Population Dynamics of *Xanthomonas campestris* pv. *vesicatoria* on Tomato Leaflets Treated with Copper Bactericides

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Florida Agricultural Research Station Journal Series No. R-00132.
Accepted for publication 26 February 1991 (submitted for electronic processing).

ABSTRACT

Jones, J. B., Woltz, S. S., Jones, J. P., and Portier, K. L. 1991. Population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets treated with copper bactericides. Phytopathology 81:714-719.

Populations of copper-resistant (Cu^r) strains of *Xanthomonas campestris* pv. *vesicatoria* were monitored in the field on nonsymptomatic tomato leaflets treated with copper or with a copper and mancozeb mixture over three and four seasons, respectively. Copper and a combination of copper and mancozeb reduced epiphytic populations of *X. c. vesicatoria*, compared to those in the untreated control. Populations of *X. c. vesicatoria* on leaflets receiving copper and the copper-mancozeb combination differed significantly in only one of three seasons. A positive correlation was observed between epiphytic populations and disease severity. In a greenhouse study, where a Cu^r strain of *X. c. vesicatoria* was applied to tomato foliage, bacterial populations were significantly less on plants treated with copper or with a copper and mancozeb mixture than on untreated plants. However, leaflets treated with the copper and mancozeb combination had significantly lower Cu^r populations than leaflets treated with copper alone. In another study, where a Cu^r and copper-sensitive (Cu^s) strain were separately inoculated on treated plants, disease severity was significantly reduced by both copper treatments compared to that of the control. However, there were no significant differences in disease severity between the two copper treatments when plants were inoculated with either a Cu^r or Cu^s strain. Ionic and total soluble copper in dew collected from bactericide-treated leaves were not significantly different between the two copper treatments. Although ionic and soluble copper may be factors in toxicity to Cu^r strains, they do not appear to be the primary components involved in the toxicity of the copper and mancozeb mixture to Cu^r strains of *X. c. vesicatoria.*

Xanthomonas campestris pv. *vesicatoria*, causal agent of bacterial leaf spot of tomato and pepper, is a major problem in Florida. This disease is difficult to control when both high temperatures and high moisture exist. Bactericides, primarily fixed coppers and streptomycin, have provided the major means of control (16,21). Streptomycin became less effective with the development of strains resistant to this antibiotic (21). Because streptomycin-resistant strains are rapidly selected on streptomycin-treated plants, emphasis has been placed on copper compounds for effective control (4,11).

Copper compounds have proven effective for controlling bacterial diseases (11,17,18). For many years in Florida, copper was shown to be more efficacious when applied in combination with mancozeb than when applied alone (4). In two in vitro studies, strains of *X. c. vesicatoria* did not appear to be sensitive to copper when exposed on agar plates to disks soaked in cupric hydroxide (1) or in solutions produced by equilibrating cupric hydroxide with water (16). These strains were determined to be copper-resistant (Cu^r). In field studies, copper applied alone was ineffective, whereas a copper and mancozeb mixture was effective for controlling bacterial spot of pepper where Cu^r strains of *X. c. vesicatoria* were used (16). The Cu^r strain, which is more prevalent than the copper-sensitive strain (Cu^s) in Florida (16), was determined to be sensitive to copper and mancozeb combinations both in vitro and in vivo. However, in field studies on tomato, copper and mancozeb combinations were no more effective than copper alone for controlling bacterial leaf spot (J. B. Jones, J. P. Jones, and S. S. Woltz, *unpublished data*).

This study was undertaken to determine the effects of copper alone and in combination with mancozeb on the population dynamics of Cu^r strains of *X. c. vesicatoria* on tomato leaflets, the relationship between epiphytic populations of Cu^r strains of *X. c. vesicatoria* and severity of bacterial spot, and the ionic and copper composition of bactericide-treated plants and its effect on populations of a Cu^r and Cu^s strain of *X. c. vesicatoria.*

MATERIALS AND METHODS

Strains and inoculum. Four Cu^r strains and one Cu^s strain of *X. c. vesicatoria* were used in this study. Sensitivity to copper was determined by growth on nutrient agar amended with $CuSO_4$ (20). These strains were grown on medium B of King et al (12) for 48 h at 28 C. Bacteria were washed from the agar plates and suspended in 0.01 M $MgSO_4 \cdot 7H_2O$. Bacterial concentration was adjusted to 10 colony-forming units (cfu) ml^{-1} by adjusting the suspension to an optical density of 0.05 at 600 nm with a spectrophotometer. Plants in the field were inoculated early in the morning with Cu^r strains when dew was still present on the foliage by misting inoculum on the top surface of all plants. In greenhouse studies, plants were inoculated on both leaf surfaces with either a Cu^r or Cu^s strain using an aerosol sprayer to generate the mist.

