

# Effects of Copper-based Compounds, Antibiotics and a Plant Activator on Population Sizes and Spread of *Clavibacter michiganensis* subsp. *michiganensis* in Greenhouse Tomato Seedlings

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## SUMMARY

Three copper-based compounds (copper hydroxide, copper oxychloride, copper sulphate), two antibiotics (streptomycin and kasugamycin) and a plant activator (ASM) significantly reduced population sizes and spread of *C. michiganensis* subsp. *michiganensis* among tomato seedlings in the greenhouse. Streptomycin had the best effect in reducing pathogen population size in all sampling regions. Moreover, this antibiotic completely stopped the spread of *C. michiganensis* subsp. *michiganensis* in the region most distant from the inoculum focus. Copper hydroxide mixed with streptomycin significantly limited the pathogen population, compared with copper hydroxide alone, the other copper-based compounds, ASM and kasugamycin. However, combining streptomycin with copper hydroxide did not contribute to its greater efficacy against the pathogen population. Copper-based compounds, in general, were less effective in limiting pathogen population sizes than the other treatments in all three sampling regions, primarily copper oxychloride and the combination of copper hydroxide and mancozeb. Among copper compounds, copper hydroxide was the most prominent in reducing the bacterial population, especially in the region closest to the inoculum focus, while its combination with mancozeb did not improve the effects. Kasugamycin significantly limited pathogen population size, compared to copper bactericides, but it was less effective than the other antibiotic compound, i.e. streptomycin. The plant activator ASM significantly reduced population density, and it was more effective when used three days prior to inoculation than six days before inoculation.

**Keywords:** Bacterial canker; Tomato; Copper-hydroxide; Copper-oxychloride, Copper-sulphate; Streptomycin, Kasugamycin, Acibenzolar-S-methyl

## INTRODUCTION

Tomato transplants for use in greenhouse or field commercial production in Serbia are usually grown in local greenhouses. However, symptomless greenhouse transplants may serve as a reservoir of populations of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker. These latent infections can result in plant wilting, stunting, reduced yields, and eventually plant death. Preventive cultural management recommendations include the use of certified seeds and healthy transplants, greenhouse disinfection, plant debris removal or plowdown and rotation with non-solanaceous plants for at least two years (Gleason et al., 1993). Resistant tomato cultivars represent the most efficient control measure against *C. michiganensis* subsp. *michiganensis*. Sources of resistance to the bacterial canker pathogen have been found in several wild relatives of *Lycopersicon esculentum*: *Lycopersicon peruvianum*, *L. hirsutum* and *L. pimpinellifolium* (Berry et al., 1989; Francis et al. 2001; Kabelka et al., 2002). In spite of the fact that numerous moderately resistant cultivars are present on the market (Poysa, 1993), currently there are no commercially available highly resistant tomato cultivars or hybrids (De Vries and Stephens, 1997).

In regard to chemical control, growers have limited options in managing bacterial canker in the field (Hausbeck et al., 2000). Some growers apply bactericides preventively while others introduce applications after symptoms have appeared. The use of bactericides ordinarily includes multiple applications of copper compounds, either alone or mixed with mancozeb (Gleason et al., 1993; Hausbeck et al., 2000), every seven to ten days. Shoemaker (1992) reported that applications of copper compounds or their mixtures with the protective fungicide mancozeb in the field every five to seven days were able to reduce less severe canker symptoms and fruit spotting in North Carolina. Recent studies have confirmed that application of copper-based bactericides on tomato seedlings in the greenhouse reduce population sizes and spread of *C. michiganensis* subsp. *michiganensis* and impact plant development and yield in the field (Hausbeck et al., 2000; Werner et al., 2002). However, long term use of these bactericides may lead to occurrences of copper-resistant bacterial strains (Cooksey, 1990). In addition, disease control is feasible by application of antibiotics such as streptomycin, although their use is forbidden in most countries (Baysal et al., 2005). In spite of the fact that *C. michiganensis* subsp. *michiganensis* strains resistant to copper compounds and anti-

biotics have not yet been reported, disease management programs that are not wholly reliant on copper compounds are desirable (Werner et al., 2002).

