Ecology and Epidemiology

Occurrence and Properties of Copper-Tolerant Strains of *Pseudomonas syringae* Isolated from Fruit Trees in California

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ABSTRACT

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Approximately 40% of the ice-nucleation-active strains of *Pseudomonas syringae* isolated from asymptomatic leaves and flowers from almond and navel orange trees from orchards with a history of copper usage were able to grow in a culture medium amended with 0.32 mM $CuSO_4$. While more than one-half of the strains were highly sensitive to cupric ions, some strains could tolerate as much as 1.12 mM $CuSO_4$ in a culture medium. Prior exposure of copper-tolerant strains to sublethal concentrations of copper in a culture medium increased the fraction of cells that could survive a higher concentration of copper by more than 1,000-fold compared with cells not receiving copper pretreatment. The mean LC_{50} of copper-tolerant strains in aqueous copper solutions (23 ppb Cu^{+2}) was about five times that of copper-sensitive strains (4.7 ppb) when cells were assayed without prior exposure to Cu^{+2} in growth medium. The LC_{50} of copper-tolerant strains increased to approximately 160 ppb when cells were grown in medium containing sublethal concentrations of $CuSO_4$. Copper-tolerant strains of *P. syringae* grew as rapidly on $Cu(OH)_2$-treated leaves as on nontreated leaves, while sensitive strains showed little growth. The size of established epiphytic populations of copper-sensitive but not copper-tolerant strains was reduced significantly on treatment of bean and almond leaves with $Cu(OH)_2$ under greenhouse and field conditions.

Copper-containing compounds, including Bordeaux mixture, cupric hydroxide, various formulations of basic copper sulfate, ammoniacal copper, and copper salts of fatty acids, have been widely used for the control of phytopathogenic bacteria and fungi (22). These materials were the first biocides used for disease control and are the only bactericides registered for use on most crops. While these materials are chemically stable and are not readily washed from plants (33,40), control often has been incomplete when they were applied as protectants (1,15,34) or as eradicants (2,12,36) of bacterial diseases. Copper compounds are considered general biocides because of the many potential sites of action of copper ions in biological systems. Copper ions may act as respiratory poisons or react with many fungal and bacterial proteins and kill the cells after sufficient exposure (16,33). Until recently, it was considered unlikely that resistance to copper ions would develop among either fungal or bacterial populations because of the general mode of action of this heavy metal.

Pseudomonas syringae can reduce the productivity of many plant species, including tree fruits such as pear, almond, and citrus,

in several ways. Pathovars (14) of this species cause diseases, including blossom and twig blast, bacterial canker, and fruit spots, of several fruit tree species (13,15,18,42). The incidence of diseases caused by *P. syringae* is correlated with the epiphytic population of this species on plants before infection (21,25). This bacterial species can also limit fruit tree production by causing ice formation, which is an important factor in frost damage to these trees (3,4,6,17,26,28,29). Ice-nucleation-active (Ice⁺) strains of *P. syringae* and other bacteria initiate ice formation at temperatures only slightly below 0 C (17,26,29,31,35) on surfaces of sensitive plants that cannot tolerate ice formation within their tissues. While nonbacterial sources of ice nuclei may exist in some fruit trees and may be important in inciting frost damage under some conditions (7,19), treatments, including copper compounds, that reduced the numbers and/or the ice-nucleation activity of bacteria reduce frost damage to tree crops (27,30). Populations of *P. syringae* are generally low on young vegetative tissues (17,27,30, 39), and copper treatments can inhibit the potential increases of the epiphytic population that would otherwise occur on plant surfaces, thereby reducing the probability of plant frost injury during subsequent periods of low temperatures (29,30). Knowledge of the sensitivity of epiphytic bacteria to copper ions

is therefore needed to determine the potential for sustained use of copper compounds for both disease and frost control.

Resistance to copper has been observed recently among both saprophytic and phytopathogenic bacteria (1,2,8,9,23,36,41,43). Some strains of *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *tomato*, and *P. s. syringae*, pathogens of pepper, tomato, and cherry, respectively, have recently been found to be resistant to the concentrations of copper ions that are sufficient to kill sensitive strains of these species (1,9,36,41,43). Copper-tolerant strains are thought to be poorly controlled by standard applications of copper compounds in some areas (1,36), although the fungicide Maneb (manganese ethylene bis-dithiocarbamate) has improved disease control when mixed with copper compounds (12,36). Only one study has provided information on the population dynamics of copper-tolerant and copper-sensitive strains in the presence or absence of copper deposits on leaves (41).

From indirect evidence, *P. syringae* strains in California appeared to be resistant to copper compounds. Incomplete control of strains of Ice$^+$ of *P. syringae* with copper compounds and significant improvement of control of Ice$^+$ strains of *P. syringae* on almond and citrus in California by copper-Maneb combinations, compared with copper alone, were observed (2,30). Therefore, a study to determine the susceptibility to copper bactericides of various Ice$^+$ strains of *P. syringae* isolated from various agricultural crops throughout California was initiated in this laboratory in 1983. Particular emphasis was placed on determining the variability of quantitative copper tolerance among strains, factors that influence the tolerance to copper ions, and the growth or survival of strains of *P. syringae* varying in tolerance to copper ions on greenhouse and field-grown plants treated with copper compounds.

MATERIALS AND METHODS

Bacterial strains. Strains of *P. syringae* were isolated from leaves and fruit spurs collected from trees in eight citrus and 14 almond orchards with a history of copper sprays and from four citrus and 12 almond orchards that had not been treated with copper bactericides within the last 3 yr. Leaf or fruit spur samples (20 g) were placed in Erlenmeyer flasks containing 200 ml of sterile washing buffer (0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% Bactopeptone), sonicated in an ultrasonic cleaner (Bransonic 52, Branson Sonic Power, Co., Danbury, CT) for 7 min, and appropriate 10-fold serial dilutions were plated onto King's medium B (KB) (24) containing 100 μg/ml of cyclo-heximide. Ice$^+$ bacteria were isolated by a replica-freezing technique from dilution platings of leaf washings and subsequent purification by several single colony transfers in a manner similar to that described by Lindow et al (28). Strains tentatively identified as *P. syringae* showed fluorescent pigment production on KB, negative arginine dihydrolase (45) and oxidase (44) reactions, and positive tobacco hypersensitivity and ice-nucleation (28) reactions, and were unable to rot potato. Strains were stored at −80 C in a solution of 15% (w/v) glycerol.

Mutant strains resistant to the antibiotic rifampicin were selected as single colonies found 4 days after 0.1 ml of a suspension of a parental strain containing 10^9 cells per milliliter was spread onto plates of KB containing 100 μg/ml of rifampicin (KBR) and incubated at 30 C. Individual mutant colonies were purified by streaking on KBR, were re-tested for the biochemical and physiological characteristics listed above, and were demonstrated to have growth rates similar to the corresponding parental strains on KB. Sources and characteristics of copper-sensitive (82-8, 80-5) and copper-tolerant (81-23, 75-3, 68-1, 83-6, E-3) strains of *X. c. vesicatoria* and copper-tolerant strain PT-23 of *P. s. tomato* as well as strain B728a of *P. s. syringae* have been reported (9,32,36).