Field plots. Tomato transplants of the cultivar Sunny were set into raised beds covered with plastic mulch. Beds were 71 cm wide and spaced 1.4 m apart from center to center. Plants were set 46 cm apart in the rows. Each plot contained 12 plants, which were spaced 1.37 m apart. A randomized complete block design with four blocks was used in all field experiments.

Bactericide application and field inoculation. In field studies, copper hydroxide alone or in combination with mancozeb was applied with a hand-held CO_2 stainless steel sprayer (Andrews and Tate Assoc., Holly Hill, FL) pressurized to 2.8 kg cm^2 during application. Sprays began 7 days after transplanting and were applied twice weekly throughout the season. Copper hydroxide was applied alone at the rate of 2.2 kg ha^{-1} ; mancozeb was applied at 1.7 kg ha^{-1}; and copper and mancozeb at 2.2 and 1.7 kg ha, respectively, were mixed before application. Control plots received no bactericide application. Field plots were inoculated 3–4 days after the first bactericide application with a suspension of four Cu^r strains of *X. c. vesicatoria.*

Leaf sampling and assay procedure. Five asymptomatic leaflets collected from recently matured leaves on five plants were harvested in each plot every 7 days. Each leaflet was placed in an individual polyethylene bag and brought to the laboratory. Leaflets were weighed, placed in separate 125-ml Erlenmeyer flasks containing 10 ml of peptone buffer (17) per gram of tissue. Flasks were shaken on a rotary shaker at 150 rpm for 30 min. Serial 10-fold dilutions were made in 0.005 M phosphate buffer containing 0.1% NaCl. A 100-μl aliquot of each dilution was plated onto each of three plates of Tween A medium (17). After incubation at 28 C for 4–5 days, colonies typical of *X. c. vesicatoria* were counted. Samples were processed within an hour after sampling.

Statistical analyses were performed on data without transformation or after transformation of individual leaflet populations by the square root or $\log_{10}$. Transformed or unaltered values were analyzed using the Shapiro-Wilk test to determine whether transformed or unaltered data best fit a normal distribution. The method of handling the data to best fit a normal distribution was used to process the data. Population differences were determined over time by analysis of variance using orthoganal contrast comparisons.

In order to determine if a relationship existed over time between epiphytic populations and disease severity, correlation coefficients were determined for these two parameters. Populations within each plot at each sampling date were correlated with disease severity in the same plot at each of the sampling dates. Each correlation coefficient represents the value of all plots.

Greenhouse studies. Tomato plants (5- to 6-wk-old) of cultivar Walter were grown in a soilless medium in pots 10 cm in diameter. The plants were treated with copper, copper in combination with mancozeb, or mancozeb alone at the rates previously described. Control plants were not treated. Plants were misted with 10^8 cfu ml^{-1} of a Cu^r strain 24 h after application of bactericides. Plants were placed in a growth room at 28 C at a relative humidity of greater than 90%. At 1, 4, 24, and 48 h after inoculation, three recently matured leaflets were removed from each of three plants in each replication from Cu-inoculated plants. Leaflets in each replication were weighed and processed to determine epiphytic populations of *X. c. vesicatoria* as described above. On a separate group of plants, which were treated with a Cu^r and Cu^s strain, the same procedure was used, but the plants were not sampled for epiphytic populations; lesions were enumerated 21 days after inoculation. Lesion numbers were transformed by $\log_{10}$ transformation before analysis. Both experiments were completely randomized block designs with five replications. The experiment on epiphytic populations was repeated three times. Data were analyzed over time and compared by contrast comparisons.

