infection by any disease (grown in Foam trays in a plastic house) were transplanted in pots (25 cm. in diameter) filled with formalin disinfested soil. Two transplants were transplanted in each pot.

The tested 9 strains of *X.c.* pv. *vesicatoria* were grown on nutrient medium for 48 h at 28±2°C. The bacterial cells were suspended in sterile distilled water and centrifuged at 3000 rpm/min for 30 min. The pellets were re-suspended in distilled water and adjusted to the density of 10^8 cfu / ml. Pepper plants (one month before transplanting) were artificially inoculated with 1×10^8 CFU ml^{-1} of the tested strains using sterilized plastic sprayers. Other pepper seedlings were sprayed with distilled water only as control treatment. The inoculated plants were put under plastic cadges for two days to maintain high relative humidity responsible for success the infection. The incidence of the infection by the tested strains was recorded 14 days after inoculation with the tested strains on randomly 10 leaflets of pepper plants for each treatment. Also, disease severity was assessed based on the disease rating scale from 0 to as described by (0-7) by Horsfall and Barratt (1945).

2.3. Management of the Disease

2.3.1. In Vitro Experiment

The antimicrobial substances of three bioagents (occasionally isolated from pepper leaves) and three fungicides were evaluated against *X. c.* pv. *vesicatoria in vitro*.

The bioagents, *B. subtilis, P. fluorescens* and *P. putida* were screened *in vitro* against *X.c.* pv. *vesicatoria*. The bacterial suspension of the tested bioagents was adjusted to 10^8 cfu ml^{-1} (Optical Density 660 = 0.06), which grew for 48 h at 28±2°C.

The fungicides, *i.e*. Efdal Bakirox 50% (copper oxchloride; 300g/100 L), Roxil 50% (metalaxil 15% + copper hydroxide 53.9%; 150 g/100 L) Tango 23% (copper sulphate 8%+ sulfur 28%; 250ml / 100 L water);. The concentrations were determined by the broth macro dilution method in 2 ml of nutrient medium. and the concentrations of each fungicide were prepared at 100, 250 and 500 ppm depending on their active ingredient. Double distilled sterile

water was used as control.

The pathogenic bacterium was cultured in a 100 ml conical flask containing 40 ml of nutrient broth at 28±2°C for 2 days on a shaker at 150 rpm. One ml of the bacterial suspension was mixed with 15 ml of molten nutrient agar medium, just before solidification and poured in each sterile Petri dish (90 mm in diameter). A Sterile filter paper discs (6 mm in diameter) were immersed in the preparation of any of the tested bio agents or fungicides. Four disks saturated with any of the tested treatments were put on each inoculated Petri-dish with the pathogenic bacterium. The plates were incubated at 28±2°C for 2 days. The diameter of the inhibition zone formed around the disc was measured. Each treatment was replicated 5 times and the results were averaged.

2.3.2. Field Experiments

Field experiments were carried out at the experimental farm of Fac. Agric. at Moshtohor, Benha Univ., Kalubia governorate during 2014 and 2015 growing seasons, where bacterial leaf spot caused by *X. campestris* pv. *vesicatoria* is annually appear with severe infection.

The land was prepared for planting pepper (cv. Balady) by diving the land into plots of 10.5 m^2 (3.5 width x3 m long of five rows). Pepper seedlings (one month old), apparently free from any infection by any disease grown in Foam trays in a plastic house were transplanted in the presence of water irrigation on one side of the row at 50 cm. between each to transplants during April, of each season respectively. The grown plants received all the recommended agricultural practices as recommendation of Min. of Agric. and Land Reclamation and left to the natural infection by the causal bacterium.

The preparations of the bioagents, *B. subtilis, P. fluorescens* and *P. putida* ($1x10^8$ cfu/ml. water) and the fungicides Efdal Bakirox 300 g./100 L water), Roxil (150 g./ 100 L water) and Tango (150 g./ 100 L water) were sprayed on the grown plants four times beginning of mid of August of each season, at beginning of appearing the symptoms of the natural infection with two weeks interval. The adherent material super film (50 ml./ 100 L water) was added to the sprayed plants to adhere and spread these material on the treated plants.

Disease severity was determined by counting lesions on each of 50 randomly sampled leaflets per replicate 10 days after each spray and the average was recorded using the devised scale (0-7) by Horsfall and Barratt (1945).

2.4. Biochemical Associated with the Infection by the Causal Bacterium

2.4.1. Estimation of Total Phenolic Compounds

One gram of pepper leaves sample was extracted with 10 ml of 80% methanol at 70°C for 15 min. Reaction mixture was containing 1 ml of methanolic extracts, 5 ml of distilled sterilized water, and 250 µl of Folin–Ciocalteau reagent (1N). This solution was kept at 25°C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Gallic acid was used as the standard. The amount of phenolic compounds was expressed as mg gallic acid per g plant material (Zieslin and Ben-Zaken, 1993).