Culture medium. A low-complexing mineral salts medium (CYE), similar to that described by Zevenhuizen et al (47), containing casitone, 1.7 g/L; yeast extract, 0.35 g/L; glycerol, 2.0 g/L; and purified agar (Difco Laboratories, Detroit, MI), 15 g/

L, was used for assays of copper tolerance in culture. Copper from a filter-sterilized (0.45 μ) stock solution of 160 mM CuSO$_4$ was added by drops to autoclaved CYE medium cooled to 50 C immediately before pouring, to achieve the desired copper concentration. Copper gradient plates were prepared by dispensing 15 ml of CYE medium containing 1.12 mM CuSO$_4$ in a 100- × 15-mm petri dish, then tilting the dish at such an angle that the medium was less than 1 mm thick on the upper side of the raised portion of the dish. After the medium solidified, the dish was returned to a horizontal position, and an additional 15 ml of CYE medium containing no copper was added. Gradient plates were stored at 4 C for at least 8 h before use.

In vitro copper sensitivity of bacterial strains. Copper sensitivity of bacteria was determined in vitro by two different methods. In all assays, bacterial strains were recovered from storage at −80 C, streaked onto KB, and incubated for 24 h at 30 C. Bacteria were harvested with a loop, suspended in sterile distilled water, and adjusted by dilution to an optical density of approximately 0.2 at a wavelength of 600 nm measured with a Spectronic 20 spectrophotometer (Bausch and Lomb). A loopful of each of these bacterial suspensions (containing about 3 × 10^8 bacterial cells per milliliter) was streaked perpendicular to the gradient of copper ions in CYE-copper-gradient plates and was incubated at 30 C for approximately 72 h. Cells harvested with a loop from the portion of CYE-copper-gradient plates containing the highest concentration of copper at which the bacteria grew were transferred to sterile distilled water, and the suspension was adjusted to the desired cell density after spectrophotometric determination of cell concentration as described previously. To measure the minimum concentration of copper that prevented cell growth, bacterial suspensions were adjusted to approximately 10^5 cells per milliliter in distilled water, and a 10-μl drop of each suspension was placed on plates of CYE medium containing the following concentrations of CuSO$_4$: 0, 0.08, 0.16, 0.32, 0.48, 0.64, 0.80, 0.96, or 1.12 mM. Twelve strains, including one well-characterized copper-sensitive strain and one copper-tolerant strain, were tested on each plate of amended CYE medium. Cultures were incubated for 96 h at 30 C, and visible confluent growth of a bacterial strain on copper-amended CYE medium was interpreted as tolerance of the corresponding concentration of CuSO$_4$ in CYE medium. Each test was repeated three times. To determine the fraction of cells of a given strain of *P. syringae* that was able to survive a given concentration of copper ions, one 10-μl drop of each of seven, 10-fold serial dilutions of a suspension of approximately 10^8 cells per milliliter was placed on each of three plates of CYE medium amended with a given concentration of CuSO$_4$ and incubated as described. Presence of individual colonies or a confluent aggregate of colonies was determined 96 h after inoculation. The fraction of surviving cells was estimated from the number of colonies found in samples from each dilution on CuSO$_4$-amended CYE medium divided by the number of colonies from corresponding samples found on unamended CYE medium. The mean fraction of surviving cells was derived from the average colony counts on the three plates of a given copper concentration in each of three replicate experiments.

Bacteria also were assayed for tolerance to copper ions when suspended in distilled water. The highest levels of copper tolerance were induced by culturing strains on CYE medium containing sublethal concentrations of copper (0.1 mM CuSO$_4$ for copper-sensitive strains or 0.36 mM CuSO$_4$ for copper-tolerant strains) before assay. "Uninduced" cells were harvested from unamended CYE medium. Cells were suspended at a concentration of 10^4 cells per milliliter in tubes of sterile distilled water that contained various concentrations of copper ions (supplied as CuSO$_4$), placed on a rotary shaker for 1 h at 30 C, and then plated on KB. The probit of the percentage of cell survival was regressed against the logarithm of copper ion concentration for each strain, and mean lethal concentration (LC$_{50}$) values were obtained by interpolation to probit = 5.0 (20). The mean of LC$_{50}$ values was determined from three replications of each strain.

Bacterial growth and survival on plants. The growth and survival of different strains of *P. syringae* on plants treated with

Cu(OH)₂ (Kocide 101, Kocide Chemical Company, Houston, TX) and a manganese and zinc coordination production of ethylene bis-dithiocarbamate (Dithane M-45, Rohm & Haas, Philadelphia, PA) were determined in both greenhouse and field studies. In greenhouse studies, common bean (*Phaseolus vulgaris* L. 'Bush Blue Lake 274') was grown, eight plants per pot, in a sand/peat (1:1) mixture in 10-cm-diameter plastic pots. Growth of bacteria on copper-treated bean leaves was determined by applying Cu(OH)₂ at concentrations of either 0.6, 1.5, or 4.8 g/L with an air-pressurized handheld sprayer to runoff 1 h before inoculation of 2-wk-old plants (~30 cm high) with aqueous bacterial suspensions. Some plants also were treated with a mixture of 0.6 g/L of Cu(OH)₂ and 0.6 g/L of manganese-zinc bis-dithiocarbamate 1 h before inoculating plants with bacteria. Rifampicin-resistant bacterial strains were transferred from CYE-copper-gradient plates to KBR and incubated for 72 h at 30 C. Bacteria were suspended in sterile distilled water at a concentration of 1×10^5 cells per milliliter and sprayed on treated bean plants to runoff with an air-pressurized atomizer. Plants were then incubated in a moist chamber at about 21 C and misted intermittently to facilitate bacterial growth. To estimate bacterial population size, 26–30 leaves from each of four pots (replicates) of plants were harvested from each treatment and placed in washing buffer, and cells were removed by sonication as described. Appropriate serial dilutions of leaf washings were plated on KBR containing 100 μg/ml of cycloheximide. To determine the toxicity of Cu(OH)₂ on populations of *P. syringae* previously established on bean plants, cells of selected strains were harvested from KB and inoculated onto plants as described, incubated for 48 h in a moist chamber at 21 C, and the plants were then sprayed with a suspension of 1.2 g/L of Cu(OH)₂. Bacterial populations were determined on KBR (as described) immediately before and 3 h after treatment with Cu(OH)₂.

