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Effects on Tomato Bacterial Canker of Resistance Inducers and Copper Compounds in Greenhouse

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ABSRACT

Bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* produces considerable economic losses in many countries because effective control measures are lacking. In this study, the effectiveness of some chemicals, a plant growth regulator (Prohexadione-Ca (PC)), two plant activators (hydrogen peroxide (HP)) and harpin protein (Hrp), fungicides, maneb+copper (MC), copper compounds (copper sulfate pentahydrate (CSP) copper hydroxide (CH) and copper oxychloride (CO)) and an antibiotic, streptomycin sulphate on the pathogen were assessed *in vitro* and *in vivo* on two tomato cultivars, Newton and Orient. In Hrp and HP treated plants, reduction in disease severity (about 45%) was correlated with suppression of bacterial growth during the time course of infection. The activities of POX, GPX and PAL enzymes were determined at 5th day as markers of resistance and applications of Hrp, PC and HP induced a progressive and significant increase of both enzymes in locally treated tissues. Only PC treatments have decreased plant heights and the effectiveness of PC in reduction of plant heigths was up to 40%. Among copper compounds, CSP and CO combination with Maneb were the most effective on the disease severity (about 50%). In addition, CSP combination with Hrp, HP and PC significantly limited the pathogen population *in vitro* and they showed considerable effects on the disease symptoms compared with CSP alone. We conclude that Hrp, HP and PC may be useful in controlling external symptoms of this disease in greenhouses, and is environmentally friendly, reducing the amount of copper applied to crops.

1. Introduction

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davis et al. (Cmm) is considered the most important bacterial disease affecting field and greenhouse tomato (*Lycopersicon esculentum*) production (OEPP / EPPO 2005), and is a quarantine organism in the European Union (EU) (Anonymous 2000).

Tomato is the most important host but in some cases natural infections have also been determined on capsicum, aubergine and several *Solanum* weeds. Infected seed is often considered to be the primary inoculum source (Thyr 1969) and the major source for outbreaks of Cmm infection (Tsiantos 1987). The pathogen can survive for several months in contaminated debris, alt-hough less in buried debris than in debris on the soil surface where decomposition is slower and interaction of other microorganisms is less (Gleason et al. 1991). Also persistence in dry conditions on equipment, boxes and glasshouse constructions is important for survival.

Seedlings infected with this seed-borne pathogen can become stunted plants that eventually succumb to the disease and soon die (Hausbeck et al. 2000). Systemic infection produces characteristic symptoms, like wilting of leaflets and cankers on stems and petioles. When the infection occurs after epiphytic spread of the pathogen, marginal necrosis of leaves and small white blister-like spots are common, and lesions, named bird's-eye spots, occasionally appear on fruits (Carlton et al. 1998). After this, the bacteria can penetrate the vascular tissues and plants develop systemic symptoms.

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Using of pathogen-free seeds and seedlings is one of the conventional control practices of the pathogen (Strider 1969; Thyr et al. 1973; Gleason et al. 1993). However, this measure does not always give satisfactory results, and the crop becomes infected with the bacteria. Disease control is difficult because of a lack of commercially acceptable resistant tomato cultivars. Studies focusing on Cmm chemical control are scarce and afford variable results. With respect to standard bactericides, secondary spread of the pathogen in the field can only be reduced by treating seedlings with streptomycin and copper compounds (Hausbeck et al. 2000; Werner et al. 2002). However, little information is available about their efficacy on greenhouse-cultivated tomatoes.

Due to the lack of publicly acceptable, effective, and non phytotoxic preparations to combat bacterial canker, there has been much interest in recent times in novel control strategies. This situation has directed so many researchers to control the disease in ecologically sound methods based on disturbing the host-pathogen relations. Plant activators and growth regulators are sought as the most promising chemicals. These chemicals for resistance induction (Soylu et al. 2003) and biocontrol employing different bacteria (El-Abyad et al. 1993; Boudyach et al. 2001; Umesha 2006) have also been proposed to improve control strategies.

The development of systemic acquired resistance (SAR) is associated with various cellular defence responses. These include synthesis of pathogenesis-related (PR) proteins and phytoalexins, accumulation of reactive oxygen species (ROS), rapid alterations in cell walls and enhanced activity of various defence-related enzymes (Ryals et al. 1996; Conrath et al. 2001). Harpin protein (Hrp) which is isolated from *E. amylovora* initiates a complex set of metabolic responses in the treated plant, causing natural gene expression and eliciting a plant's natural defense and growth systems. It binds to plant receptors, initiating a set of complex signaling pathways: activating a well-defined series of systemic acquired resistance (SAR) genes, inducing the jasmonic acid/ethylene dependent pathway and eliciting plant growth-related systems. These responses protect plants against a wide variety of pests on multiple crops, while at the same time improving growth, crop yield and quality (Jones 2001). Prohexadione-Ca (PC) is a growth retardant used to control the excessive vegetative growth and to improve fruit quality in apple, pear and cherry trees. PC inhibits the biosynthesis of the biological active gibberellins A1, A4, and A7 (Evans et al. 1997 and 1999; Rademacher 2000). These chemicals activate natural growth systems, improving crop yield, quality and food safety while simultaneously triggering defense systems to protect against diseases and some pest damages (Wei et al. 1992; Wei and Beer 1996; Momol et al. 1999; Jones 2001).

Hydrogen peroxide (H_2O_2) (HP) is primarily used as a topical disinfectant due to its ability to release a single oxygen molecule, which is very reactive. It is prefered

as alternative to chlorine based disinfectants and used as a disinfectant for greenhouse surfaces, equipment, benches, pots, trays and tools, as well as plants (Newman 2004).

Recent studies have confirmed that application of copper-based bactericides on tomato seedlings in the greenhouse reduce population sizes and spread of Cmm and impact plant development and yield in the field (Hausbeck et al. 2000; Werner et al. 2002).