Copper determination. Greenhouse-grown tomato plants of the cultivar Walter were sprayed with copper hydroxide alone or copper hydroxide applied in combination with mancozeb, or were left unsprayed. Twenty-four hours after the sprays had dried, the plants were placed in a dew chamber (Percival, Inc., Boone, IA) at 25 C and incubated for 15 h. Dew was shaken from each plant onto polyethylene, collected, and filtered through a 0.22-μm filter. Approximately 10^5 cfu ml^{-1} of a Cu^r or Cu^s strain were added to an aliquot of each dew sample. The suspensions were incubated for 0 and 4 h at 28 C, diluted, and duplicate-plated on Tween medium A. The number of cfu ml^{-1} and total and ionic copper was determined. Treatments were arranged in a randomized complete block and replicated three times. The experiment was repeated twice.

Ionic copper was determined with an Orion cupric specific ion electrode (Orion Research, Boston, MA). Total copper was determined by an adaptation of the method of Spencer (19) based on the principle of spectrophotometric measurement of the copper salt of sodium diethyldithiocarbamate with the inclusion of ammonium citrate and EDTA to prevent interference of other ions (3), which could react with the carbamate. The copper reagent was prepared by mixing 8 g of Na_2 EDTA, 0.25 g of Na diethyldithiocarbamate, and 80 ml of ammonium citrate buffer. The solution was then adjusted to a final volume of 100 ml by adding deionized water. Ammonium citrate buffer was prepared by combining 22 g of diammonium citrate with 130 ml of concentrated NH_4OH and adjusting to 1:1 volume. Copper determination was performed on a dew sample volume of 6 ml combined with 5 ml of reagent and reacted 7.5 min for color development. Standards of 0–25 μg of copper as cupric sulfate were included. Optical density was determined spectrophotometrically at 440 nm. Copper was calculated from a standard curve.

RESULTS

Epiphytic populations of *X. c. vesicatoria* on the leaf surface most closely followed a lognormal distribution. Log_{10}-transformed values followed a normal distribution better than square-root-transformed or nontransformed values in 26 and 27 data sets, respectively. Thus, population data were log-transformed before analysis. In the spring of 1986, the copper and mancozeb combination effectively reduced epiphytic populations of *X. c. vesicatoria* compared to those of the control (Table 1). Epiphytic populations on the bactericide-treated leaves remained low (2.5×10^2 cfu g^{-1}) throughout the experiment (Fig. 1A). In fall 1986, leaflets of control plants had the greatest populations of *X. c. vesicatoria* (Table 1 and Fig. 1B). There was a gradual reduction in epiphytic populations in all three treatments over the course of the experiment. In comparison to the control, both the copper and the copper and mancozeb combinations significantly reduced populations of *X. c. vesicatoria* on the leaflets (Table 1). Populations on leaflets treated with the two copper treatments did not differ significantly. In spring 1987, populations of *X. c. vesicatoria* on the control and copper-treated plants reached approximately 10^5 cfu g^{-1} of leaf tissue early in the experiment, then gradually declined (Fig. 1C). The copper and mancozeb treatment maintained significantly lower populations than the control and copper treatments throughout the experiment (Table 1), and the copper-treated plants had significantly lower populations than the control. In fall 1987, the treatments of copper and of copper and mancozeb had significantly lower populations of *X. c. vesicatoria* than the control (Table 1). Populations of *X. c. vesicatoria* did not decline during the season as in previous seasons (Fig. 1D).

TABLE 1. Differentiation of the effects of bactericide treatments on epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on tomato

Contrast	Spring 1986	Fall 1986	Spring 1987	Fall 1987
Copper hydroxide vs. control	ND[a]	**	*	**
Copper hydroxide vs. copper hydroxide + mancozeb	ND	NS	*	NS
Copper hydroxide + mancozeb vs. control	**	**	**	**

[a] ND = not determined; NS = not significant; * = significant at $P < 0.05$; ** = significant at $P < 0.01$ as determined by contrast comparisons for population differences over the season.

In general, disease severity was not significantly affected by the bactericides for most sampling periods (Table 2). In spring 1986, the copper and mancozeb treatment significantly reduced disease severity compared to the control. Occurrence of target spot, incited by *Corynespora cassiicola* (Berk. & M. A. Curtis) C. T. Wei, made it impossible to evaluate bacterial spot from 22 days after inoculation in fall 1986. Disease severity in spring 1987 was very low in all plots, and leaflets treated with copper and the combination of copper and mancozeb had significantly less disease than the control early in the season. In fall 1987, disease severity was low in all treatments early in the experiment, but late in the season leaflets on the copper and the copper and mancozeb treatments had significantly fewer lesions than the control.