Survival of selected strains of *P. syringae* (differing in copper tolerance in culture) was determined on Cu(OH)₂-treated almond (*Prunus dulcis* 'Nonpareil') at the University of California, Westside Field Station, located near Five Points in the San Joaquin Valley of California. The experimental plot consisted of a randomized complete block design with four replications. Each replication consisted of one mature almond tree. At least one nontreated tree separated each treated tree within and between blocks. Strains of *P. syringae* grown for 3 days on KBR plates at 24 C were harvested by washing, suspended in tap water, and applied at a concentration of approximately 5×10^7 cells per milliliter (~3 L/tree) to trees at approximately 50% bloom on 16 March 1986. The application was done within 30 min of harvest with a backpack mist-blower similar to that used in a previous study (30). Cupric hydroxide (1.2 g/L) or a mixture of Cu(OH)₂

and manganese-zinc ethylene bis-dithiocarbamate (0.6 g/L) was applied to a subset of trees at the rate of 26 L/tree with a piston-pressurized gun-sprayer within 4 h after inoculation with bacteria. Population sizes of rifampicin-resistant strains of *P. syringae* were determined from dilution-plating of washings from 20 bulked fruiting spurs for each replication on KBR containing 100 μg/ml of cycloheximide as described.

Statistical methods. Statistical computations were made by using software provided by Statistical Analysis Systems (SAS) (release 5.16; SAS Institute, Inc., Cary, NC). The SAS general linear models procedure was used to perform analysis of variance on LC_{50} estimates and on log-transformed bacterial population sizes. Mean comparisons were made with Fisher's unprotected LSD test. This test controls the comparison-error rate.

RESULTS

In vitro copper tolerance of strains of *P. syringae*. Strains of *P. syringae* isolated as epiphytes from healthy leaves and flowers of almond and navel orange in California exhibited variable levels of tolerance to copper ions added to CYE medium, a culture medium that has limited cupric ion binding capacity. Thirty-three percent and 45% of the 126 strains of *P. syringae*, collected from navel orange and almond, respectively, were tolerant to more than 0.32 mM CuSO₄ in this medium (Fig. 1). Although a few strains were unable to tolerate the presence of even 0.16 mM CuSO₄ in CYE medium, no strains grew in CYE medium containing over 1.12 mM CuSO₄. Frequency of copper tolerance among the strains of *P. syringae* tested was distinctly bimodal. Whereas, over one-half of the tested strains were highly sensitive to cupric ions, the remaining strains had a high mean tolerance to cupric ions in CYE medium (Fig. 1). Qualitatively similar variations in copper tolerance also were observed in strains of *P. syringae* isolated from pear orchards in California (data not shown). While copper-tolerant strains of *P. syringae* were detected on both almond and citrus, a higher percentage of almond strains exhibited a high level of tolerance to CuSO₄ in CYE medium (>0.6 mM CuSO₄).

Cell populations of different strains of *P. syringae* showed distinct copper-concentration thresholds on which cell growth stopped with further addition of CuSO₄ to CYE medium. A large fraction of cells of most strains survived concentrations of CuSO₄ in CYE medium that approached the threshold concentration at which cell death began (Figs. 2 and 3). For example, while over 10% of the cells in populations from strains Al513R, Al489, Al529, and Al436 survived 0.8, 0.8, 0.48, and 0.08 mM CuSO₄
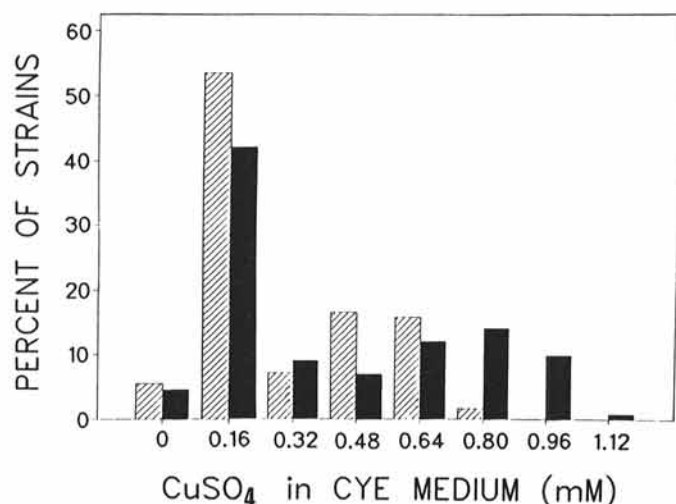

Fig. 1. Frequency distribution of maximum concentrations of CuSO₄ in CYE medium at which different strains of *Pseudomonas syringae* collected from almond (solid bars) or navel orange (hatched bars) grew.
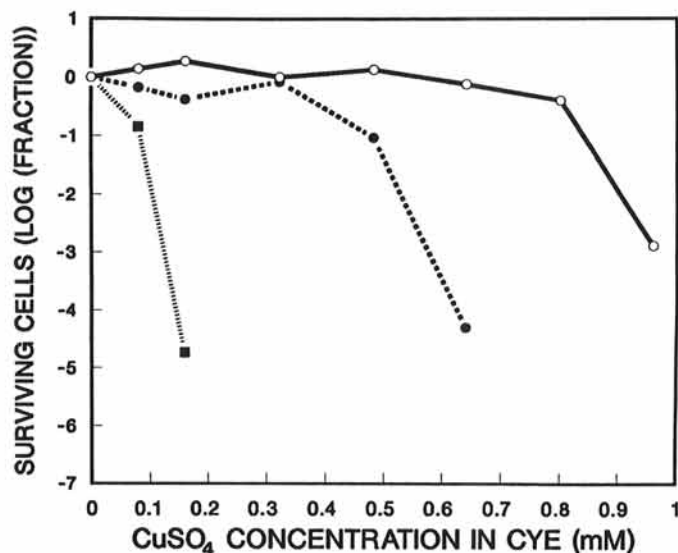

Fig. 2. Fraction of cells in populations of strains Al513R (○), Al529 (●), and Al436 (■) of *Pseudomonas syringae* that survived exposure to the increasing amounts of copper ions (as CuSO₄) added to CYE medium.