Peroxidases have been implicated in a variety of defence-related processes, including the hypersensitive response, lignification, cross-linking of phenolics and glycoproteins, suberization and phytoalexin production (Nicholson and Hammerschmidt 1992; Wojtaszek 1997; Bestwick et al. 1998; Baysal et al. 2005). Phenylalanine ammonia-lyase (PAL) is one of the biochemical markers of induced resistance. It is considered to be the principal enzyme of the phenyl propanoid pathway, which is the prime intermediary in the biosynthesis of phenolics and flavonoids (Hahlbrock and Scheel 1989; Dixon and Lamb 1990). It catalyses the conversion of L phenylalanine to trans-cinammic acid in the first step of the phenylpropanoid pathway and regulates the production of precursors for lignin biosynthesis along with other phenolic protectants in plant cells. This pathway has been associated with the initiation of resistance in plants (Sticher et al. 1997; Baysal et al. 2005).

The objective of this study was to compare the effectiveness of copper-based compounds, streptomycin sulphate and the resistance inducers in reducing populations and symptoms of Cmm on different tomato cultivars *in vitro* and *in vivo*. Levels of defense-related enzymes in tomato tissue, POX, GPX and PAL were also assessed to determine possible relationships between the activation of these enzymes and the protection of seedlings following treatment with the resistance inducers.

2. Material and Method

2.1. Plant Material

The experiments were carried out with tomato cultivars, Orient and Newton, susceptible to bacterial canker. Plants were grown under controlled conditions (at 25 $^0C\pm5$ with 65-80% RH and under 12000-14000 Lux from tungsten-filament lamps for a 16-h photoperiod), in 20 cm pots in a soil mix containing sand, perlite, peat compost and a fertilizer (20-20-20, N-P-K) in the glasshouse. Plants were fertilized twice a week with 2.5 ml/L of NPK Fertilizer (Gubretas, Turkey) solution.

2.2. Bacterial Strains and Growth Conditions

The strains of *C. m.* subsp. *michiganensis*, CmmKD22 (from Selcuk University, Dept. of Plant Protection, Bacterial Culture Collection) and Cmmt4 (West Mediterranian Agricultural Research Institute), CMM3 (from Prof. H. Saygili, Aegean University) and cmmADM (from Prof. S. Maden, Ankara University) were tested and CMM3 was determined as the highest

virulent strain. The strain was used in all experiments and maintained on yeast–peptone–glucose agar (YPGA: yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; agar, 15 g; in 1 L of distilled water) and incubated at 25±1 ^{0}C. Yeast–peptone–glucose broth (YPGB: yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; in 1 L of distilled water) was used for liquid cultures.

2.3. *Chemical Compounds Used in the Experiments*

The chemical compounds used in the experiment were Prohexadione-Ca (PC), Harpin protein (Hrp), Hydrogen peroxide (HP), Copper sulphate pentahydrate (CSP), Copper hydroxide (CH), Copper oxychloride (CO), Maneb + Copper oxychloride (MC) and Streptomycin sulphate (Str). These compounds and their properties are shown in Table 1.

Table 1

Active ingredients, application rates, formulations of chemical compounds used in the experiments

Commercial Name and Firm	Active Ingradient and Percentage		Formulation	Application Rate (/ 100 L water)
BAS 125 10 W / BASF	Prohexadione-Ca	10%	WG*	125 g
Messenger / Eden Bioscience	Harpin protein	3%	Powder	50 g[a] + 20 ml adjuvant[b]
Herkul / Hektas Company	Maneb+Copper oxychloride	20% + 37,5%	Powder	400 g
Peras / Timsel Chem.	Hydrogen peroxide+Perasetic acid	260% + 90%	Liquid	500 ml
Mastercrop / Agrikem	Copper sulphate pentahyrate	65,82%	Liquid	150 ml
Champion / Nufarm Agr. Ltd	Copper hydroxide	77%	WP**	250 ml
Cupravit Ob21 / Bayer	Copper oxychloride	50%	WP	400 g
Streptomycine / I.E. Ulagay	Streptomycin sulphate	100%	Powder	59 g

* WG: wettable granule

**WP: wattable powder

[a] It was diluted with distilled water

[b] Non ionic adjuvant, KINETIC® was manufactured by Helena Chemical Company (225 Schilling Blvd. Collierville, TN 38017, USA)

Table 2

Application of the chemicals used in the experiments according to shoot length of plants

Chemicals	Shoot Lengths					Cmm Inoculation	1 day after the inoculation
	6-10 cm	12-15 cm	18-20 cm	22-25 cm	1 day before the inoculation		
Hrp[1]		+	+			+	
Hrp + CSP		+	+			+	
Hrp[2]		+	+				
PC[1]	+	+				+	
PC + CSP	+	+				+	
PC[2]	+	+					
HP[1]		+	+			+	
HP + CSP		+	+			+	
HP[2]		+	+				
MC[1]		+	+	+		+	
MC[2]		+	+	+			
CSP[1]		+	+	+		+	
CSP[2]		+	+	+			
CO[1]		+	+	+		+	
CO[2]		+	+	+			
CH[1]		+	+	+		+	
CH[2]		+	+	+			
Str[1]					+	+	+
Str[2]					+		+
Control[1]	+	+	+	+		+	
Control[2]	+	+	+	+			

[1] Chemical or water treatments + Cmm inoculation

[2] Chemical or water treatments

Hrp: Harpin protein, PC: Prohexadione-Ca, HP: Hydrogen peroxide, MC: Maneb+Copper oxychloride,

CSP; Copper sulphate pentahydrate, CO: Copper oxychloride, CH: Copper hydroxide, Str: Streptomycin sulphate

Cmm; *Clavibacter michiganensis* subsp. *michiganensis*

2.4. *Experimental Design*

The experiment was set up in a completely randomized block design with three replicates (Duzgunes et al. 1987). Tomato cultivars, Orient and Newton, were assigned in equal numbers (520 plants from each cultivar) to four treatment groups. Plants in the first group were treated with the chemicals and inoculated with Cmm. Plants in the second group were treated with water and inoculated with Cmm (control). Plants in the third and fourth groups were treated to effects of the chemicals but not inoculated with Cmm.