One of the potential methods of reducing the severity of disease caused by the pathogen is an induction of plant resistance. Improvement of resistance in plants pretreated with avirulent pathogens (biotic inducers) is a well-known phenomenon called systemic acquired resistance (SAR) (Ryals et al., 1996; Wallad and Goodman, 2004).

In addition to biotic inducers, SAR may be induced by some synthetic chemicals, such as salicylic acid, potassium salts, amino butyric acid and the recently discovered benzothiadiazole derivative - acibenzolar-S-methyl (ASM). Induction of SAR by ASM has been reported in many plants against a broad spectrum of fungal, viral and bacterial pathogens (Siegrist et al., 1997; Cole, 1999; Godard et al., 1999; Ishii et al., 1999; Anfoka, 2000; Brisset et al., 2000). Recent studies of the efficacy of ASM in limiting populations and spread of *C. michiganensis* subsp. *michiganensis* have shown that this compound may be helpful in disease management of tomato bacterial canker, especially when combined with copper-hydroxide and resistant cultivars (Werner et al., 2002). In similar experiments, Baysal et al. (2003) reported a reduction in disease severity (up to 76.3%) in plants pretreated with ASM, and enhancement of resistance of ASM-treated plants associated with significant increases in the activities of POX and chitinase. More recent investigations of resistance induced by DL- $\beta$ -amino butyric acid against *C. michiganensis* subsp. *michiganensis* have shown suppression of disease development of up to 54% (Baysal et al., 2005).

Another management option may be available by the application of biocontrol agents such as avirulent strains of bacterial pathogens or antagonistic microorganisms (Wilson et al., 2002). *Pseudomonas fluorescens* is one of the most important antagonists of certain fungi and bacteria, and numerous greenhouse studies have been conducted to show its efficacy in the control of several plant diseases (Parke et al., 1991; Kraus and Loper, 1992; Umeha et al., 1998). Results of a novel study of Umeha (2006) showed that biological seed treatment with the antagonistic *P. fluorescens* strain drastically reduced bacterial canker incidence in the field. The objective of this study was to compare the effectiveness of copper-based compounds, antibiotics and the plant activator ASM in reducing populations and spread of *C. michiganensis* subsp. *michiganensis* among greenhouse tomato seedlings.

## MATERIAL AND METHODS

### Plant material

Tomato seedlings (cv. Saint Pierre) in the second true leaf stage were used for all experiments. Plants were grown in a growth chamber in sterile plant growing substrate "B medium course" (Floragard, Germany) in plastic plug sheets with 102 cells in total at 26°C and 60% relative humidity. Each treatment was applied in a separate plug sheet with three replicates per treatment. Plants were not additionally fertilized.

### Bacterial strain and inoculum preparation

The P-8 strain of *C. michiganensis* subsp. *michiganensis* was maintained on Nutrient agar (NA) at 4°C. Inoculum was prepared from the bacterial strain grown on Nutrient Broth Yeast extract agar (NBY) at 26°C for 48h and suspended in sterile distilled water. Inoculum concentration was adjusted to approximately 10<sup>8</sup> cfu/ml using McFarland's scale and confirmed by plate count technique on NA after 72 h (Klement et al., 1990). Tomato seedlings in the second true leaf stage, which were to be inoculated, were removed and sprayed with bacterial suspension (10<sup>8</sup> cfu/ml) using a hand-sprayer. The first true leaf of each individual seedling was then removed by clipping the petiole next to the stem with scissors dipped in the same bacterial suspension. Inoculated seedlings were covered with plastic bags and incubated overnight in a separate growth chamber at 26°C.

### Application of bactericides and ASM

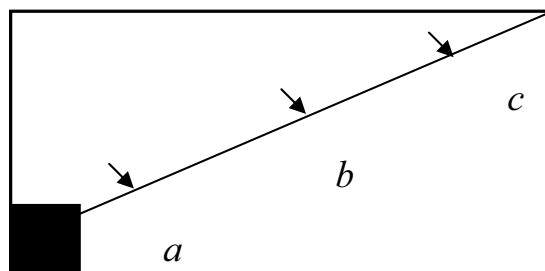
Active ingredients, product trade names and applied concentrations are shown in table 1. Bactericide sprays were initiated two hours before inoculation and subsequent sprays were applied every seven days until sam-

pling. Acibenzolar-S-methyl was introduced three days prior to inoculation in one treatment, and six days before inoculation in the second treatment. Subsequent ASM sprays were applied every seven days until sampling. All treatments were applied using a hand sprayer. Tomato seedlings sprayed with water prior to inoculation served as a positive control, while untreated uninoculated plants were used as negative control.