in CYE medium, respectively, only less than about $10^{-4}-10^{-5}$ of the population of cells of each strain survived when an additional 0.16 mM $CuSO_4$ was added to the medium (Figs. 2 and 3). In contrast, the proportion of the cells of strain B728a that tolerated increasing concentrations of $CuSO_4$ in CYE medium decreased progressively with i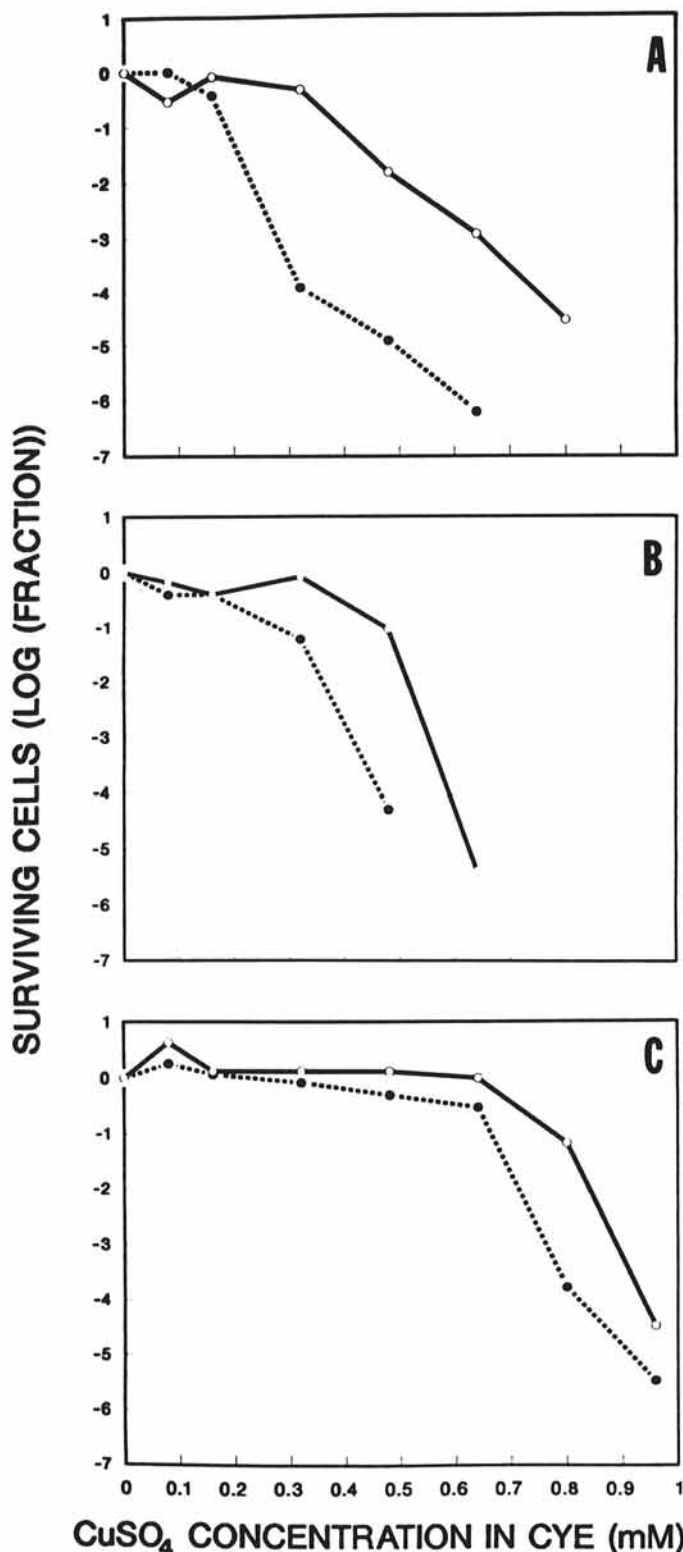ncreasing concentration of $CuSO_4$ above 0.24 mM and did not exhibit a distinct threshold of tolerance of $CuSO_4$ as did most other strains (Fig. 3).

Prior exposure of several strains of *P. syringae* to sublethal concentrations of $CuSO_4$ (0.2 mM) on CYE medium caused a proportion of the induced cells to survive concentrations of copper that killed nontreated (uninduced) cells (Fig. 3). More than a 1,000-fold increase was observed in the fraction of induced cells of most copper-tolerant strains that grew on CYE medium containing a given concentration of $CuSO_4$ compared with cells not receiving copper pretreatment (Fig. 3). However, previous exposure to $CuSO_4$ had no effect on the ability of several copper-tolerant strains and all sensitive strains to grow in CYE medium containing near lethal amounts of $CuSO_4$ (data not shown).

Strains of *P. syringae* differed greatly in their survival in aqueous solutions of $CuSO_4$. Strains that grew only on CYE medium containing low concentrations of $CuSO_4$ were highly sensitive to $CuSO_4$ in distilled water. Conversely, the most tolerant strains on copper-amended CYE medium also survived in the highest concentrations of $CuSO_4$ tested in distilled water. The mean LC_{50} of copper-tolerant strains of *P. syringae* (23 ppb) was about five times higher than that of copper-sensitive strains (4.7 ppb) when cells were assayed without prior exposure to Cu^{+2} in the growth medium (Table 1). However, the mean LC_{50} of copper-tolerant strains increased to about 160 ppb when cells were grown on a medium containing sublethal concentrations of $CuSO_4$. In contrast, the LC_{50} of copper-sensitive strains treated in this way was no higher than cells not previously exposed to $CuSO_4$ in growth media (Table 1). Exposure to $CuSO_4$ in growth media therefore induced cells to express a sevenfold increase in the level of copper ions that could be tolerated compared to uninduced cells of copper-tolerant strains. Strain B728a of *P. syringae*, isolated from bean in Wisconsin, was significantly more tolerant of copper ions than sensitive fruit tree strains when induced by exposure to sublethal doses of $CuSO_4$ in CYE medium, but was much less tolerant of Cu^{+2} than other copper-tolerant strains (Table 1). The most copper-tolerant strains of *P. syringae* survived exposure to concentrations of copper ions in distilled water as high as those tolerated by most copper-tolerant strains of *X. c. vesicatoria* and higher than that of a copper-tolerant strain of

SURVIVING CELLS (LOG (FRACTION))

CuSO₄ CONCENTRATION IN CYE (mM)

Fig. 3. Fraction of cells in populations of strains B728a (**A**), Al529 (**B**), and Al489 (**C**) of *Pseudomonas syringae* that survived exposure to the increasing amounts of copper ions added to CYE medium when inoculum was harvested from medium containing no added copper ions (○), or containing 0.2 mM $CuSO_4$ (●).

TABLE 1. Sensitivity of induced and uninduced cells of different bacterial strains to copper ions in distilled water

Strain tested	Copper ion tolerance[x] LC_{50} (ppb)	
	Uninduced[y]	Induced[z]
Pseudomonas syringae		
Al563R	3.6 de	4.2 ef
Al555R		5.1 ef
Al423R		3.7 ef
Al539R	4.1 de	3.1 f
Al487R	6.5 cd	6.8 e
B728a	8.5 cd	18.1 d
Al489R	40.6 a	157.0 b
Al513R	12.6 abc	183.8 b
Al444R	14.2 abc	138.0 b
P. syringae pv. *tomato*		
PT-23	3.5 dc	54.1 c
Xanthomonas campestris pv. *vesicatoria*		
82-8	1.2 e	3.7 ef
80-5	1.4 e	3.3 f
81-23	8.8 bcd	207.7 ab
75-3		155.3 b
68-1	30.8 ab	114.6 b
83-6	15.1 abc	177.0 b
E-3	32.1 ab	408.9 a

[x]Means in each column followed by the same letter do not differ significantly by Fisher's unprotected LSD-means comparison test.
[y]Cells were harvested from unamended CYE medium.
[z]Cells of copper-sensitive strains ($LC_{50} < 10$ ppb) were harvested from CYE medium containing 0.1 mM Cu^{+2} (as $CuSO_4$). Cells of copper-tolerant strains were harvested from CYE medium containing 0.36 mM Cu^{+2} (as $CuSO_4$).