The relationship between epiphytic populations and disease severity was not consistently correlated (Table 3), but there were strong indications of a positive relationship between epiphytic populations and disease severity over the course of the study. In the spring of 1986, epiphytic populations at days 1 and 22 correlated positively with disease severity at days 8, 15, 22, and 29. In the fall of 1986, there was a positive correlation (0.33 at $P < 0.01$) between epiphytic populations at the first sampling date and disease severity 1 wk later. In the fall of 1987, there was a positive correlation between epiphytic populations at 8 days and disease severity at days 15 and 22. Epiphytic populations at day 22 were positively correlated with disease severity at days 15, 22, and 50. Late in the season there were several positive correlations between epiphytic populations and disease severity.

In the greenhouse study, epiphytic populations of the Cu^r strain were reduced significantly by copper alone and by the copper

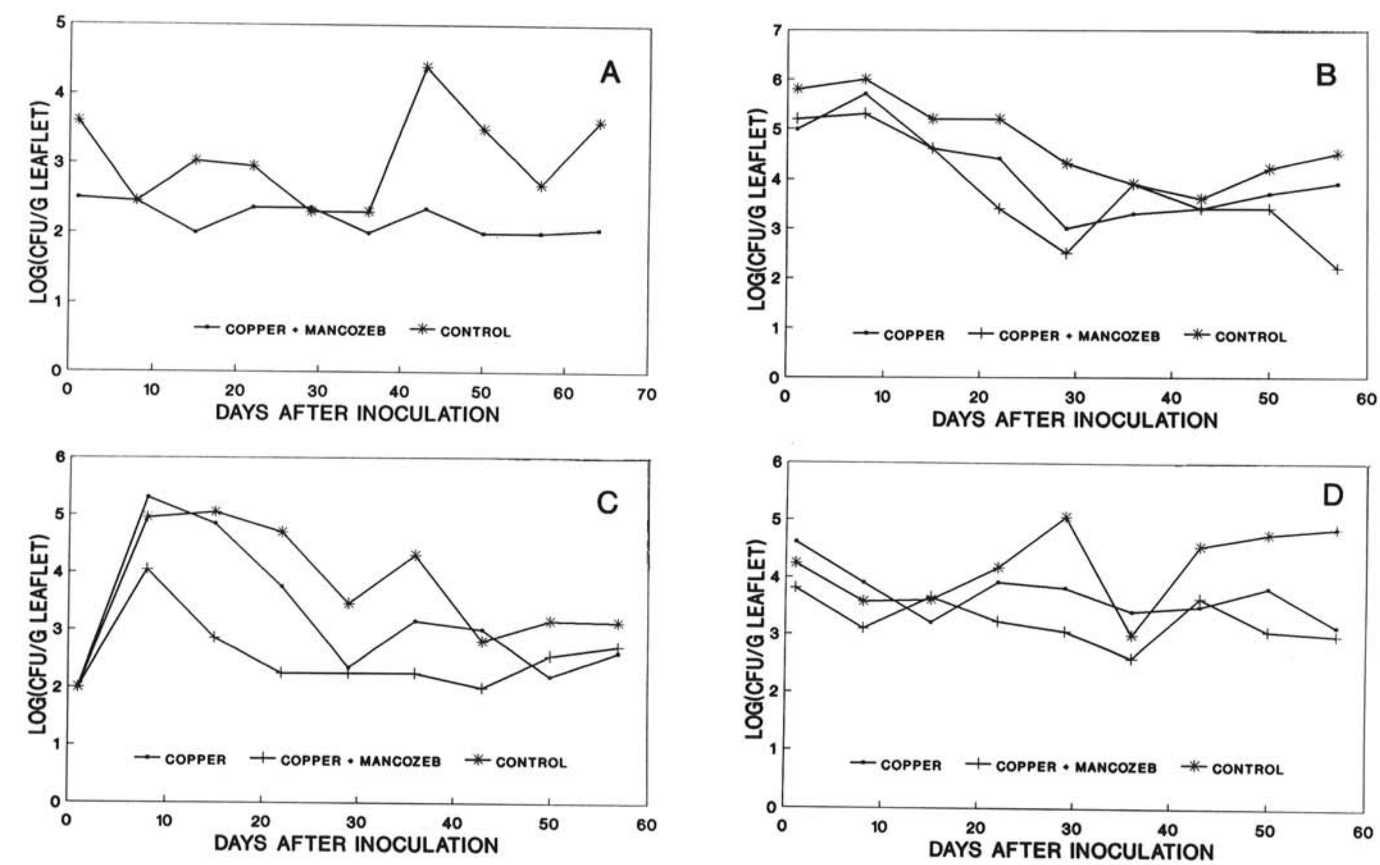

Fig. 1. Effect of bactericides on epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on tomato in **A**, spring 1986; **B**, fall 1986; **C**, spring 1987; and **D**, fall 1987.

and mancozeb mixture compared to those of the control (Fig. 2); however, the copper and mancozeb combination reduced populations significantly more than copper alone. In comparison to the control, copper applied alone and copper applied in combination with mancozeb were equally effective in reducing the number of lesions on plants inoculated with either Cu^r or Cu^s strains of *X. c. vesicatoria* (Table 4), whereas mancozeb reduced disease severity significantly on plants inoculated with the Cu^s strain. The Cu^r strain produced significantly more disease than the Cu^s strain; however, there was no interaction between bactericide and strain.

Dew collected from plants treated with copper or a copper and mancozeb mixture were highly inhibitory in vitro to Cu^s and Cu^r strains (Table 5). Ionic and total copper in the solutions did not differ between the copper treatments, although they did differ significantly from the control.

DISCUSSION