To initiate the infection, two seedlings were removed from each treatment from one side of the plug sheet and replaced with the same number of previously inoculated seedlings in order to establish inoculum focus (Figure 1).

### Sampling of tomato seedlings

The sampling of seedlings for estimation of population densities was conducted five weeks (35 days) after the introduction of inoculated plants. Two plants were removed from each sampling region (*a*, *b* and *c*) following the diagonal from inoculum focus (Figure 1). The inoculated plants used to initiate disease within each treatment were not included in the foliar samples since they were dead by the time of sampling. Samples from each region were stored individually in plastic bags and processed separately. One gram of plant material from each sample (stems and leaves) was macerated in 2 ml



**Figure 1.** Sampling design – seedlings were sampled following the diagonal from the inoculum focus (regions *a* to *c*)

**Table 1.** Investigated treatments

Active ingredients (a. i.)	Product trade names	Applied concentrations (a.i. %)
Copper-hydroxide	Blauvit	0.3
Copper-hydroxide + Mancozeb	Blauvit + Dithane	0.3 + 0.3
Copper-oxchloride	Cuprozin 35 WP	0.3
Tribasic copper-sulphate	Cuproxat	0.35
Streptomycin sulphate	Agri-mycin 17	0.025
Streptomycin sulphate + Copper-hydroxide	Agri-mycin 17 + Blauvit	0.025 + 0.3
Kasugamycin	Kasumin	1.25
Acibenzolar-S-methyl	Bion	0.02

0.05 M phosphate buffer amended with Tween-20 detergent (0.02%). Each homogenate was subjected to a 10-fold serial dilution and the diluted samples spread on NBY medium. Population densities were estimated based on the number of bacterial colonies that grew from the serial dilutions on NBY medium. The number of bacterial colonies per gram of plant material (cfu/g) was calculated, the mean values were log-transformed and separated in ANOVA by  $t$ -test ( $P < 0.05$ ).

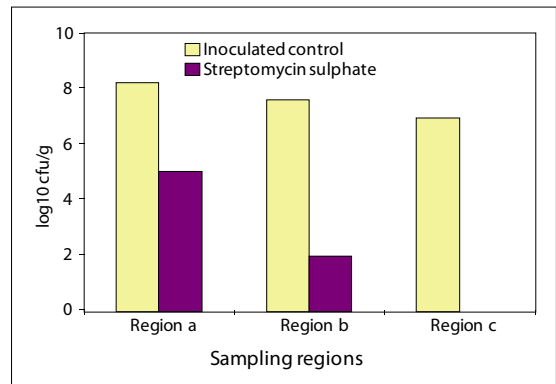
Samples collected from the untreated uninoculated control plants were also macerated and their serial dilutions were plated on to NBY medium.

## RESULTS

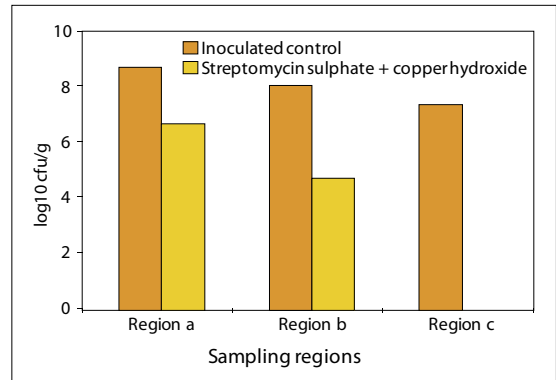
Symptoms on seedlings that were directly inoculated with bacterial suspension and introduced into treatment plug sheets to initiate the disease, included canker, wilt, leaf firing and eventually plant death. Populations of *C. michiganensis* subsp. *michiganensis* were detected in all inoculated treatments, but not in the negative control (uninoculated, untreated plants). Pathogen population density in all treatments differed significantly from the untreated, inoculated positive control (Figures 2-10).