2.4.2. Determination of Vitamin-C

Vitamin-C or ascorbic acid method (A.O.A.C., 1975) is based on measurement of the extent to which a 2,6-dichlorophenol-indophenol dye solution is decolorized by the presence of ascorbic acid.

Procedure:

Two dry test tubes, pipette the requisite volume of sample or standard ascorbic acid solution and make up to 5 ml with 2% HPO_3. Add 10 ml of the dye solution (0.02%) with rapid delivery pipette, shake and take the reading within 15-20 sec. Measure the red color at 518 nm against blank consisting of 5 ml 2% HPO_3 and 10 ml of water.

2.4.3. Determination of Chitinase Activity

a. Substrate preparation:

Colloidal chitin was prepared according to Bade and Stinson (1981) as follows: 4.0 gm of purified chitin powder (sigma) was suspended in 100 ml water at 4°C and stirred in cold. concentrated H_2SO_4 (30 ml)at 4°C was added drop wise to the suspension. The cold viscous chitin solution was filtered through glass wool into 1800 ml ice-cold 50% ethanol with rapid stirring. The precipitated colloidal chitin was washed with distilled water to pH 5. It was buffered with phosphate buffer (pH 6.5, 0.2 M) before use as substrate.

b. Enzyme assay:

The reaction mixture according to Ishaaya and Casida (1974), with some modifications consisted of: 1 ml phosphate buffer (0.2 M, pH 6.5), 200 ml 0.5% colloidal chitin and 200 ml enzyme solution. After 1.5 hour incubation at 37°C,. Enzyme activity was terminated by boiling test tub. Undigested chitin was sediment by centrifugation for 15 min at 8.000 r.p.m.. The supernatant was taken for determination of N-acetyl glucose amine that produced as a result of chitin digestion by chitinase.

2.4.4. Determination of Peroxidase

Peroxidase activity was determined according to Vetter *et al*. (I958).

The sample (200µl), in which the color is to be formed, the following reagents are added:

1 ml. of 1% o-phenylenediamine (in 95%ethyl alcohol; fresh every 4 hours)and 1 ml of 0.3% hydrogen peroxide (in distilled water). The reaction is allowed to proceed for 5 minutes at which time it is stopped by adding 2 ml of saturated sodium bisulfate.

The reagent blank for each sample is prepared by adding the dye, followed by the sulphite, and then the hydrogen peroxide. The enzyme is inhibited by the sulfite so that it is inactive when the hydrogen peroxide is added.

The starch in the sample and the blank is flocculated by adding 25 ml of 95%ethyl alcohol. The starch suspension must be swirled continuously during addition of alcohol, so that good flocculation occurs.

The samples were then centrifuged at approximately 3000 r.p.m. for 5 minutes. The clear supernatant is decanted into a

colorimeter tube and its absorbance recorded at 430 mμ. The colorimeter is set at 100% transmittance with the corresponding blank for each sample.

The enzyme activity was expressed as the change in absorbency at 430 m(ΔOD430)/minute/gm fresh weight.

2.4.5. Determination of Polyphenoloxidase Activity

Polyphenoloxidase activity was determined according to a modification of Ishaaya (I971), in a reaction mixture consisting of 0.5 ml phosphate buffer (0.1 M, PH 7), 200 µl enzyme solution and 200 µl catechol solution (2%). Prior to the initiation of the reaction, the substrate and other ingredients of the reaction mixture were separately incubated at the optimum temperature of the reaction (25°C). Enzyme reaction was initiated by adding catechol solution. then after exactly 1 min, the optical density was determined. Zero adjustment was against sample blank. The phenol oxidase activity was determined as O.D. units ×10 3 at an absorbency of 405 nm.

2.5. Disease Assessment

Disease severity ratings were recorded using assessing lesion numbers on 10 leaflets in case of pathogenicity test and 50 in case of field experiments of disease management using the Horsfall- Barratt scale (Horsfall and Barratt, 1945) as in Table (1).

Table 1. *Scale ranging content eight degrees from 0-7.*

Rating	No. of spots	Description
0	0	Represented a sparse plant canopy
1	1-10	Necrotic spots on the leaves/plant
2	11-20	Necrotic spots on the leaves/plant
3	21-30	Necrotic spots on the leaves/plant
4	31-40	Necrotic spots on the leaves/plant
5	41-50	Necrotic spots on the leaves/plant
6	51-60	Necrotic spots on the leaves/plant
7	>61	lack of epinasty on new growth

Disease severity was assessed as follows:

Disease severity % = £ (nxv) / 7 N X 100

Where:

n = Number of infected leaves in each category.

v = Numerical values of each category.

N = Total number of the infected leaves.

2.6. Statistical Analysis

Data were statistically analyzed using the standard procedures for complete randomize block and split designs as mentioned by Snedecor and Cochran (1989). The averages were compared at 5% level using least significant differences (L.S.D) according to Fisher (1948).