P. s. tomato (Table 1). The maximum concentration of $CuSO_4$ in aqueous solutions in which bacterial strains survived was not directly proportional to the maximum concentration of $CuSO_4$ in CYE medium in which growth occurred (Table 1; Figs. 2 and 3).

Growth of strains of *P. syringae* on copper-treated plants. Strains of *P. syringae* varied in their ability to grow on plants treated with a copper-containing bactericide. Copper-tolerant strains grew as rapidly on plant surfaces treated with 1.5 g/L of $Cu(OH)_2$ (a typical field rate of this bactericide) as on plants not treated with $Cu(OH)_2$ (Fig. 4). However, about 90–95% more of the cells of copper-tolerant strains died shortly after application to plants treated with $Cu(OH)_2$ compared to cell survival on non-treated leaves (Fig. 4). Population sizes of copper-tolerant strains were similar on $Cu(OH)_2$-treated and nontreated leaves within 3–4 days after inoculation. In contrast, copper-sensitive strains of *P. syringae* were unable to grow on plants treated with $Cu(OH)_2$ or exhibited growth rates much lower than those on untreated plants (Fig. 5). The population sizes of copper-tolerant strains were from 100 to more than 10^5 times higher than those of copper-sensitive strains after 4 days of growth on $Cu(OH)_2$-treated plants (Figs. 4 and 5).

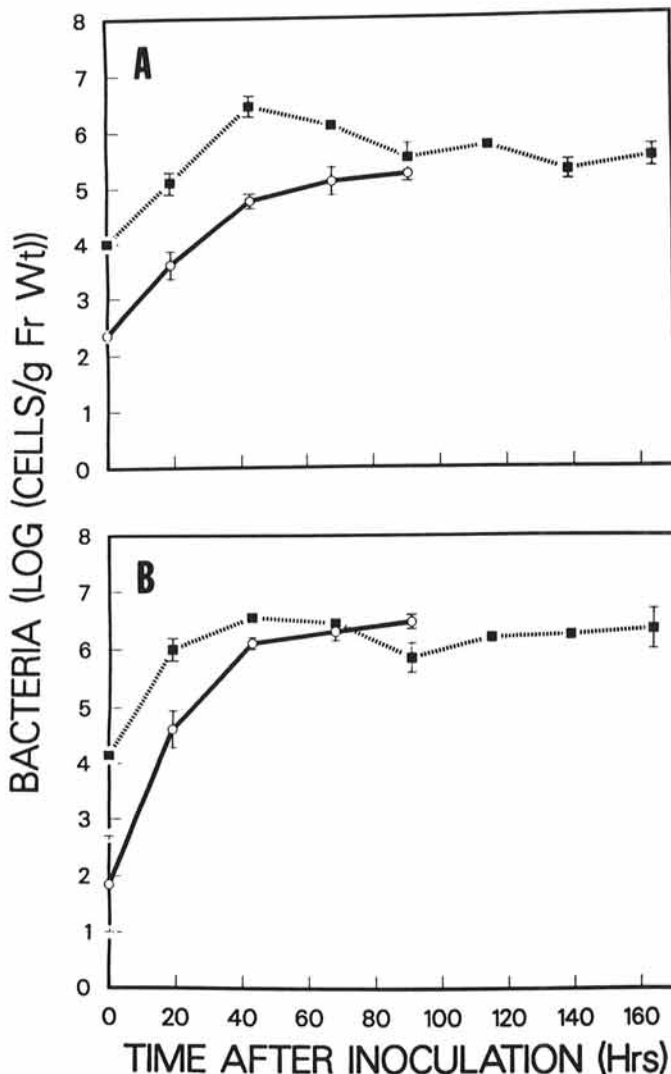The ability of copper-tolerant strains to grow on $Cu(OH)_2$-treated plants could not be overcome simply by increasing the amount of $Cu(OH)_2$ applied to leaves (Fig. 6). Little difference was observed in either the growth rate of copper-tolerant strains of *P. syringae* on leaves or the population size of such strains after 3 days of growth on nontreated leaves and on leaves treated with 0.6 g/L or 4.8 g/L of $Cu(OH)_2$ (0.5 and four times the recommended disease control concentrations, respectively) (Fig. 6). The fraction of cells of copper-tolerant strains that survived initially on $Cu(OH)_2$-treated plants decreased with increasing amounts of $Cu(OH)_2$ applied, but surviving cells rapidly colonized treated plants (Fig. 6). In contrast, increasing the amount of $Cu(OH)_2$ applied to leaves decreased the maximum population size that copper-sensitive strains attained on treated plants (Fig. 7). Both copper-tolerant and copper-sensitive strains of *P. syringae* grew slowly and attained much lower population sizes on plants treated with a mixture of $Cu(OH)_2$ and manganese-zinc ethylene bis-dithiocarbamate than on plants treated with an equivalent rate of $Cu(OH)_2$ alone (Figs. 6 and 7).

Killing of strains of *P. syringae* previously established on plants by $Cu(OH)_2$. While large decreases in the population size of copper-sensitive *P. syringae* occurred when $Cu(OH)_2$ was applied to leaves before application of bacterial strains, $Cu(OH)_2$ was much less effective at killing established populations of such strains. Viable populations of copper-sensitive strains of *P. syringae* that were recovered from colonized bean leaves in a greenhouse decreased to about 80% of original levels within 3 h after treatment of leaves with a $Cu(OH)_2$ suspension (Table 2). No measurable death of copper-tolerant strains occurred on leaves after application of $Cu(OH)_2$.

$Cu(OH)_2$ sprays were an effective eradicant of copper-sensitive but not copper-tolerant strains of *P. syringae* on plants under field conditions. The population size of copper-sensitive *P. syringae* on almond trees was generally reduced over 90% after treatment with $Cu(OH)_2$ under field conditions (Table 3). In contrast, little, if any, reduction of population size of copper-tolerant strains occurred after application of $Cu(OH)_2$ unless manganese-zinc ethylene bis-dithiocarbamate was also applied (Table 3). Similar results were observed in other field trials on almond, navel orange, and pear trees (data not shown).