2.5. Applications of the Chemicals

Chemical application timing and schedule were based on Momol et al. (1999) (Table 2). Hrp and HP were applied when the shoots measured 12-15 cm and 18-20 cm. PC was applied twice when the shoots were 6-10 cm and 12-15 cm long. CSP, CH, CO and MC were applied three times when the shoots were 6-10 cm, 12-15 cm and 18-20 cm long. Streptomycin was applied twice, 1 d before and 1 d after inoculation. The chemicals were sprayed per treatment until runoff (approximately 300 ml), using a hand-held spray. After being sprayed, plants were allowed to dry until no liquid droplets were visible. Control plants were sprayed with the same volume of sterile tap water.

2.6. Inoculation of the Plants with Cmm

Bacterial cells were obtained from a 48 h culture at 25 ^{0}C in YPGB, after centrifugation at 2000 g for 5 min at 4 C. The pellet was rinsed twice in sterile distilled water and adjusted to OD640 nm 0.15 (10^8 CFU ml^{-1}) for inoculations. The plants, four to six fully expanded-leaf stage (8-week old), were inoculated with the virulent strain of CMM3 by pricking with an insulin needle and placing a drop (20 µl) of bacterial suspension in the node of the first true leaf with the stem. After inoculation, plants were covered with polyethylene bags for 5 d, and kept in the proportion of 80-90% humidity for 10 days plants and (Meier 2001).

2.7. Disease Assessment

The percentage of wilting leaves was calculated to assess disease severity. The level of resistance induced in seedlings against Cmm was evaluated at 25th day after inoculation using a 0–5 arbitrary scale (0, leaves showing no sign of wilting; 1, slight marginal wilting, 1–10% of leaves with wilt; 2, 11–25% of leaves with wilt; 3, sectored wilting, 26–49% of leaves showing wilting associated with chlorosis; 4, pronounced leaf collapse, 50–74% of leaves showing wilting; and 5, whole leaf wilted). A mean disease severity (%) (Anfoka 2000) and percent effectiveness of the chemicals according to Abbott formula (Anonymous, 1996) were calculated for each treatment from the scores of 30 plants (three replicates of 10 plants) for each treatment.

2.8. Re-isolation of the Pathogen from Diseased Tissues

For pathogen isolation, small pieces from the samples were soaked in 1ml of sterile water and streaked on YPGA plates, which were then incubated for 5 d at 25 ^{0}C. To confirm the identity of putative *C. m. subsp. michiganensis* colonies, randomly selected ones were tested by biochemical and physiological tests according to Schaad et al. (2001), and PCR assay using *pat*-1 gene spesific primers, CMM-5 (5′GCGAATAAGCCCA-TATCAA3′), CMM-6 (5′CGTCAGGAGGTCGCTA-ATA3′) developed by Dreier et al. (1995).

2.9. Determination of Bacterial Population in Tomato Tissues

Cmm was isolated from inoculated tissues, treated with the chemicals or water, by removing 5-mm-diameter leaf discs aseptically from the region of inoculation. Excised discs were homogenized in 1 mL of sterile 0,06% NaCl solution and each homogenate were subjected to 10-fold serial dilution. Aliquots of alternate dilutions (100 µl) were plated onto nutrient yeast agar (NYA) medium plates. Plates were incubated at 26 ^{0}C for 48 h, and emerging colonies were counted on all dilution plates showing bacterial growth. Samples collected from the untreated uninoculated control plants were also macerated and their serial dilutions were plated on to NYA medium.

2.9. Determination of Enzyme Activities

From leaves treated with the chemicals and inoculated with Cmm and, non-inoculated control plants, tissues were taken at the actual site of inoculation with Cmm and 5th day. To avoid possible side effects of leaf cutting, both cut edges (about 2 mm) of the leaf segments were removed and the adjacent tissue was immersed in liquid nitrogen. The frozen leaf segments were homogenized (1:5 w/v) in an ice-cold mortar using 50 mM potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1% polyvinylpyrrolidone, 1 mM EDTA and 10 mM b-mercaptoethanol. Thereafter, the homogenates were centrifuged at 17 000 g^{-1} for 20 min at 4 ^{0}C and finally, the supernatant (crude enzyme extract) was collected and divided into 1.5 ml portions. Enzyme extracts were stored at -20 ^{0}C until using.

Peroxidase, POX (EC 1.11.1.7), activity was determined from the crude extract according to Maehly and Chance (1954) using guaiacol as a common substrate for peroxidases. Enzyme activity was calculated from the change in absorbance and was expressed as A 480 mm tetraguaiacol produced (in mg protein^{-1} min^{-1}). Sodium acetate buffer was used as a blank.

Glutathione peroxidase, GPX (EC 1.11.1.9), activity was determined according to the method described by Kampranis et al. (2000) with cumene hydroperoxide as a substrate by monitoring the absorbance at 340 nm As above the GPX activity was measured as units of GPX mg of protein. Bovine erythrocyte GPX was used as a positive control.

Phenylalanine ammonia-lyase, PAL (EC 4.3.1.24) activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson et al. 1984). The amount of trans-cinnamic acid synthesized was calculated (Bradford 1976). Enzyme activity was expressed as nmol *trans*-cinnamic acid min mg protein^{-1}.

2.10. Statistical Analyses

Data obtained after inoculation were analysed using MINITAB Software, version 13 (Minitab Inc., State College, PA, USA). The means (expressed as percent

disease severity) were used to determine significant treatment differences. Data were analyzed using MSTAT software (Michigan State University, MI, USA) and the differences between treatments were determined by Duncan's New Multiple Range Test at p< 0.05.