3. Results

3.1. Isolation and Frequency of the Isolated Bacteria from Pepper Leaves Showing Leaf Spot

Isolation trials from pepper leaves showing bacterial spot yielded 50 bacterial isolates, Table (2). The isolated bacteria were purified and identified as *Bacillus* spp., *Pseudomonas* spp., *P. fluorescens* and *Xanthomonas campestris* pv. *vesicatoria*, being 13, 12, 6 and 9 isolates, respectively. The frequency of the isolated bacteria was not greatly differed in the three governorates, *i.e*. Giza, Kalubia and Behera, being 14, 13 and 13 isolates respectively.

3.2. Confirmation the Identification of X. campestris pv. vesicatoria

The 9 strains of the presumptive pathogen were yellow Gram negative of short rods producing Xanthomonadins, Table (3) shows the following the tested isolates were positive for growth on potato sucrose peptone agar medium, gas of glucose, relation to o_2, starch hydrolysis and catalase activity, but negative for KOH 3%, pigment in K. B, utilization of arabinose, gas of arabinose, utilization of glucose, utilization of maltose and gas of maltose. The strains also induced typical bacterial spot lesions on Balady pepper cultivar.

Table 2. *Frequency of the isolated bacteria from pepper leaves collected from three governorates, during 2013 growing season.*

The isolated bacteria	Frequency of the isolated bacteria from			Total	%Frequency
	Giza	Kalubia	Behera		
Bacillus spp.	4	3	6	13	26
Pseudomonas spp.	5	4	3	12	24
P. fluorescens	3	2	1	6	12
X.c. pv. *vesicatoria*	2	4	3	9	18
Total	14	13	13	50	--

Table 3. *Tests carried out for the identification of 9 isolates of X.c.pv. vesicatoria.*

Identification test	Isolates of *X.c.* pv. *vesicatoria*								
	Giza. 1	Giza. 2	Kalubia. 3	Kalubia. 4	Kalubia. 5	Kalubia. 6	Behera. 7	Behera. 8	Behera. 9
KOH 3%	-	-	-	-	-	-	-	-	-
Gram reaction	-	-	-	-	-	-	-	-	-
Size	short	Short	short	Short	short	short	Short	short	short
Growth on PSPA (potato sucrose peptone agar)	+	+	+	+	+	+	+	+	+
Spore	-	-	-	-	-	-	-	-	-
Pigment in K. B	-	-	-	-	-	-	-	-	
Yellow pigment	+	+	+	+	+	+	+	+	+
Utilization of arabinose	-	-	-	-	-	-	-	-	-

Identification test	Isolates of *X.c.* pv. *vesicatoria*								
	Giza. 1	Giza. 2	Kalubia. 3	Kalubia. 4	Kalubia. 5	Kalubia. 6	Behera. 7	Behera. 8	Behera. 9
Gas of arabinose	-	-	-	-	-	-	-	-	-
Utilization of glucose	-	-	-	-	-	-	-	-	-
Gas of glucose	+	+	+	+	+	+	+	+	+
Utilization of maltose	-	-	-	-	-	-	-	-	-
Gas of maltose	-	-	-	-	-	-	-	-	-
Relation to o_2	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+	+
Another bacterial genera	X	X	X	X	X	X	X	X	X

3.3. Pathogenicity Test of the Tested Isolates of X. c. pv. vesicatoria

Table (4) shows pathogenicity test of the 9 isolates of *X.c.*pv. *vesicatoria* on Balady pepper cv. Results indicate that *X.c.*pv. *vesicatoria* isolate No.3 of Kalubia governorate resulted in the highest incidence of the infection to the leaves of the inoculated pepper plants, being 51.7% as well as disease severity, being 12.9%. Meanwhile, isolate No.2 of Giza governorate resulted in the lowest incidence and severity of the infection, being 36.2 and 7.8%, respectively. The other isolates caused intermediate figures of incidence and severity of the disease. No infection was observed on the un-inoculated pepper plants.

Table 4. *Pathogenicty test of the 9 isolates of X.c.pv. vesicatoria on pepper plants (cv.Balady), greenhouse experiment.*

The tested isolates	% Disease incidence	% Disease severity
Giza-1	42.8	9.8
Giza-2	36.2	7.8
Kalubia-1	39.4	8.5
Kalubia-2	38.8	8.1
Kalubia-3	51.7	12.9
Kalubia-4	39.0	8.3
Behera-1	37.8	7.8
Behera-2	38.3	8.0
Behera-3	38.8	8.1

* No infection was observed on the un inoculated pepper plants.

3.4. Inhibitory Effect of Some Bioagents and Fungicides on the Causal Bacterium

The inhibitory effect of three bioagents and three fungicides on the causal bacterium is shown in Tables (5 and 6).