DISCUSSION

A bimodal distribution of copper tolerance was discovered among strains of *P. syringae* tested for copper tolerance on CYE
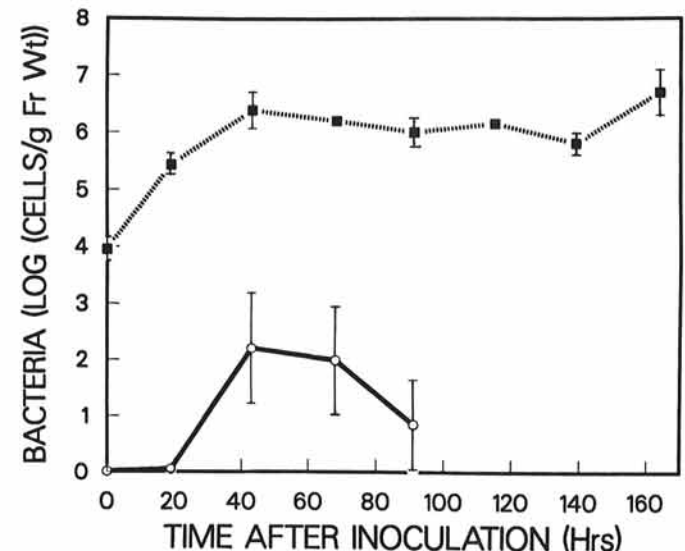

Fig. 4. Population size of copper-tolerant strains Al513R (A) and Al114 (B) of *Pseudomonas syringae* on $Cu(OH)_2$-treated and nontreated bean leaves at different times after inoculation. Plants were sprayed with a suspension of 1.2 g/L of $Cu(OH)_2$ (○) or water alone (■) 1 h before spray inoculation with bacterial suspensions. The vertical bars represent the standard error of the determination of the mean log population size.

Fig. 5. Population sizes of copper-sensitive strain Al105 of *Pseudomonas syringae* on $Cu(OH)_2$-treated (○) and nontreated (■) bean leaves at different times after inoculation. Plants were sprayed with a suspension of 1.2 g/L of $Cu(OH)_2$ 1 h before spray inoculation with a suspension of strain Al105. The vertical bars represent the standard error of the determination of the mean of log bacterial population size.

medium, indicating that at least two separate classes of resistance exist. Although a majority of the strains were tolerant only of low concentrations of $CuSO_4$ (<0.16 mM) in CYE medium, a number of strains, especially those isolated from almond fruiting spurs, were tolerant to much higher concentrations of added
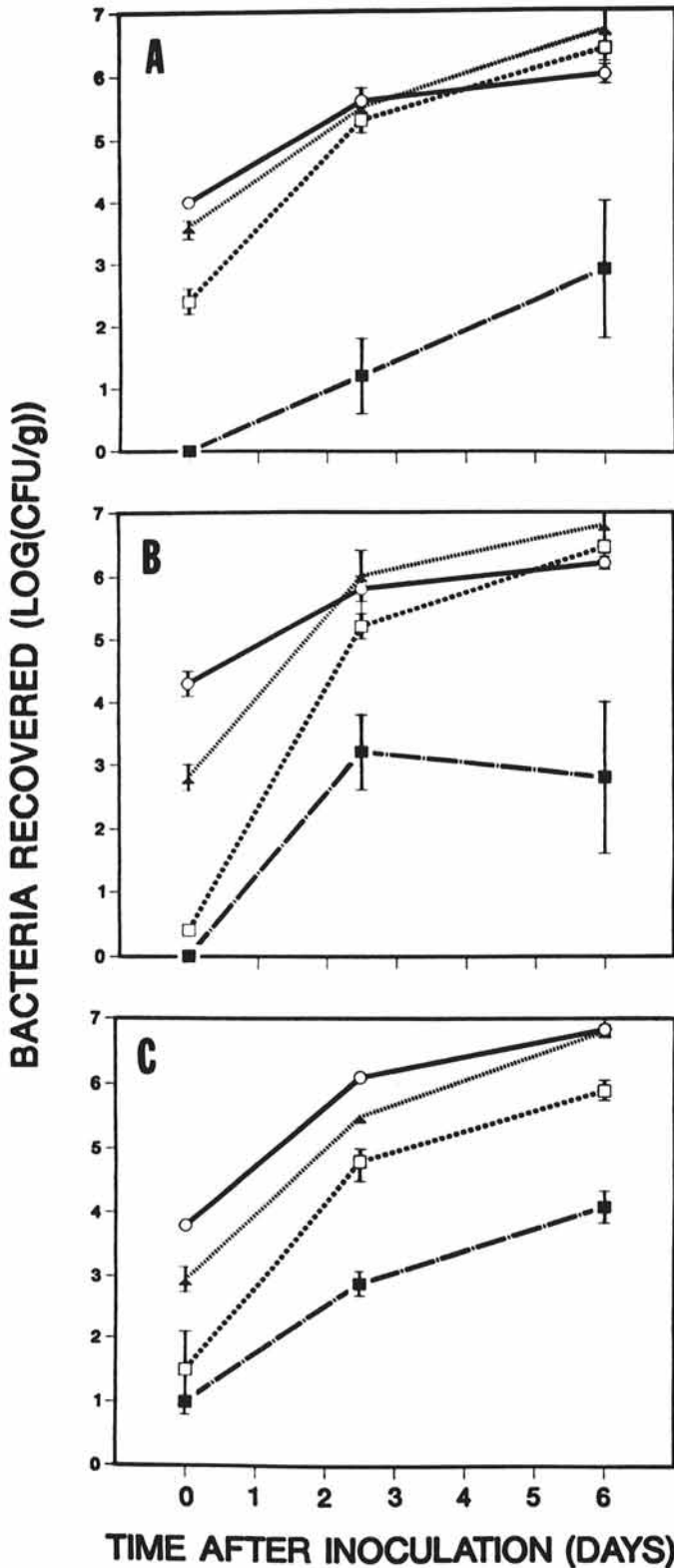
copper. Up to 40% of the strains of *P. syringae* isolated from almond were able to tolerate concentrations of $CuSO_4$ in CYE medium that were inhibitory to the most tolerant strains isolated from citrus leaves (Fig. 1), even though higher amounts of copper compounds were applied to citrus compared with almond trees. Differences in copper tolerance may, therefore, reflect ecological variations in the strains selected by the host plant. Substantial differences in the growth and survival characteristics of various epiphytic strains of *P. syringae* have been documented (17,18). The wide range of distinct, reproducible tolerances to $CuSO_4$ on CYE medium exhibited by the different strains suggests either that more than one gene is responsible for this phenotype, as observed in *P. s. tomato* (38), or that other properties of the strains conditioned the tolerance to copper conferred by such genes. For example, the strains could differ in the amount and type of extracellular polysaccharide, which has been shown to confer partial copper tolerance (11).