The number of bacterial colonies per gram of plant material (CFU g^{-1} of plant tissue) was calculated, the mean values were log-transformed and separated in ANOVA by t-test (p<0.05).

3. Results

In present study, the effectiveness of plant growth regulator, Prohexadione-Ca, plant activators, Harpin protein and Hydrogen peroxide, and Copper compounds (copper sulfate pentahydrate, copper oxychloride, copper hydroxide and maneb+copper oxychloride) in comparison with streptomycin sulphate were determined *in vitro* and *in vivo* on two tomato cultivars 'Newton' and 'Orient'.

3.1. Bacterial Population in Plant Tissues

The population size of Cmm differed significantly from the untreated and Cmm inoculated positive control, but not in the negative control (uninoculated, untreated plants) (*p*<0,05). Obtaining to data, all chemicals prevented bacterial population compared to controls on plant tissues (Table 3). After streptomycin treatment (1,8 x 10^2 in cv. Newton and 0,6 x 10^3 in cv. Orient, Hrp (6,3 x 10^5 and 5,1 x 10^5) HP (4,2 x 10^5 and 5,6 x 10^6) and their combination with CSP had the best effects in reducing pathogen populations compairing to control plants (3,9 x 10^8 and 2,7 x 10^8) (Table 3). In general, copper compounds were less effective in limiting pathogen populations than the other treatments, primarily

CH and CO. Among alone copper compounds, CSP applications had the lowest Cmm population size in plant tissues (Table 3).

Table 3
Population of Cmm (CFU g^{-1} of plant tissue) on tomato cvs. Newton and Orient in 3rd day after inoculation (p<0.05)

Chemicals	Tomato Cultivars / Population Size (CFU g^{-1} of plant tissue)	
	cv. Newton	cv. Orient
Hrp	6.3±0.71 x 10^5 f*	5.1±1.98 x 10^5 f
Hrp+CSP	4.1±0.84 x 10^5 ef	3.7±3.69 x 10^5 ef
HP	4.2±1.93 x 10^5 ef	5.6±1.19 x 10^6 d
HP+CSP	2.1±2.60 x 10^5 e	3.4±1.62 x 10^5 ef
PC	2,7±1.44 x 10^6 c	2,3±1.66 x 10^6 c
PC+CSP	1.8±3.60 x 10^5 e	1.4±2.32 x 10^5 e
CSP	6.7±0.91 x 10^6 d	4.9±2.01 x 10^6 cd
MB+CO	2.2±0.47 x 10^6 c	3.8±0.47 x 10^6 c
CO	0.4±2.20 x 10^7 b	0.2±2.20 x 10^7 b
CH	1.1±0.73 x 10^7 b	1.3±0.18 x 10^7 b
Str	1.8±0.47 x 10^2 h	0.6±1.42 x 10^3 g
Control	3.9±3.60 x 10^8 a	2.7±0.83 x 10^8 a

Hrp: Harpin protein, CSP: Copper sulfate pentahydrate, PC: Prohexadione-Ca, HP: Hydrogen peroxide, MB+CO: Maneb+ Copper oxychloride, CO: Copper oxychloride, CH: Copper hydroxide, Str: Streptomycin, Control: water application to the plants
*In a column, values followed by the same letter are not significantly different (P < 0.05) as determined by Duncan's New Multiple Range Test.

3.2. Effects of the Chemicals on Plant Growing

Shoot lengths of tomato plants treated with PC were significantly shortened, measuring 21,25 cm and 23,12 cm in cvs. Newton and Orient, respectively, in comparison with the untreated control (38,77 cm and 40,14 cm in cvs. Newton and Orient, respectively) (p<0,05) (Table 4).

Table 4
Effectiveness of the chemicals on reducing plant heights of tomato cvs. Newton and Orient

Chemicals	Tomato Cultivars			
	cv. Newton		cv. Orient	
	Plant heights at 30th day after treatment (cm)	Efficacy of Chemical (%)	Plant heights at 30th day after treatment (cm)	Efficacy of Chemical (%)
PC	**21,25** c*	45,18	**23,12** c	42,40
Hrp	40,78 a	-	41,21 a	-
HP	39,01 ab	-	38,67 ab	3,66
MB+CO	38,73 ab	0,10	39, 26 ab	2,19
CO	37,23 b	3,97	38,53 ab	4,01
CSP	39,26 ab	-	39,76 ab	0,94
CH	38,93 ab	-	38,22 ab	4,78
Str	39,46 ab	-	40,22 a	-
Control	38,77 ab	-	40,14 a	-

Hrp: Harpin protein, CSP: Copper sulfate pentahydrate, PC: Prohexadione-Ca, HP: Hydrogen peroxide,
MB+CO: Maneb+ Copper oxychloride, CO: Copper oxychloride, CH: Copper hydroxide, Str: Streptomycin,
Control: water application to the plants
*In a column, values followed by the same letter are not significantly different (P < 0.05) as determined by Duncan's New Multiple
Range Test

3.3 Effects of the Chemicals on Disease Severity

Symptoms on tomato cultivars inoculated with Cmm included canker, wilt, leaf firing and eventually plant death. Resistance induced and growth regulated in tomato cultivars by Hrp, HP and PC are shown respectively in Table 5. Initial symptoms appeared on control plants as wilting along leaf margins. The progress of the disease in control plants increased with time and by 25 dai, most of the plant leaves developed severe wilting. The mean DS in control plants were about 70%. The most effective chemical Hrp (about 45%) and its mixing

with CSP (about 48%) were in all tomato cultivars. CSP mixed with Hrp significantly (37%) limited the disease severity compared with CSP alone, the other copper-based compounds. However, combining HP and PC with CSP did not contribute to its greater efficacy against the disease severity. Copper-based compounds, in general, were less effective in limiting pathogen population sizes than the other treatments, primarily copper oxychloride and the combination of copper hydroxide and mancozeb. Among copper compounds, CSP was the most prominent in reducing the disease severity (about 52%).