Results reveal that the three tested bioagents, *i.e. B. subtilis, P. fluorescens* and *P. putida* and fungicides, *i.e*. Efdal Pakirox, Raxil and Tango caused great inhibition to the causal bacterium comparison with the control treatment.

The bacterium *P. putida* was the most efficient bioagent followed by *P. fluorescens* and *B. subtilis* which were the lowest efficient, being 3.4, 2.9 and 1.9 mm., respectively (Table, 5).

Table 5. *Inhibition effect of three bioagents ($1x10^8$ cfu/ml) on X.c.pv. vesicatoria., two days after incubation at 28± 2º.*

Treatments	Inhibition zone (mm)
B. subtilis	1.9
P. fluorescens	2.9
P. putida	3.4
Control	0.0
L.S.D. at 5%	1.5

Table 6. *Inhibition effect of three fungicides at different conc. on growth of X.c. pv. vesicatoria, two days after incubation at 28± 2º.*

Treatments	Concentration (ppm)	Inhibition zone (mm)
Efdal Bakirox	100	1.9
	250	2.9
	500	3.8
Roxil	100	0.0
	250	0.2
	500	0.4
Tango	100	0.0
	250	0.0
	500	0.2
Control	0.0	0.0
L.S.D. at 5%		1.8

The fungicide Efdal Bakirox was the most efficient and superior one in inhibiting the causal bacterium more than the fungicides Roxil and Tango (Table, 6). In addition, the fungicide Tango failed to cause any inhibition to the causal bacterium at 100 and 250 ppm and caused low inhibition at 500 ppm. (0.2 mm). Also, the fungicide Roxil failed to cause any inhibition to the causal bacterium at 100 ppm. and caused low inhibition at 250 and 500 ppm., being 0.2 and 0.4 mm., respectively.

3.5. Effect of Three Bioagents and Three Fungicides on Management of Pepper Leaf Spot and the Produced Fruit Yield

Data of the field experiments on managing pepper leaf spot during 2014 and 2015 growing seasons are shown in Table (7).

Table (7) indicates that spraying pepper plant with the tested bioagents, i.e. *B.subtilis, P. fluoresence* and *P. putida* and fungicides Efdal Bakirox, Roxil and Tango four times resulted in significant reduction to the severity of the natural infection by bacterial spot caused by *X.c.*pv. *vesicatoria* with significant increase to the produced fruit yield compared with the control. In general, the tested fungicides were more efficient in this regard than the bioagents. In addition, Roxil was the most efficient treatment in reducing the severity of the disease (1.8%) and increasing the produced pod yield (22.4 kg./plot of 10.5 m^2). Meanwhile, the bioagent *B. subtilis* was the lowest efficient treatment, being 6.4% and 19.1 Kg., respectively. The other treatments recorded intermediate figures. The plants of the control recorded 23.1% and 17.3 Kg., respectively.

3.6. Biochemical Changes Associated with the Infection by Bacterial Leaf Spot

Data presented in Table (8) reveal that total phenols and vitamin-c content as well as the activity of chitinase, peroxidase and polyphenoloxidase in pepper leaves infected by *X.c.*pv. *vesicatoria* were greatly higher in the infected leaves compared with the uninfected leaves. In this respect, bioagent the *P. putida* resulted in the highest increase in the total phenols (245.33 ug GAE/g fresh weight), the content of vitamin-c (659.33 ug A.A./g fresh weight) and the activity of the enzyme chitinase (2123.67 ug NAGA x 10^3/min/g fresh weight) compared with the other treatments. Meanwhile, the fungicide Efdal Bakirox resulted in the lowest increase in the activity of the enzymes of peroxidase and polyphenoloxidase, being 571.33 and 617.0 ug NAGA x 10^3/min/g fresh weight, respectively compared with the other treatments. On the other hand, the fungicide Tango resulted in the highest increase in the total phenols (1256.67 ug GAE/g fresh weight), the activity of the enzyme chitinase (1238.33 ug NAGA x 10^3/min/g fresh weight) and the enzymes of polyphenoloxidase (292.0 ug NAGA x 10^3/min/g fresh weight) compared with the other treatments. The fungicide Roxil and the bioagent *B. subtilis* resulted in the highest increase in of vitamin-c (319.33 ug A.A./g fresh weight) and the activity of the enzyme polyphenoloxidase (292.0 ug NAGA x 10^3/min/g fresh weight). Uninfected plants by the causal bacterium recorded high figures for total phenols, vitamin-c and the three enzymes.