Streptomycin had the best effect in reducing pathogen populations in all sampling regions (*a*, *b* and *c*). Moreover, this antibiotic completely stopped the spread of *C. michiganensis* subsp. *michiganensis* in the most distant region (region *c*) (Figure 2). Copper hydroxide mixed with streptomycin significantly limited pathogen populations, compared with the copper hydroxide treatment alone, other copper compounds, ASM and kasugamycin. However, combining streptomycin with copper hydroxide did not contribute to its higher effectiveness against pathogen populations. On the contrary, it was less effective than streptomycin alone, but still significantly limited bacterial populations compared to other treatments (Figure 3).

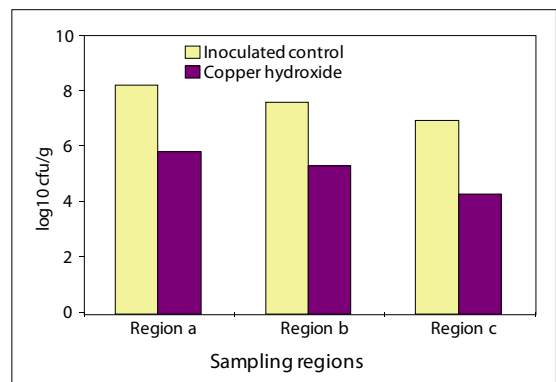
In general, copper compounds were less effective in limiting pathogen populations than the other treatments in all three sampling regions, primarily copper oxychloride and the combination of copper hydroxide and mancozeb. Among copper compounds, copper hydroxide showed the highest effectiveness in reducing bacterial population, especially in region *a* (closest to the inoculum focus), while its combination with mancozeb did not improve the effects. Kasugamycin significantly limited pathogen populations, compared



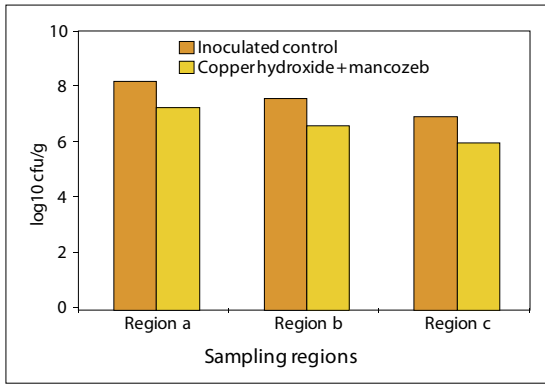
**Figure 2.** Population size of *C. michiganensis* subsp. *michiganensis* (log<sub>10</sub>cfu/g) in inoculated control and streptomycin sulphate treatment



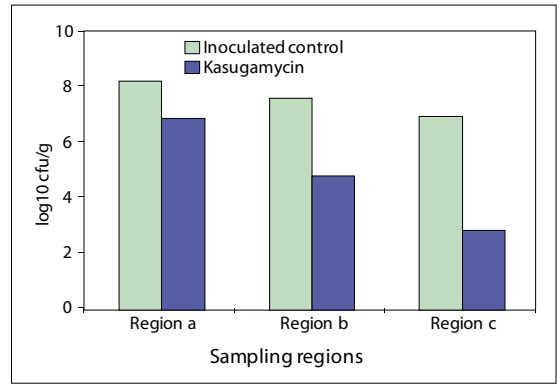
**Figure 3.** Population size of *C. michiganensis* subsp. *michiganensis* (log<sub>10</sub>cfu/g) in inoculated control and streptomycin sulphate + copper hydroxide treatment



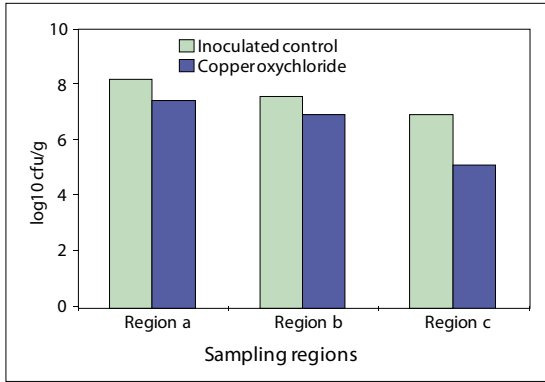
**Figure 4.** Population size of *C. michiganensis* subsp. *michiganensis* (log<sub>10</sub>cfu/g) in inoculated control and copper hydroxide treatment