Table 5
Effects of the chemicals in the experiments used on the bacterial canker disease severities and enzyme activities of tomato cultivars against the disease

	Tomato Cultivars									
	cv. Newton					cv. Orient				
Chemicals	DS	Efficacy of Chemical (%)	Enzyme Activities**			DS	Efficacy of Chemical (%)	Enzyme Activities**		
			POX	GPX	PAL			POX	GPX	PAL
Hrp	40,4±0.8 f *	45,10	4.8 ± 2,0	5.2±0,4	5.3±0,9	39,3±1.4 f *	43,12	5.2 ± 1,4	4.8±1,1	6.4±1,3
Hrp+ CSP	37,8±1.2 g	48,64	5.3± 1,2	5.5±0,3	6.7±1,4	37,6±2.3 g	45,58	6.1± 2,6	5.7±0,9	6.6±0,7
HP	41,7±1.9 f	43,34	4.2±2,3	5.8±0,7	6.1±2,6	43,8±3.1 f	36,61	4.9±0,9	5.1±1,5	5.1±1,9
HP+ CSP	40,2±2.4 f	45,38	4.6±1.6	5.3±1,2	5.1±1,8	39,6±2.7 f	42,69	4.8±1,8	5.2±0,8	5.7±2,4
PC	51,3±0.6 de	30,29	4.1±2.8	4.2±1,4	4.2±0,6	50,9±1.6 de	26,33	3.6±0,7	3.8±2,1	5.2±2.1
PC+CSP	47,8±0.9 e	35,05	4.4±2.4	4.9±0,5	4.8±0,8	48,5±2.2 e	29,81	4.1±1,6	4.6±1,4	4.3±0,5
CSP	52,8±1.1 d	28,26	3.5±1,6	3.7±0,8	4.1±1,5	51,7±2.7 d	25,18	4.0±2.2	3.7±0,8	4.0±2.2
MB+CO	50,6±1.8 de	31,25	2.7± 2,1	4.1±0,6	3.9±1,7	51,2±1.3 de	25,90	3.9± 1,2	4.1±0,7	3.9±1.4
CO	57,3±0.7 c	22,14	2.0±2,7	3.2±2,1	3.4±1,9	54,9±1.9 cd	20,54	3.1±1,3	3.2±1.2	3.1±1.6
CH	61,4±2.3 b	16.57	2.6±1,3	2.7±1,7	2.9±2,1	60,7±2.6 b	12,15	2.2±0,8	2.1±0.6	1.8±1,3
Str	3,2±2.8 h	95,65	2.8±1,9	2.9±0.9	3.2±0,9	2,8±1.8 h	95,94	1,9±1,7	2.4±1,7	2.7±2.6
Control	73,6±1.7 a	-	2.4± 1,1	1.9±0,2	2.8±0,7	69,1±1.7 a	-	2.2± 0,9	2.1±1,6	2.8±1,6

Hrp: Harpin protein, CSP: Copper sulfate pentahydrate, PC: Prohexadione-Ca, HP: Hydrogen peroxide, MB+CO: Maneb+ Copper oxychloride, CO: Copper oxychloride, CH: Copper hydroxide, Str: Streptomycin, Control: water application + Cmm inoculation, DS: Disease Severity, POX: Peroxidase, GPX: Glutathione peroxidase, PAL: Phenylalanine ammonia-lyase
*In a column, values followed by the same letter are not significantly different (P < 0.05) as determined by Duncan's New Multiple Range Test.
** Enzym activities were determined in mg protein^{-1} min^{-1}

3.4. Re-isolation of C. m. subsp. michiganensis from Tomatoes

Cmm was easily isolated in samples obtained from symptomatic leaves and cankers. Obtaining pure colonies on GYPA medium were Gram positive and, oxidative metabolism of glucose; catalase and aesculin hydrolysis were positive; oxidase negative; acid produced aerobically from mannose, but not from mannitol; sodium acetate and sodium succinate used as carbon sources; growth in presence of 6% NaCl; potato starch hydrolysed. H$_2$S produced from peptone. The identity of strains isolated from diseased tomato plants was confirmed using the PCR protocol and primer set, CMM-5 and CMM-6, designed by (Dreier et al. 1995). PCR products of expected size (614-bp) for Cmm were amplified from all investigated strains. Based on these characteristics, the isolated all strains were identified as *C. m.* subsp. *michiganensis*.

3.5. POX, GPX and PAL enzyme activities in Tomato Cultivars

In tomato plants treated with Hrp, HP, PC the expression of induced resistance was associated with enhanced POX, GPX and PAL activities (Table 5). The seedling treated with these chemicals and their combinations with CSP challenged at 5th day and they had significantly higher levels of POX, GPX and PAL activities than the plants from the other treatments. Peroxidase activity respectively Hrp application 4.8-5.2 mg ml^{-1} min^{-1}, Hrp+CSP applications 5.3-6.1 mg ml^{-1} min^{-1}, HP applications 4.2-4.9 mg ml^{-1} min^{-1}, HP+CSP 4.6-4.8 mg ml^{-1} min^{-1}, PC applications 4.1-3.6 mg ml^{-1} min^{-1}, PC+CSP applications 4.4-4.1 mg ml^{-1} min^{-1}, CSP applications 3.5-4.0 mg ml^{-1} min^{-1} were found only in control given this rate 2.4-2.2 mg ml^{-1} min^{-1} in cvs. Newton and Orient, respectively (p <0.05). GPX and PAL activities showed similar results in all applications.