Distribution of *X. c. vesicatoria* on tomato leaflets generally followed a lognormal distribution, which is typical of bacterial pathogens on leaf surfaces (10,13). A sample size of five leaflets per plot was large enough to demonstrate differences among treatments in epiphytic populations. In preliminary studies, bulking 10 leaflets per plot masked differences (J. B. Jones, *unpublished data*). Crosse demonstrated that the bulking of leaves could effectively be used for demonstrating differences in epiphytic populations among plots (5,6). He showed that bulked samples of 192 leaves were necessary to demonstrate differences in populations of *Pseudomonas mors-prunorum* on cherry leaves. However, bulk sampling of this magnitude creates logistic problems when working in small plots where samples are collected periodically.

Both copper alone and in combination with mancozeb reduced epiphytic populations of the Cu^r strains. The two bactericide treatments did not differ significantly, although populations on plants treated with the copper and mancozeb mixture were generally lower than those on copper-treated plants. Although resistant strains are not as sensitive in the laboratory as the sensitive strains (16), the former do have a degree of sensitivity to copper in the field that is expressed on plants treated with copper. This was substantiated by the greenhouse studies with Cu^r and Cu^s strains. Both copper alone and copper in combination with mancozeb effectively reduced lesion number compared to untreated plants. However, copper treatments did not differ significantly. Albeit Conover and Gerhold (4) observed considerable improvement in the control of bacterial spot of tomato with the combination of copper and maneb, and Marco and Stall (16) observed a pronounced effect of combinations of copper and mancozeb compared to the negligible effect of copper alone in reducing bacterial spot of peppers induced by Cu^r strains of *X. c. vesicatoria*, our field study findings clearly demonstrate that

TABLE 2. Effect of bactericides on severity of bacterial spot of tomato

		Days after inoculation							
Season	Treatment[v]	8	15	22	29	36	43	50	57
Spring 1986	Copper + mancozeb	1.9[w] a[x]	2.5 a	1.2 a	0.0	0.0	0.0	0.0	0.0
	Control	14.0 b	19.6 b	1.3 a	0.0	0.0	0.0	0.0	0.0
Fall 1986	Copper	2.0 a	3.3 a[y]	...[z]	...	...	...	...	...
	Copper + mancozeb	4.3 a	5.4 a	...	...	...	...	...	...
	Control	3.1 a	9.6 a	...	...	...	...	...	...
Spring 1987	Copper	4.1 ab	4.7 b	2.2 ab	1.2 a	1.1 a	0.0	1.1 a	...
	Copper + mancozeb	2.5 b	3.0 c	2.0 b	1.1 a	1.1 a	0.0	1.1 a	...
	Control	4.6 a	7.0 a	3.9 a	1.4 a	1.5 a	0.0	1.4 a	...
Fall 1987	Copper	...	4.7 a	1.9 a	3.0 a	...	1.2 b	1.8 b	2.8 b
	Copper + mancozeb	...	2.9 a	1.7 a	4.3 a	...	2.7 a	1.9 b	3.1 b
	Control	...	4.2 a	2.2 a	5.2 a	...	2.9 a	7.4 a	14.0 a