Table 7. *Effect of three bioagents and fungicides on management of pepper leaf spot and the produced fruit yield, field experiments at the experimental farm of Fac. Agric. at Moshtohor, Kalubia governorate during 2014 and 2015 growing seasons.*

Treatment	%, Disease severity during		Mean	Average weight of the pods / plot (10.5m^2)		Mean
	2014	2015		2014	2015	
B. subtilis	6.2	6.6	6.4	19.2	18.9	19.1
P. fluorescens	5.0	5.3	5.2	20.7	20.1	20.4
P. putida	5.0	5.5	5.3	21.5	20.7	21.1
Efdal Bakirox	3.0	3.2	3.1	22.3	21.1	21.7
Roxil	1.7	1.9	1.8	22.9	22.0	22.4
Tango	2.1	2.4	2.3	22.7	21.1	21.9
Control	23.8	24.3	23.1	17.5	17.0	17.3
Mean	6.7	7.0	---	20.4	18.6	---

LSD at 5% for: Treatments (T) = 2.1 1.9
Season(S) = n.s. 1.6
T X S = 2.8 3.1

Table 8. *Estimation of total phenols and vitamin-c content as well as the activity of chitinase, peroxidase and polyphenoloxidase in pepper leaves infected by X.c.pv. vesicatoria.*

Treatment	Total phenols (ug GAE/g fresh weight)	Vitamin C (ug A.A./g fresh weight)	Chitinase (ug NAGA x 10^3/min/g fresh weight)	Peroxidase (Δ_{405} O.D.x10^3/min/g fresh weight)	Polyphenoloxi-dase (O.D. unitsx10^3/min/g fresh weight)
B.subtilis	1930.67	467.33	1917.00	313.00	536.67
P.fluorescens	1759.67	500.67	1741.67	365.67	417.00
P. putida	2450.33	659.33	2123.67	495.67	542.67
Efdal Bakirox	2359.00	584.00	2100.00	571.33	617.00
Roxil	1554.00	319.33	1353.33	421.00	314.33
Tango	1256.67	431.33	1238.33	330.33	292.00
Control *	1207.78	298.65	1200.21	298.07	278.90
Control **	2510.67	672.00	2157.00	589.33	647.67

* Infected plants by the causal bacterium, ** Uninfected plants by the causal bacterium.

4. Discussion

Symptoms of pepper leaf spot caused by *Xanthomon campestres pv*. vesicatoria appear as water-soaked spots that change from green to dark-brown as infection progresses. The spots or lesions are often surrounded by yellow zones called halos. The size of lesions is fairly variable. On some cultivars, leaves may display several small lesions (0.25 to 0.5 cm) covering over 80% of leaf area, whereas on others fewer large lesions (larger than 0.5 cm) may be visible. In some cases a combination of small and large lesions may be found on the leaves. A general yellowing may occur on infected leaflets and often leads to premature defoliation. Stem lesions are narrow, elongated and raised expanding up to 6 mm. The lesions become light brown and rough in appearance. Fruit lesions are prominent beginning as green spots. As a spot enlarges, it becomes brown in color, and raised with cracked, roughened, wart-like appearance. The lesions range in size, up to 5 mm. in diameter. During periods of high moisture, fruit rot may develop around the lesions (Roberts *et al.,* 2004). Affected fruit may not be marketable. The disease agent survives on pepper seeds (Bashan *et al.,* 1982), volunteer plants, plant debris, and weeds as epiphytes, as well as on pepper leaves (Jones *et al.,* 1991).

Isolation trials from pepper leaves showing typical symptoms of bacterial spot collected from Giza, Kalubia and Behera governorates yielded 50 bacterial isolates. The

isolated bacteria was purified and identified as *Bacillus* spp., *Pseudomonas* spp., *P. fluorescens* and *Xanthomonas campestris* pv. *vesicatoria*. The frequency of the isolated bacteria was not greatly differed in the three governorates.

Identification of the isolated 9 bacterial strains proved to be *Xanthomonas campestris* pv. *vesicatoria*. Identification of the 9 strains was initially confirmed by morphological, biochemical and physiological tests as described by (Schaad *et al.,* 2001). The causal bacterium was isolated by may authors from pepper plants showing bacterial leaf spot (Bashan and Okon, 1986; Jones *et al.,* 1991; Mirik *et al.*,2007 and 2008 and Ju-Hee *et al*., 2015).

Results of pathogenicity test indicated that *X.c*.pv. *vesicatoria* isolate No.3 of Kalubia governorate resulted in the highest incidence and severity of the infection to the inoculated pepper plants meanwhile, isolate No.2 of Giza governorate resulted in the lowest incidence and severity of the infection. Mirik *et al.* (2007) reported that a total of 67 bacterial strains were isolated and purified. In pathogenicity tests, pepper plants inoculated with bacterial suspensions of the 67 strains and reference strains (GSPB 224) gave characteristic bacterial spot symptoms on pepper leaves in 7 to 14 days. No symptoms appeared on negative control plants. All strains were pathogenic on pepper plants cv. Bursa Yaglik. Re-isolations made from artificially infected plants yielded the bacterium originally inoculated.