4. Discussion

Chemical activation of disease resistance in plants represents an additional option for growers to protect their crops from losses due to plant diseases. Against some pathogens, it may be the best option for chemical control where genetic resistance is not available or not sufficient. Improvement of resistance in plants pretreated with some synthetic chemicals, such as salicylic acid and harpin protein are a well-known phenomenon called SAR (Ryals et al. 1996; Wallad and Goodman 2004). Induction of SAR by Hrp has been reported in many plants against a broad spectrum of fungal, viral and bacterial pathogens (Anonymous 2002; Fontanilla et al. 2005; Jones 2001; Momol et al. 1999; Wei and Beer 1996; Bastas and Maden 2004; Bastas et al. 2010). The harpin has been extensively tested on more than 40 crop groupings plus turf and ornamentals throughout the world. It has been shown to be effective on a wide variety of economically important crops, such as cotton, wheat, cucumber, citrus, tobacco, strawberry, tomato and peppers (Jones 2001).

Unlike traditional chemical pesticides, Hrp and PC do not kill or otherwise adversely affect pests or pathogens, and hence it does not exert the selection pressure that promotes the development of resistance in pest populations, thus reducing the likelihood of resistance or cross-resistance development. Harpin is ideally suited to controlling pests that have developed resistance to conventional chemical treatments and to being used as a partner with highly pest-specific, lower risk products. Resistance induced by SAR agents requires an induction period that is an interval of time between application of the agent and the challenge from the pathogen. The timing of chemical sprays during the period of host susceptibility to infection should be the main concern of growers and advisers, since it is very important for the efficacy of sprays and the optimization of applications. Baysal et al. (2003) reported that acibenzolar S methyl treatment three days prior to inoculation had the best effect on reducing Cmm population, and that the later application did not show better results in disease management. This may result in saving for the grower as well as in preventing environmental problems. Even so it would be worthwhile to determine the optimum time of application.

PC has been affected on different rosaceous plants in the control of fire blight disease (Evans et al. 1997; Greene 1999; Rademacher and Kober 2003; Yoder et al. 1999). This reduction is attributed to the inhibition of biological active GAs (Costa and Rademacher 2006). It is observed an increment in lycopene in fruits from PC plants. The values of lycopene are also higher as the concentration of PC increased (Nunez et al. 2005). The content of vitamin C is also increased with PC at any evaluated concentration in fruits of tomato plants (Costa and Rademacher 2006). Vitamin C as an antioxidant is a compound which plays an important role in detoxification of activated oxygen and reacts directly with reactive oxygen molecules (Padayatt et al. 2001). Therefore, the increase in vitamin C by this growth retardant offers a good alternative for tomato in modern horticulture (Ramireza et al. 2012).

The increased production of both the superoxide radical and H_2O_2 is a common feature of defense responses in plants to challenge by avirulent pathogens and elicitors (Lamb and Dixon 1997). There is enough evidence indicating that H_2O_2 performs several important functions in disease resistance (Mehdy et al. 1996). H_2O_2 has been implicated not only in triggering hypersensitive cell death, but also in limiting the spread of cell death by inducing cell protectant genes in surrounding cells (Levine et al. 1994; Newman 2004). In our experiments, disease severity and Cmm population in leaves of plants treated with H_2O_2 was considerably lower than in those not treated. There was no apparently phytotoxic effect on plants at the dose applied.

In this study, streptomycin sulphate was effective in preventing wilting and canker symptoms of the disease on tomato and this antibiotic was included in our trials only as a standard antibiotic. Regardless of the excellent effects of streptomycin, application of this compound is banned in most European countries and the USA in greenhouse conditions. The use of this antibiotic should be limited to high disease pressure conditions.

Application of intensive copper treatments to commercial crops over many years can lead to copper accumulation in soils, with subsequent negative effects on plants and the environment (Ninot et al. 2002). Furthermore, copper tolerance of plant-pathogenic bacteria seems to have increased since the 1980s (Andersen et al. 1991; Scheck et al. 1996; Sholberg et al. 2001). The application of copper compounds on tomatoes in the greenhouse reduces population sizes and spread of Cmm (Hausbeck et al. 2000; Werner et al. 2002). Products containing copper have been reported to significantly reduce foliar blight and/or fruit spotting produced by this pathogen (Gleason et al. 1993).

According to the findings of Werner et al. (2002), mixing copper hydroxide and mancozeb did not prevent the spread of the pathogen, although in some earlier studies (Marco 1983; Jones et al. 1991a and 1991b; Hausbeck et al. 2000) it was reported that a combination of these two compounds showed better effects in reducing populations of bacterial pathogens. Baysal et al. (2003) have also shown positive results with acibenzolar S methyl alone or combined with copper hydroxide for management of bacterial canker in greenhouse tomato seedlings. Our results show that copper compounds alone may be useful as 'protective' to prevent the pathogen spreading, or even as a post-infection treatment to reduce the risk of new external infections in greenhouse cultivated tomatoes. However, CSP combined with PC and HP, except Hrp, were not more effective statistically than CSP, CH and CO alone treatments in reducing symptoms in plants inoculated with Cmm.

Obtaining moderately disease control by various chemicals can be attributed to artificial inoculation by hypodermic injection, use of high inoculum's density and susceptible host plants in the experiments. Better results might be obtained in the natural infections. Besides continuation of the treatments with this kind of chemicals after inoculation should be considered. These positive effects should be further tested under natural conditions in the fields and greenhouses. It is important to mention that these treatments that interfere with host susceptibility (growth regulation and/ or SAR) should be applied some weeks before the real infections occur. This allows the plants to switch on their natural defense system in time. It will be required to find the right strategy for the applications of these compounds in different areas.

Hrp, HP and PC applications should be seen as a complementary action in the whole process of bacterial canker control measures. These chemicals could provide a new approach of control of the disease but its practical use needs further investigation. When these situations are taken into consideration, improved performance may occur in natural infections with repeated applications during the growing season. Therefore, repeated applications should be considered in situations where disease epidemics are anticipated.