[v] See Materials and Methods.
[w] Number of lesions per leaflet.
[x] Numbers in a column for each year followed by letters that are not similar are significantly different at $P = 0.05$ according to LSD.
[y] Only two readings were made because of target spot, which masked symptoms of bacterial spot.
[z] Readings not made.

TABLE 3. Coefficients of correlation among epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets and severity of bacterial spot

Sampling day (disease severity)[a]	Sampling day (epiphytic populations)[a]								
	1	8	15	22	29	36	43	50	57
Spring 1986									
8	0.60***[b,c]	0.17	0.08	0.61***	0.08	...	...	...	...
15	0.52***	0.08	0.04	0.54***	0.10	...	...	...	...
22	0.43***	−0.04	−0.13	0.43***	−0.06	...	...	...	...
29	0.27*	0.04	0.08	0.30*	0.04	...	...	...	...
Fall 1987									
15	−0.29	0.84***	0.11	0.73**	0.31	0.08	0.33	0.26	0.19
22	−0.26	0.61*	0.20	0.61*	0.65*	0.17	0.58*	−0.06	0.07
29	−0.47	0.33	0.03	0.42	0.43	−0.36	0.38	−0.08	0.19
43	0.02	−0.07	0.33	0.17	0.14	−0.01	0.78**	−0.05	0.26
50	−0.12	0.27	−0.04	0.65*	0.66*	0.14	0.66**	0.51	0.77**
57	−0.10	0.19	0.06	0.54	0.60*	−0.04	0.45	0.59*	0.84***

[a] Days after inoculation.
[b] Correlations were made by comparing epiphytic populations within plots with disease severity within the same plot. Values represent the correlation of data from all plots.
[c] *** = Significant at $P < 0.001$; ** = significant at $P < 0.01$; * = significant at $P < 0.05$.

the differences between the two treatments were not of as great a magnitude for controlling epiphytic populations of *X. c. vesicatoria* on tomato as had previously been reported (4,16).

Although copper alone or in combination with mancozeb consistently reduced epiphytic populations of Cu^r strains of *X. c. vesicatoria*, plants treated with either of the two copper treatments did not consistently have lower disease ratings than the untreated plants in field experiments. Although Lindemann et al (14) observed a relationship between threshold populations of *P. syringae* pv. *syringae* on bean leaves and the occurrence of disease, disease severity was not always positively correlated with epiphytic populations in our studies; however, the general reduction in *X. c. vesicatoria* populations on the leaf surface appears to have been related to the overall reduction in disease in those plots.

The inhibitory effect of a heavy metal on the growth rate of a resistant bacterium has been reported with cadmium and *P. putida* (8). Copper has been shown to inhibit the growth rate of a Cu^r strain of *X. c. vesicatoria* in vitro (22). Under field conditions the bacterium is not continuously exposed to copper, whereby the bacterium's physiology is continually altered to adjust to periods of exposure to copper. Thus, with fluctuating exposures to a heavy metal, the bacterium may be in a state of reduced growth much of the time compared to the situation of continuous exposure to copper.