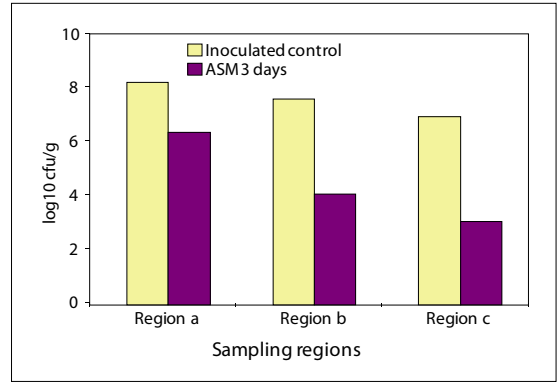
**Figure 5.** Population size of *C. michiganensis* subsp. *michiganensis* ( $\log_{10}$ cfu/g) in inoculated control and copper hydroxide + mancozeb treatment



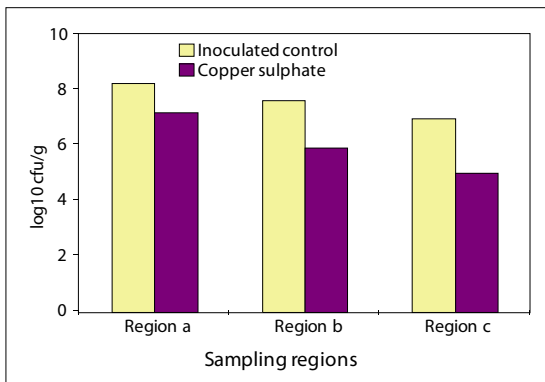
**Figure 8.** Population size of *C. michiganensis* subsp. *michiganensis* ( $\log_{10}$ cfu/g) in inoculated control and kasugamycin treatment



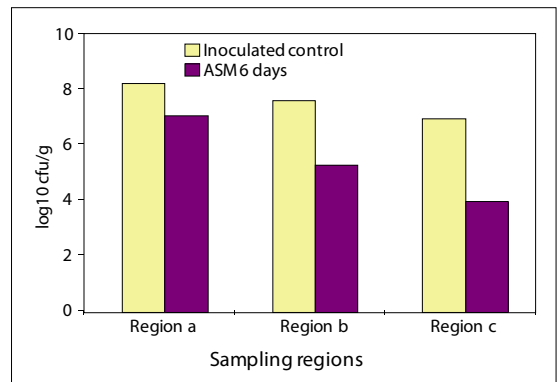
**Figure 6.** Population size of *C. michiganensis* subsp. *michiganensis* ( $\log_{10}$ cfu/g) in inoculated control and copper oxychloride treatment



**Figure 9.** Population size of *C. michiganensis* subsp. *michiganensis* ( $\log_{10}$ cfu/g) in inoculated control and ASM treatment applied 3 days prior to inoculation



**Figure 7.** Population size of *C. michiganensis* subsp. *michiganensis* ( $\log_{10}$ cfu/g) in inoculated control and copper sulphate treatment



**Figure 10.** Population size of *C. michiganensis* subsp. *michiganensis* ( $\log_{10}$ cfu/g) in inoculated control and ASM treatment applied 6 days prior to inoculation

with copper compounds, but it was less effective than the other antibiotic compound, i.e. streptomycin. The plant activator ASM significantly reduced population density, particularly in region *b*, and it was more effective when used three days than six days prior to inoculation (Table 2).

## DISCUSSION

Long-term and multiple applications of copper-based bactericides may lead to occurrences of bacterial strains resistant to copper compounds (Cooksey, 1990). There are numerous reports on this subject, regarding bacterial diseases of tomatoes, yet most of them concern the other two devastating tomato bacterial pathogens, *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* strains (Marco and Stall, 1983; Adaskaveg and Hine, 1985; Ritchie and Dittapongpitch, 1991; Pernezny et al., 1995). Although *C. michiganensis* subsp. *michiganensis* strains resistant to copper compounds have not been reported yet, which was also confirmed in our previous studies (Milijašević, 2008), bacterial canker management programs that are not entirely reliant on copper compounds are favored (Werner et al., 2002).