POX plays several important roles in the disease resistance expressed against a number of pathogens and is responsible for the generation of reactive oxygen species (Wojtaszek 1997). Increases in POX activity are often associated with the progressive incorporation of phenolic compounds within the cell wall during incompatible plant-microbe/elicitor interactions. In tomato, POX is one of the enzymes believed to catalyse the last step in lignification (Brisson et al. 1994). The association between resistances induced by POX, GPX and PAL and increased deposition of cell wall phenolics and enhanced enzyme activity of peroxidase was apparent in other pathosystems (Brisset et al. 2000; Resende et al. 2002). Development of induced resistance in plants is also associated with the coordinate expression of a complex set of PR proteins, so-called 'SAR genes' (Conrath et al. 2001).

It was found a correlation between increases of PAL and POX concentrations in plants and the reduction of disease in H_2O_2, PC and Hrp treated plants. In plants treated with Hrp, PC and HP, activities of POX, GPX and PAL were determined as markers of resistance. Applications of Hrp, PC and HP induced a progressive and significant increase of both enzymes in locally treated tissues. Such responses were expressed earlier and with a much higher magnitude when POX, GPX and PAL treated seedlings were challenged with the pathogen, thus providing support to the concept that a signal produced by the pathogen is essential for triggering enhanced synthesis and accumulation of these enzymes. Therefore, the slower symptom development and reduction in bacterial growth in POX, GPX and PAL treated

plants might be due to the increase in activity of both oxidative and antioxidative protection systems in planta. In several studies, GPX was shown to increase in plants under stress conditions, such as exposure to HP, salt and pathogen infection (Levine et al. (1994). In addition, the increment in POX and GPX activities is observed in PC treated in tomato plants at 125 mg L^{-1}. This chemical promotes peroxidase synthesis, perhaps linked to the production of antioxidants (Ramireza et al. 2006). PC treatment to tomato reduces plant height and increases yield. PC provokes increments in lycopene, vitamin C and capsaicin in fruits. These effects seem to be related to an increase in catalase and peroxidase activity during fruit maturity (Ramireza et al. 2012).

In plant health management programs, plant activators and growth regulators can be useful on plant health and growth. The current study assessed the effect of two plant activators, Hrp and HP, a growth regulator, PC, and copper compounds on the disease development by Cmm. The low disease control obtained from treatments with Hrp, HP and PC can be attributed to the inoculation method, high inoculation density, and host/cultivar susceptibility. Although not more effective than streptomycin they should have a role in Integrated Pest Management as they can reduce the applications of streptomycin and thus lessen the possibility of development of streptomycin-resistant strains. Unlike Hrp and HP, the height of plants treated with PC significantly was less than untreated plants. It is concluded that Hrp, HP and PC seem to be efficient resistance inducers of several plant defense mechanisms and may be useful in controlling external symptoms of this disease in greenhouses, and is environmentally friendly, reducing the amount of copper applied to tomatoes.

5. References

Anonymous (1996). Standard pesticide experiment methods to fire blight disease [*E. amylovora* (Burr.) Winslow et al.] on pears, quinces and apples. Standard Pesticide Experiment Methods for Agricultural Control. Vol. 2, Plant Diseases. TAGEM, Ankara. 261 p.

Anonymous (2000). Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the community of organisms harmful to plants or plant products and against their spread within the community. Office for Official Publications of the European Communities. Consolidated Text, pp. 1–39.

Bastas KK, Maden S (2004). Control of Fire Blight (*Erwinia amylovora* (Burr.) Winslow et al.) by Prohexadione-Ca (BAS 125 10 W) and Benzothiadiazole + Metalaxyl (BION MX 44 WG). *Selcuk University Journal of Agriculture Faculty* 18 (33): 49 – 58.

Bastas KK, Maden S, Katırcioglu YZ, BoyrazN (2010). Determining of Application Time for Chemical Control of Fire Blight Disease in Pear Varieties. *Journal*

of Agricultural Sciences Ankara University Faculty of Agriculture 16 (3): 150-161.

Baysal O, Gursoy YZ, Ornek H, Duru A (2005). Induction of oxidants in tomato leaves treated with DL-*b*-Amino butyric acid (BABA) and infected with Clavibacter michiganensis ssp. Michiganensis. *European Journal of Plant Pathology* 112: 361–369.

Bestwick CS, Brown IR, Mansfield JW (1998). Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a non-host hypersensitive reaction inlettuce. *Plant Physiology* 118:1067 – 1078.

Boudyach EH, Fatmi M, Akhayat O, Benizri E, Ait Ben Aoumar A (2001). Selection of antagonistic bacteria of *Clavibacter michiganensis* subsp. *michiganensis* and evaluation of their efficiency against bacterial canker of tomato. *Biocontrol Science and Technology* 11:141–149.

Bradford M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Brisson LF, Tenhaken R, Lamb CJ (1994). Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *Plant Cell* 6: 1703–1712.

Byers RE, Yoder KS (1997).The effect of Bas 125 W on apple tree growth, fruit quality and fire blight suppression. *HortScience* 32(3): 557

Carlton WM, Braun EJ, Gleason ML (1998). Ingress of *Clavibacter michiganensis* subsp. *michiganensis* into tomato leaves through hydathodes. *Phytopathology* 88: 525–529.

Costa G, Rademacher W (2006). Prohexadione-Ca: More than a growth regulator for pome fruit trees. *Acta Horticulturae* 727:107-116.

Dixon RA, Lamb CJ. (1990). Molecular communication in interaction between plants and microbial pathogens. *Annual Review of Plant Physiology and Plant Molecular Biology* 41: 339-367.

Dreier J, Bermpohl A, Eichenlaub R (1995). Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology* 85:462–468.

El-Abyad MS, El-Sayed MA, El-Shanshoury AR, El-Sabbagh SM (1993). Towards the biological control of fungal and bacterial diseases of tomato using antagonistic Streptomyces spp. *Plant Soil* 149:185–195.

Evans JR, Evans R R, Regusci CL, Rademacher W (1999). Mode of action, metabolism and uptake of BAS 125W, prohexadione-calcium. *HortScience* 34 (7): 1200-1201.