The level of $CuSO_4$ tolerated by a given strain was highly reproducible under a given set of conditions, but was greatly influenced by culture conditions and by the assay procedure itself. A more quantitative assessment of variations in copper tolerance among cells of a given genotype was obtained in this study, because the probit survival response of a population of cells exposed to different concentrations of Cu^{+2} was measured. The LC_{50} of other
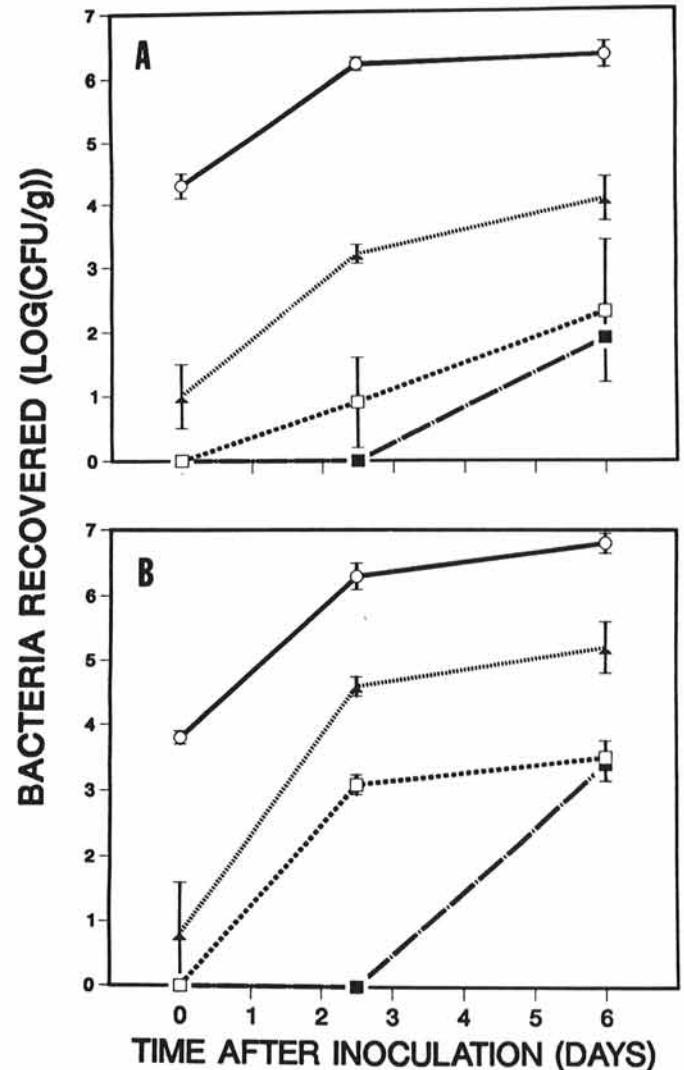

Fig. 6. Population size of copper-tolerant strains Al489 (**A**), Al513R (**B**), and B728a (**C**) of *Pseudomonas syringae* on nontreated bean leaves (○), leaves treated with 0.6 g/L of $Cu(OH)_2$ (▲), 4.8 g/L of $Cu(OH)_2$ (□), or with 0.6 g/L $Cu(OH)_2$ plus 0.6 g/L of Maneb (■) at the times after inoculation shown on the abscissa. The vertical bars represent the standard error of the determination of mean log bacterial population size.

Fig. 7. Population size of copper-sensitive strains Al423 (**A**), and Al487 (**B**) of *Pseudomonas syringae* on nontreated bean leaves (○) leaves treated with 0.6 g/L of $Cu(OH)_2$ (▲), 4.8 g/L of $Cu(OH)_2$ (□), or with 0.6 g/L of $Cu(OH)_2$ plus 0.6 g/L of Maneb (■) at the times after inoculation shown on the abscissa. The vertical bar represents the standard error of the determination of mean log bacterial population size.

copper-tolerant bacterial species that were measured in this study in distilled water were significantly lower (up to 80-fold) than reported (9,36). Methodological differences may account for the discrepancy in the reported tolerances of Cu^{+2}. Increasing the concentration of *X. c. vesicatoria* cells to the level tested by Marco and Stall (5×10^5 cells ml^{-1}) (36) in aqueous solutions resulted in threshold copper tolerances (concentrations at which at least one cell survived) similar to the reported values. The cupric ion has been reported to be the toxic form of copper (5,16,47). The highly reactive cupric ion can bind with a multitude of chemical species present in whole and lysed cells, and in tap water, to form nontoxic copper complexes. With higher numbers of bacterial cells in an aqueous suspension, a higher concentration of $CuSO_4$ is necessary to overcome the complexing ability of the bacteria. The apparent tolerance of strains of *P. syringae* to more than 100 times the concentration of Cu^{+2} in amended CYE medium compared to that in distilled water is probably due to the complexing of the toxic cupric ion by components of the medium itself into copper species that are not toxic to the bacterium. Increasing the concentration of $CuSO_4$ added to this medium probably causes the amount of toxic-free cupric ions present to increase in a more than proportional manner (47). While the true sensitivity of a bacterial strain to cupric ions is best measured in distilled water, a rapid estimate of the relative sensitivity of different bacterial strains to copper may be obtained by scoring for growth on CYE medium amended with various concentrations of $CuSO_4$. Accurate measurements of the amount of free Cu^{+2} in the CYE medium have been made and should permit the rapid quantitative estimation of the copper tolerance of test strains by testing growth on amended CYE medium (O. Menkissoglou and S. E. Lindow, *unpublished data*).

Exposure of cells of copper-tolerant strains to sublethal concentrations of $CuSO_4$ in a growth medium resulted in an increase in tolerance to copper ions in distilled water. This could be because of amplification of a conjugative plasmid in response to copper stress, as has been reported by Bender and Cooksey (9) for *P. s. tomato* and by Stall et al (43) for *X. c. vesicatoria*. Mellano and Cooksey (38) recently reported that a copper-resistant operon from *P. s. tomato* is inducible by copper ions. We, however, found that, while copper-tolerant strains of *P. syringae* and of *X. c. vesicatoria* showed significantly higher tolerance to copper ions than sensitive strains even in the absence of prior exposure to copper ions, strain PT-23 of *P. s. tomato* did not (Table 1). Genes involved in copper tolerance and/or their manner of regulation appear to differ between these species and pathovars (10; S. E. Lindow, *unpublished data*). A low constitutive level of tolerance of Cu^{+2} ions shown by cells of *P. syringae* may be

important in the process by which cells are induced to express higher levels of copper tolerance upon sensing high concentrations of copper ions. It will be necessary to study this induction phenomenon further in culture and on leaves to elucidate the basis for survival of at least a part of the cell population after the sudden deposition of large amounts of copper on leaves.