The results from this study indicated that all treatments significantly limited populations of *C. michiganensis* subsp. *michiganensis* among tomato seedlings in the greenhouse, compared with the untreated, inoculated control. Significant differences between treatments were also defined.

In spite of the fact that in our study streptomycin was the most effective in limiting populations of *C. michiganensis* subsp. *michiganensis* in all three sampling regions (*a*, *b* and *c*), and also completely stopped pathogen spread in the most distant region, confirming the findings of Hausbeck et al. (2000) and Werner et al. (2002), this antibiotic was included in our trials only as a standard. However, regardless of the excellent effects of streptomycin, application of this compound is banned in most European countries, including Serbia, and it is forbidden even in the USA in greenhouse conditions and therefore cannot be recommended for use.

Although less effective than streptomycin, the other antibiotic, kasugamycin, was more effective in population reduction than the copper compounds. These satisfying effects of kasugamycin, especially in the region with the lowest population density, make this antibiotic a good alternative for streptomycin in cases of early disease diagnosis.

This study verified the findings of Werner et al. (2002) that premixing copper hydroxide and mancoz-

**Table 2.** *C. michiganensis* subsp. *michiganensis* population density (cfu/g) in treatments, untreated inoculated control (K+) and untreated uninoculated control (K-)

Region a		Region b		Region c	
Treatment	(cfu/g)	Treatment	(cfu/g)	Treatment	(cfu/g)
STR	2,2 x 10 <sup>5</sup> <i>i</i>	STR	1,3 x 10 <sup>2</sup> <i>b</i>	STR	0,0 <i>i</i>
STR+Cu(OH) <sub>2</sub>	0,5 x 10 <sup>7</sup> <i>b</i>	ASM 3	2,3 x 10 <sup>4</sup> <i>g</i>	STR+Cu(OH) <sub>2</sub>	0,0 <i>i</i>
Cu(OH) <sub>2</sub>	1,6 x 10 <sup>6</sup> <i>g</i>	STR+Cu(OH) <sub>2</sub>	5,4 x 10 <sup>4</sup> <i>f</i>	KAS	1,1 x 10 <sup>3</sup> <i>b</i>
ASM 3	6,0 x 10 <sup>6</sup> <i>f</i>	KAS	1,3 x 10 <sup>5</sup> <i>e</i>	ASM 3	1,9 x 10 <sup>3</sup> <i>g</i>
ASM 6	3,1 x 10 <sup>7</sup> <i>e</i>	ASM 6	4,2 x 10 <sup>5</sup> <i>d</i>	ASM 6	1,7 x 10 <sup>4</sup> <i>f</i>
KAS	2,0 x 10 <sup>7</sup> <i>e</i>	Cu(OH) <sub>2</sub>	4,5 x 10 <sup>5</sup> <i>d</i>	Cu(OH) <sub>2</sub>	3,8 x 10 <sup>4</sup> <i>e</i>
CuSO <sub>4</sub>	4,2 x 10 <sup>7</sup> <i>d</i>	CuSO <sub>4</sub>	1,9 x 10 <sup>6</sup> <i>c</i>	CuSO <sub>4</sub>	2,1 x 10 <sup>5</sup> <i>d</i>
Cu(OH) <sub>2</sub> +Mn	5,3 x 10 <sup>7</sup> <i>c</i>	Cu(OH) <sub>2</sub> +Mn	1,1 x 10 <sup>7</sup> <i>bc</i>	CuOCl	2,9 x 10 <sup>5</sup> <i>c</i>
CuOCl	8,1 x 10 <sup>7</sup> <i>b</i>	CuOCl	2,4 x 10 <sup>7</sup> <i>b</i>	Cu(OH) <sub>2</sub> +Mn	2,4 x 10 <sup>6</sup> <i>b</i>
K+	5,5 x 10 <sup>8</sup> <i>a</i>	K+	1,2 x 10 <sup>8</sup> <i>a</i>	K+	2,4 x 10 <sup>7</sup> <i>a</i>
K-	0	K-	0	K-	0

The values in columns followed by different letters differ significantly (t-test, p<0,05)

STR - Streptomycin sulphate; STR+Cu(OH)<sub>2</sub> - Streptomycin sulphate + Copper hydroxide; Cu(OH)<sub>2</sub> - Copper hydroxide;