X. c. pv. *vesicatoria* is widespread and damaging to pepper and tomatoes in field-grown crops in warm-temperate and tropical countries, especially under overhead irrigation. Losses of fruit yield are greatest when infection occurs early (Bashan and Okon, 1986). The fruits rarely show symptoms, but may drop if infected early. But, the damage of the leaves tends to expose fruits to the sun and increasing sunscald.

Field experiments during 2014 and 2015 growing seasons indicated that spraying pepper plant with the tested bioagents, *i.e. B.subtilis, P.fluoresence* and P.putida and fungicides Efdal Bakirox, Roxil and Tango four times resulted in significant reduction to the severity of the natural infection by bacterial spot caused by *X.c*.pv. *vesicatoria* with significant increase to the produced fruit yield compared with the control. In general, the tested fungicides were more efficient in this regard than the bioagents. In addition, Roxil was the most efficient treatment in reducing the severity of the disease and increasing the produced pod yield. Meanwhile, the bioagent *B. subtilis* was the lowest efficient treatment and the other treatments recorded intermediate figures.

Regarding bioagents, Byme *et al.* (2005) found that under field experiments the highest mean reductions in severity of bacterial spot on foliage, averaged across all locations, were provided by *P. syringae* Cit7 and *Pseudomonas putida* B56]. They added that unfortunately, neither the bacterial strains nor the standard copper bactericides consistently reduced disease incidence on fruit.

Mirik *et al.* (2008) reported that bacterial leaf spot development was decreased by 11-62 and 38-67% in pepper plants inoculated with the 3 *Bacillus* strains alone and in combination, respectively, in greenhouse and field experiments. Also, they found the *Bacillus* species were important agents for decreasing disease development through increasing biological activity They added that disease severity was decreased by *Bacillus* strains in the field experiments, where about 38-67% reduction in disease development was observed.

According to Salerno and Sagardoy (2003), it was found that *B. subtilis* 210 was resistant to rifampicin at 20 µg ml- 1 and showed the highest degree of antibiosis against *Xanthomonas campestris* pv. *glycines* under greenhouse conditions. Also, members of multiple *Bacillus* species are known as very efficient producers of antibiotic molecules. *B. subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). According to Salerno and Sagardoy (2003), it was found that *B. subtilis* 210 was resistant to rifampicin at 20 µg ml^{-1} and showed the highest degree of antibiosis against *Xanthomonas campestris* pv. *glycines* under greenhouse conditions.

Copper bactericides have provided control of copper-sensitive strains, although the presence of copper-tolerant strains makes control with copper compounds extremely difficult. Copper-tolerant strains are more effectively controlled by the combination of a copper bactericide and mancozeb or maneb (Stall *et al*., 1986).

Fixed copper is often in combination maneb and mancozebn have been the principle chemicals for managing bacterial spot caused by *X.c*.pv. *vesicatoria* on pepper (Mirik *et al.,*2007). Similar results were obtained by Marco and Stall(1983); Mc Carter (1992) and Ju-Hee *et al.*(2015).

Wilson *et al.* (2006) mentioned that foliar bacterial biological control agents and plant growth promoting rhizobacteria (PGPR) have been tested for control of bacterial spot of tomato. In field trials foliar biological control agents and PGPR strains controlled bacterial spot although they provided variable results. PGPR strains may induce plant resistance under field conditions, providing effective suppression of bacterial spot of tomato. As a result of that study, there may be some benefit for integrating rhizosphere-applied PGPR and foliar-applied biological control agents into a bacterial spot management program.

Ascorbic acid, or vitamin C, is the most common sulfite alternative. It acts as a reducing agent, preventing quinone accumulation by reducing it back to the original phenolic form before they are able to polymerize and form pigments (Queiroz *et al*., 2008).

It has been found that the obtained data revealed that total phenols and vitamin-c content as well as the activity of chitinase, peroxidase and polyphenoloxidase in pepper leaves infected by *X.c*.pv. *vesicatoria* and treated with the tested bioagents and fungicides were greatly higher than those of the infected plants and untreated with any treatment

The obtained data revealed that spraying of pepper plants with the tested bioagents and fungicides resulted in

considerable increased in the activity of chitinase, peroxidase and polyphenol oxidase. It is known that these enzymes can play an important role in plant defense mechanisms against the infection by plant pathogens. Data showed that the enzymatic activity in treated pepper plants was increased than those in the control. Many plant enzymes are involved in defense mechanisms against plant pathogens. Oxidative enzymes such as peroxidase and polyphenol oxidase enhance formation of lignin, while other oxidative phenols contribute in formation of defense barriers for reinforcing the cell structure (Avdiushko *et al*., 1993) In addition, experimentally supported the idea that peroxidase and chitinase play a defense role against invading pathogens (Yang *et al*., 2008 and Fan *et al*.,2016). Also, phenolic compounds are among the most influential and widely distributed secondary products in the plants. Such compounds govern disease resistance in many crop plants. There are many researches established that higher level of phenolic content was positively proportional to the degree of plant resistant against plant diseases.