Leaf surface chemistry may be important in the interaction between copper bactericides and copper-resistant strains. Components of leachates on the tomato leaf surface may modify the bacterium's sensitivity to copper. The phylloplane of pecan contained certain leachates that were toxic and certain ones that were promotive to the pecan scab fungus (23). Considerable work has demonstrated the effects of organic and inorganic compounds on the availability of copper (2) and the effects they have on the toxicity of copper to bacteria (7,9,22). Lukezic et al (15) observed that the source of protein used for growth of *P. s. tomato* affected the sensitivity of bacterial cells to copper. In an in vitro study, the addition of magnesium to a solution containing copper was beneficial to the growth of *X. c. vesicatoria* (22). Metal toxicity may also be modified by interacting with organic compounds to increase (9) or reduce (7) the metal toxicity. It was previously shown that amino acids vary dramatically in the avidity for copper (2) and may affect copper toxicity. Why a mixture of copper and mancozeb was not more effective than copper on tomato plants for controlling copper-resistant strains of *X. c. vesicatoria* in the field is not known. Leachates on tomato leaves may modify the copper and mancozeb combination or the bacterium itself and result in reduced efficacy.

Neither ionic copper (24) nor soluble copper (16) appeared to be the critical components in the copper and mancozeb mixture that affected the toxicity of copper to the Cu^r strain. The copper and mancozeb combination, which apparently is more toxic to Cu^r strains than copper alone, did not result in significantly more ionic copper in dew than copper alone. In preliminary studies, washings from leaves treated with a mixture of copper and mancozeb was toxic to a Cu^r strain of *X. c. vesicatoria* at a 200-fold dilution of the washing (S. S. Woltz and J. B. Jones, *unpublished data*). This would tend to discount the importance of ionic copper and total soluble copper in the increased toxic effect of the copper and mancozeb combination compared to copper alone.

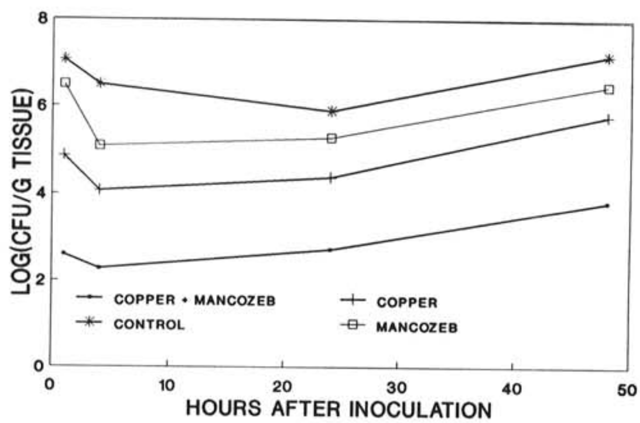

Fig. 2. Effect of bactericides on epiphytic populations of a copper-resistant strain of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets.

TABLE 4. Effect of bactericides on severity of bacterial spot of greenhouse-grown tomato plants inoculated with copper-resistant (Cu^r) or copper-sensitive (Cu^s) strains of *Xanthomonas campestris* pv. *vesicatoria*

Treatment	Rate	Lesions per plant	
		Cu^s	Cu^r
Copper hydroxide	2.2[y]	26.5 b[z]	63.5 b
Copper hydroxide + mancozeb	2.2 + 1.7	14.7 b	55.4 b
Mancozeb	1.7	38.0 b	106.6 ab
Control	...	129.9 a	147.7 a

[y] Value represents kilograms per 950 L of water applied per hectare.
[z] Values in the same column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan-Waller test. Data were transformed by $\log_{10}(X + 1)$ before analysis.

TABLE 5. Sensitivity of copper-resistant (Cu^r) and copper-sensitive (Cu^s) *Xanthomonas campestris* pv. *vesicatoria* strains to dew collected from tomato leaves treated with bactericides

Treatment	Rate	Exposure incubation period				pH	Copper ($\mu g\ ml^{-1}$)	
		0 h		4 h			Total	Ionic
		Cu^r	Cu^s	Cu^r	Cu^s			
Copper	2.2[x]	1.0[y] b[z]	0.0 b	0.0 b	0.0 b	7.7	4.1 b	0.0084 b
Copper + mancozeb	2.2 + 1.7	0.0 b	0.0 b	0.0 b	0.0 b	7.5	2.9 b	0.0061 b
Control	...	4.3 a	4.9 a	4.4 a	4.9 a	7.6	0.643 a	0.0001 a

[x] Value represents kilograms per 950 L of water per hectare.
[y] $\log_{10}$ (cfu ml^{-1}).
[z] Values at 0 or 4 h followed by letters that are not similar are significantly different at $P < 0.01$ according to LSD.

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