ASM 3 - Acibenzolar-S-methyl applied 3 days prior to inoculation; ASM 6 - Acibenzolar-S-methyl applied 6 days prior to inoculation;

KAS - Kasugamycin; CuSO<sub>4</sub> - Tribasic copper sulphate; Cu(OH)<sub>2</sub>+Mn - Copper hydroxide + Mancozeb; CuOCl - Copper oxychloride;

K+ - untreated inoculated control; K- - untreated uninoculated control

eb did not prevent the spread of the pathogen, although in some earlier studies (Marco, 1983; Jones et al., 1991) it was reported that a combination of these two compounds showed better effects in reducing populations of bacterial pathogens.

Considering that ASM significantly reduced populations of *C. michiganensis* subsp. *michiganensis*, the results of this study indicate that ASM may be helpful in disease management. Other studies have also shown positive results with ASM alone or combined with copper hydroxide for management of bacterial canker in greenhouse tomato seedlings (Werner et al., 2002; Baysal et al., 2003). Induction of resistance by the plant activator ASM requires the application of SAR inducers at a certain interval before the pathogen challenge. In most cases this interval was reported to last 1-7 days (Baysal et al., 2003). According to Siegrist et al. (1997), a minimum interval period of 96 h was necessary for ASM to induce resistance on bean leaves against fungal or bacterial pathogens. Similarly, Baysal et al. (2003) reported that ASM treatment three days prior to inoculation had the best effect on reducing *C. michiganensis* subsp. *michiganensis* population, and that the later application did not show better results in disease management. The results of our study showed that ASM was more effective when applied three than six days prior to inoculation, thereby verifying the findings of Baysal et al. (2003).

In conclusion, the results of this study implied that bacterial canker control strategies should focus primarily on greenhouses in order to prevent severe bacterial canker incidence and losses in the field.

## ACKNOWLEDGEMENT

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# Efekti bakarnih jedinjenja, antibiotika i aktivatora otpornosti biljaka na populaciju i širenje *Clavibacter michiganensis* subsp. *michiganensis* na rasadu paradajza u zaštićenom prostoru

## REZIME

Jedinjenja na bazi bakra (bakar-hidroksid, bakar-oksihlorid, bakar-sulfat trobazni), antibiotici (streptomycin i kasugamicin) i aktivator otpornosti biljaka (acibenzolar-S-metil, ASM) značajno su redukovali veličinu populacije i širenje *C. michiganensis* subsp. *michiganensis* na rasadu paradajza u zaštićenom prostoru. Streptomycin je imao najbolji efekat u smanjenju populacije patogena u svim regionima uzorkovanja. Takođe, ovaj antibiotik je potpuno zaustavio širenje *C. michiganensis* subsp. *michiganensis* u regionu najudaljenijem od žarišta inokuluma. Mešavina bakar-hidroksida i streptomicina je značajno smanjila populaciju patogena u poređenju sa tretmanom bakar-hidroksidom primenjenim samostalno, drugim bakarnim jedinjenjima, acibenzolar-S-metilom i kasugamicinom. Međutim, kombinovanje streptomicina sa bakar-hidroksidom nije doprinelo poboljšanju njegovog efekta na smanjenje populacije patogena. Bakarni preparati su imali najslabiji efekat u smanjenju populacije patogena na sva tri mesta uzorkovanja, što se pre svega odnosi na bakar-oksihlorid i kombinaciju bakar-hidroksida sa mankozebom. Među bakarnim jedinjenjima najbolji efekat imao je bakar-hidroksid, posebno u regionu najbližem žarištu inokuluma, dok njegova kombinacija sa mankozebom nije poboljšala efekat. Kasugamicin je bio efikasniji od bakarnih jedinjenja, ali znatno slabiji u smanjenju populacije bakterije od drugog antibiotika-streptomicina. Aktivator otpornosti ASM je ispoljio značajan efekat u smanjenju zaraze, pri čemu je bolji efekat ovog jedinjenja ostvaren primenom tri dana pre inokulacije u odnosu na primenu šest dana pre inokulacije.

**Ključne reči:** Bakteriozni rak; paradajz; bakar-hidroksid; bakar-oksihlorid; bakar-sulfat trobazni; streptomycin; kasugamicin; acibenzolar-S-metil