Evans RR, JR Evans, Rademacher W (1997). Prohexadione calcium for suppression of vegetative growth in eastern apples. *Acta Horticulturae* 451: 663-666.

Gleason ML, Braun EJ, Carlton WM, Peterson RH (1991). Survival and Dissemination of *Clavibacter michiganensis* subsp. *michiganensis* in Tomatoes. *Phytopathology* 81(12): 1519-1523.

Gleason ML, Gitaitis RD, Ricker MD (1993). Recent Progress in Understanding and Controlling Bacterial Cancer of Tomato in Eastern North America. *Plant Disease* 77(11): 1069-1076.

Hahlbrock K, Scheel D (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology Plant Molecular Biology* 40:347–369.

Hausbeck MK, Bell J, Medina-Mora CM, Podolsky R, Fulbright DW (2000). Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganensis* on tomatoes in the greenhouse and on disease development and crop yield in the field. *Phytopathology* 90: 38–44.

Jones J (2001). Harpin, Biopesticides, Pesticide Outlook – August 2001The Royal Society of Chemistry DOI: 10.1039/b106934a.

Lamb CJ, Dixon RA (1997). The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology Plant Molecular Biology* 48: 251–275.

Levine A, Tenhaken R, Dixon R, Lamb C (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Plant Cell* 79: 583–593.

Maehly AC, Chance B (1954). The assay of catalases and peroxidases, 1: 357-424. In D. Glick (Ed.), Methods of Biochemical Analysis. Interscience Publishers, Inc.

Mehdy MC, Sharma YK, Sathasivan K and Bays NW (1996). The role of activated oxygen species in plant disease resistance. *Plant Physiology* 365–374.

Momol MT, Ugine JD, Norelli JL, Aldwinckle HS (1999).The effect of prohexadione-Ca SAR inducers and calcium on the control of shoot blight caused by *E. amylovora* on apple. *Acta Horticulturae* 489: 601-605.

Newman SE, (2004). Disinfecting Irrigation Water for Disease Management 20th Annual Conference on Pest Management on Ornamentals February 20-22, 2004. *Society of American Florists* San Jose, California, 1-10.

Nicholson RL, Hammerschmidt R (1992). Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology* 30: 369–389.

Nunez NE, García N, Medina MG, Miranda R, Rodriguez A, Hernandez D (2005). Efecto sobre el contenido de licopeno de tomate (*Lycopersicon esculentum* var. Gironda) sembrado en invernadero bajo diferentes sistemas de cultivo con y sin injerto. *VII Congreso Nacional de Ciencia de los Alimentos y III Foro Nacional de Ciencia y Tecnología de Alimentos*, p.340-345.

OEPP/EPPO, 2005. *Clavibacter michiganensis* subsp. *michiganensis*. *Bull. OEPP-EPPO Bull*. 35, 275–283.

Padayatt SJ, Daruwala R, Wang Y, Eck PK, Song J, Koh WS, Levine M (2001). Vitamin C: From molecular actions to optimum intake, p.117-145. In: E. Cadenzad and I. Packer (eds.), Handbook of Antioxidants., 2nd ed. Washington. CRC Press.

Rademacher W (2000). Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. *Annual Review of Plant Physiology Plant Molecular Biology* 51: 501-531.

Rademacher W (2004). Prohexadione-Ca induces resistance to fire blight and other diseases. *EPPO Bull*. 34: 379-384.

Ramireza H, Rancaño AJH, Benavides MA, Mendoza VR, Padron CE (2006). Influencia de promotores de oxidación controlada en hortalizas y su relación con antioxidantes. *Journal Revista Chapingo Serie Horticultura* 12(2): 189-195.

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996). Systemic acquired resistance. *Plant Cell* 8: 1809–19.

Sherf BA, Bajar AM, Kollattukudy PE (1993). Abolution of an inducible highly anionic peroxidase activity in transgenic tomato. *Plant Phsiology* 101:201-208.

Soylu S, Baysal O, Soylu EM (2003). Induction of disease resistance by the plant activator, acibenzolar-S-methyl (ASM), against bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) in tomato seedlings. *Plant Science* 165: 1069–1075.

Sticher L, Mauch-Mani B, Metraux JP (1997). Systemic acquired resistance. *Annual Reiew of Plant Pathology* 35: 235-270.

Strider DL (1969). Bacterial canker of tomato caused by Corynebacterium michiganense. A literature review and bibliography. *N. C. Agric. Exp. Snt. Tech. Bull*. 193.

Thyr BD (1969). Assaying Tomato Seed for *Corynebacterium michiganense*. *Plant Dis. Reptr*. 53: 858-860.

Thyr BD, Webb RE, Jaworski CA, Ratcliffe TJ (1973). Tomato bacterial canker: control by seed treatment. *Plant Disease Reporter* 57: 974–977.

Tsiantos J (1987). Transmission of bacterium *Corynebacterium michiganense* pv. *michiganense* by seeds. *Journal of Phytopathology* 119: 142–6.

Umesha S (2006). Occurrence of bacterial canker in tomato fields of Karnataka and effect of biological seed treatment on disease incidence. *Crop Protection* 25: 375–381.

Umesha S (2006). Occurrence of bacterial canker in tomato fields of Karnataka and effect of biological seed treatment on disease incidence. *Crop Protection* 25: 375–381.

Wei ZM, Beer SV (1996). Harpin from Erwinia amylovora induces plant resistance. *Acta Horticulturae* 411: 223-225.

Werner NA, Fulbright DW, Podolsky R, Bell J, Hausbeck MK (2002). Limiting populations and spread of *Clavibacter michiganensis* subsp. *michiganensis* on seedling tomatoes in the greenhouse. *Plant Disease* 86: 535–542.

Wojtaszek P (1997). The oxidative burst: A plant's early response against infection. *Biochemistry Journal* 322: 681–692.