Phenolic compounds are ubiquitous secondary metabolites in plants, which are crucial in many aspects of plant life, especially during their interactions with the environment (Lattanzio, 2013). Some phenolics play key roles in plant defense responses to pathogen or insect attacks.

References

[1] Abolmaaty, S. M. and Abd El-Ghafar, N. Y. (2010). The relationship between the incidence of bacterial spot disease of pepper and some environmental conditions. J. of Plant Protec. and Pathol., 1 (2): 103–110.

[2] Akram, W.; Mahboob A. and Javel A. A. (2013). *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against Fusarium wilt. Eur. J. of Mirobiol. and Immun., 275-280.

[3] Albersheim P. and Valent B. S. (1978). Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. J. Cell Biol., 78 (3): 627–643.

[4] Avdiushko, S. A.; Ye, X. S. and Kuc, J. (1993). Detection of several enzmetic activities in leaf prints of cucumber plants. Physiol. and Mol. Plant Pathol., 42: 441-454.

[5] A. O. A. C. (1975). Ascoric acid. In analysis of fruit and vegetable products. 94-101.

[6] Bade, M. L. and A. Stinson (1981). Biochemistry of insect differentiation. A system for studying the mechanism of chitinase activity *in vitro*. Archs Biochem. Biophyscs., 206: 213-221

[7] Bashan, Y.; Diab, S. and Okon, Y. (1982). Survival of *Xanthomonas campestris* pv. *vesicatoria* in pepper seeds and roots in symptomless and dry leaves in non-host plants and in the soil. Plant Soil, 68: 161-170.

[8] Bashan, Y. and Okon, Y. (1986). Internal and external infection of fruits and seeds of peppers by *Xanthomonas campestris* pv. *vesicatoria*. Canad. J. of Bot., 64, 2865-2871.

[9] Byme, J. M.; Cuppels, D. A.; Louws, F. J.; Miller, S. A.; Jones, J. B. and Wilson, M. (2005). Biological control of bacterial spot of tomato under field conditions at several locations in North America. Biol. Cont., 32 (3):408:418.

[10] Diab, S.; Henis, Y.; Okon, Y. and Bashan, Y. (1982). Effects of relative humidity on bacterial scab caused by *Xanthomonas campestris* pv. *vesicatoria* on pepper. Phytopathology, 72:1257-1260.

[11] Durrant, W. E. and Dong, X. (2004). Systemic acquired resistance. Ann. Rev. Phytopathol., 42, 185–209.

[12] Fan, S.; Tian, F. l.; Li, J.; Hutchins, W. I.; Chen, H.; Yang, F.; Yuan, F.; Cui, Z; Yang, Ching-Hong and He, C. (2016). Identification of phenolic compounds that suppress the virulence of *Xanthomonas oryzae* on rice via the type III secretion system. Mol. Plant Pathol., 9: 1-14, BSPP And John Wiley & Sons LTD.

[13] Fisher R. A. (1948). Statistical Methods 6th ed. Iowa State Univ. Press, Ames, Iowa, USA.

[14] Gore, J. P. and O'Garro, L. W. (1999). *Xanthomonas campestris* pv. *Vesicatoria* from bell pepper and tomato in Barbados undergoes changes in race structure, virulence and sensitivity to chemical control agents. J. of Phytopathol., 147, 397-402.

[15] Gupta, S. K. and Durga, P. (2011). Studies on bacterial spot of bell pepper caused by *Xanthomonas campestris* pv. *vesicatoria*. Plant Dis. Res., 26: 161.

[16] Holt, J. G. and Krieg, N. R. (1984). Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore, USA.

[17] Horsfall, J. G. and Barratt, R. W. (1945). An improved grading system for measuring plant diseases. Phytopathology, 35: 655.

[18] Ishaaya, I. (1971) In the armored scale *Aonidiella aurantii* and observation on the phenoloxidase system *Chrysomphalus aonidum.* Comp. Biochem. Physiol., 39 B,935-943

[19] Ishaaya, I. and. Casida, J. E. (1974). Dietary TH- 6040 alters composition and enzyme activity of housefly larval cuticle. Pestici. Biochem. Physiol., 4:484-490.

[20] Jones, J. B.; Jones, J. P..; Stall, R. E. and Zitter, T. A. (1991). Bacterial spot. in: Compendium of Tomato Disease. The American Phytopathological Society, St. Paul, Mensit National, 73 p.

[21] Ju-Hee, K.; Seong-Soo, C.; Ki-Kwon, L.; Ju-Rak, Y. and Wang-Hyu, L. (2015). Determination of economic control thresholds for bacterial spot on red pepper caused by *Xanthomonas campestris* pv. *vesicatoria*. Res. in Plant Dis., 21(2): 89-93.

[22] Lattanzio, V. (2013). Phenolic compounds: Introduction. In: Handbook of Natural Products (Ramawat, K. G. and Merillon, J. M., eds), pp. 1543–1580. Berlin, Heidelberg: Springer.

[23] Li, Y.; Peng, Q. L.; Selimi, D.; Wang, Q.; Charkowski, A. O.; Chen, X. and Yang, C. H. (2009). The plant phenolic compound p-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. Appl. Environ. Microb., 75:1223–1228.

[24] Li, Y.; Hutchins, W.; Wu, X.; Liang, C.; Zhang, C.; Yuan, X.; Khokhani, D.; Chen, X.; Che, Y.; Wang, Q. and Yang, C. H. (2015). Derivative of plant phenolic compound inhibits the type III secretion system of *Dickeya dadantii* via HrpX / HrpY two component signal transduction and Rsm systems. Mol. Plant Pathol. 16: 150–163.

[25] Marco, G. M. and Stall, R. E. (1983). Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Dis., 67: 779–781.

[26] Mc Carter, S. M. (1992). Effect of bactericide treatment on bacterial spot severity and yield of different pepper genotypes and on population of certain insects. Plant Dis., 76:1042-1045.

[27] Mirik, M.; Aysan, Y. and Cinar, O. (2007). Copper-resistant strains of *Xanthomonas axonopondis* pv. vesicatoria (Doidge) Dye in the eastern Mediterranean region of Turkey. J. of Plant Pathol., 89 (1), 153-154. 2

[28] Mirik, M.; Aysan, Y. and Cinar, O. (2008). Biological control of bacterial spot disease of pepper with *Bacillus* Strains. Turk. J. Agric., 32: 381-390.

[29] O'Garro, L. W. and Tudor, S. (1994). Contribution of four races of *Xanthomonas campestris* pv. *vesicatoria* to bacterial spot in Barbados. Plant Dis., 78 88-90.

[30] Parry, J. M.; Turnbull, P. C. B. and Gibson, J. R. (1983). A colour atlas of Bacillus species, Wolfe Medical Publications Ltd.

[31] Pernezny, K. and Collins, J. (1997) Epiphytic Populations of *Xanthomonas campestris* pv. *vesicatoria* on pepper: Relationships to host-plant resistance and exposure to copper sprays. Plant Dis., 791-794.

[32] Pernezny, K. L.; Roberts, P. D.; Murphy, J. F. and Goldberg, N. P. (2003). Compendium of Pepper Diseases. The American Phytopathological Society. St. Paul, MN, 73 p.

[33] Queiroz, C.; Lopes, M. L. M.; Fialho, E. and Valente-Mesquita, V. L. (2008). Polyphenol oxidase: characteristics and mechanisms of browning control. Food Reviews International, 24: 361-375.

[34] Roberts, P. D.; Adkins, S.; Pernezny3, K. and Jones, J. B. (2004). Diseases of Pepper and their Management. in: Diseases of Fruits and Vegetables. Kluwer Academic Publishers. Printed in the Netherlands, 333-387.

[35] Salerno, C. M. and Sagador, M. A. (2003). Short communication: Antagonistic activity by *Bacillus subtilis* againts *Xanthomonas campestris* pv. *glycines* under controlled condition. Spanish J. of Agric. Res., 1(2):55-58.

[36] Schaad, N. W.; Jones, J. B. and Lacy, G. H. (2001). Gram negative bacteria, Xanthomonas., In: Schaad N. W., Jones J. B., Chun W. (eds.). Laboratory Guide for Identification of Plant Pathogenic Bacteria, pp. 175-193. APS Press. St. Paul, Minnesota, USA.

[37] Snedecor G. W. and Cochran W. G. (1989). Statistical Methods. 8th Ed. Iowa State. Univ. Press, Ames, Iowa, USA.

[38] Stall, R. E.; Loschke, D. D. and Jones, J. B. (1986). Linkage of copper resistance and a virulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology, 76:240-243.

[39] Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol., 56 (4):. 845-857.

[40] Vetter, S. D. (1958), Quantitative determination of peroxidaese in sweet corn. Agric. and Food Chem., 6(1):39-41.

[41] Yang, S., Peng, Q., San Francisco, M., Wang, Y., Zeng, Q. and Yang, C. H. (2008) Type III secretion system genes of Dickeya dadantii 3937 are induced by plant phenolic acids. PLoS One, 3, e2973.

[42] Zieslin, N. and Ben-Zaken, R.(1993). Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. Plant Physiol. Biochem., 31:333–339.

[43] Ward, H. P. and O'Garro, L. W. (1992). Bacterial spot of pepper and tomato in Barbados. Plant Dis. 76, 1046–1048.

[44] Wilson, M.; Campbell, H. L.; Jones, J. B. and Cuppels, D. L (2006). Biological control of bacterial speck of tomato under field conditions at several locations in North America. Phytopathology, 92:1284-1292.