Threshold levels of copper tolerated by strains of *P. syringae* on copper-amended CYE medium and their mean tolerance of copper in distilled water correlated well with their ability to survive and grow on plants treated with various copper compounds. Only copper-tolerant strains of *P. syringae* ($LC_{50} > 15$ ppb Cu^{+2}) grew after inoculation onto $Cu(OH)_2$-treated leaves (Figs. 4–7) or survived when treated with $Cu(OH)_2$ after colonization of plants had occurred (Tables 2 and 3). Strains of copper-tolerant *P. s. syringae* from cherry were recently shown to be less effectively eradicated from $Cu(OH)_2$-sprayed leaves than sensitive strains (41), but growth of these strains after application to copper-treated leaves has not been previously demonstrated.

A transient reduction in the viable population size of copper-tolerant strains of *P. syringae* occurred immediately after inoculation onto copper-treated bean leaves with high concentrations of copper (Figs. 4 and 5). The $Cu(OH)_2$ deposits on such leaves were probably not uniform. Small areas of the leaf may have had little or no $Cu(OH)_2$ deposited, and the amount of $Cu(OH)_2$ found in leaf openings such as stomata, which may harbor bacteria, is likely to be low. A small percentage of inoculant cells may have survived on areas of the leaf where the copper deposit was low, and the maximum concentration of free copper ions was less than the maximum level tolerated by the bacteria. Cells inoculated onto plants in our experiments were not induced to maximum levels of copper tolerance before inoculation. The surviving cells, however, were probably induced to express a high level of copper tolerance by the sublethal concentrations of copper to which they were exposed. These induced cells probably then multiplied at an equivalent rate and reached similar maximum populations as cells on bean leaves not treated with copper. Because the concentration of cupric ions, and not the amount of $Cu(OH)_2$, determines toxicity to the bacterial cell, increasing the amount of $Cu(OH)_2$ applied to leaves to four times the recommended rate did not inhibit the growth of copper-tolerant strains of *P. syringae* inoculated onto treated bean leaves (Fig. 6). The concentration of cupric ions on leaves is not directly

TABLE 2. Viable population sizes of resident copper-tolerant and copper-sensitive strains of *Pseudomonas syringae* on bean leaves immediately before and 3 h after treatment of leaves with $Cu(OH)_2$

Bacterial strain	Copper tolerance[x]	Bacterial cells recovered[w] (log [cells per gram fresh weight])	
		Before $Cu(OH)_2$[y]	After $Cu(OH)_2$[z]
Al436	−	5.86 a C	4.99 b D
Al423	−	6.29 a BC	5.61 a C
Al487	−	6.16 a BC	5.52 a CD
Al448	+	6.87 a A	6.82 a A
Al489	+	6.41 a B	6.22 a B
Al513R	+	6.27 a BC	6.21 a B

[w] Means in each column followed by the same upper-case letter and means in each row followed by the same lower-case letter are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test.

[x] Copper-tolerant strains grew on CYE medium containing >0.36 mM Cu^{+2} (as $CuSO_4$) and had an LC_{50} for Cu^{+2} of >50 ppb when assayed in distilled water.

[y] Bacterial populations were measured 48 h after inoculation and immediately before treatment of plants with $Cu(OH)_2$.

[z] $Cu(OH)_2$ (1.2 g/L) was applied to plants colonized with each bacterial strain 3 h before sampling.

TABLE 3. Survival of copper-tolerant and copper-sensitive *Pseudomonas syringae* strains on almond trees in the field

Strain[v]	Copper tolerance[w]	$Cu(OH)_2$ treatment[x]	Bacteria recovered (log [cells per gram])[y]	
			24 March	6 May
Al423	−	−	4.47 a	4.32 abc
Al423	−	+	2.04 c	2.54 efg
Al436	−	−	2.57 abc	4.69 a
Al436	−	+	1.49 bc	2.08 fg
Al487	−	−	3.77 ab	4.37 abc
Al487	−	+	2.06 bc	3.54 abcdef
Al448	+	−	4.24 a	3.04 bcdef
Al448	+	+	3.98 a	4.15 abcd
Al489	+	−	2.54 abc	4.48 ab
Al489	+	+	3.19 abc	1.50 g
Al513	+	−	3.80 ab	3.06 bcdef
Al513	+	+	4.06 a	3.65 abcde
Al513	+	+[z]	3.33 abc	2.67 defg

[v] Rifampicin-resistant *P. syringae* strain applied to trees.

[w] Copper-tolerant strains grew on CYE medium containing >0.36 mM Cu^{+2} (as $CuSO_4$) and had an LC_{50} for Cu^{+2} of >50 ppb when assayed in distilled water containing $CuSO_4$.

[x] $Cu(OH)_2$ (1.2 g/L) was applied to a subset of the trees 4 h after inoculation with bacterial strains on 23 March and again on 10 April 1986.

[y] Numbers in the same column followed by the same letter are not significantly different ($P = 0.05$), according to Duncan's multiple range test.

[z] Manganese-zinc ethylene bis-dithiocarbamate was applied (0.6 g/L) 1 h after application of $Cu(OH)_2$.

proportional to the amount of $Cu(OH)_2$ present (O. Menkissoglou and S. E. Lindow, *unpublished data*). In contrast, there was a concentration-dependent increase in death of copper-sensitive strains with increasing amounts of $Cu(OH)_2$ applied to leaves (Fig. 7). The cupric ion concentration in most habitats on treated leaves where *P. syringae* exists appears to be higher than the concentrations tolerated by these sensitive strains. Between 0.1 and 1% of the cells of copper-sensitive strains appear to occur at sites where the concentration of cupric ions, after uniform application of $Cu(OH)_2$ at the standard rate, is less than the LC_{50} (Fig. 7). The LC_{50} of cupric ions at about 90–95% of such protected sites appears to be exceeded when the rate of $Cu(OH)_2$ applied is increased fourfold (Fig. 7).

P. syringae cells that have colonized leaves (even in the absence of applied copper) appear to be less sensitive to copper ions than laboratory-cultured cells. While, on average, about 90% of laboratory-cultured cells of copper-tolerant strains of *P. syringae* were killed when applied to $Cu(OH)_2$-treated plants (Figs. 4–6), no significant death occurred when $Cu(OH)_2$ was applied to plants previously colonized by these strains (Tables 2 and 3). Similarly, over 99.9% of laboratory-cultured cells of copper-sensitive strains were killed when applied to $Cu(OH)_2$-treated leaves (Figs. 5 and 7), while only about 90% of cells of these strains were killed when $Cu(OH)_2$ was applied to plants previously colonized by these strains (Table 2). Some, but probably not all, of the cells of copper-sensitive and tolerant strains that survived eradicative applications of $Cu(OH)_2$ may have colonized portions of leaves not readily accessible to chemical applications. Cells on colonized plants also may survive better than laboratory-cultured cells if they are physiologically different, such as by accumulating extra-cellular polysaccharides in a slime layer around the cells. Such a slime layer may have reduced the concentration of cupric ion to which plant-grown cells are exposed. The exposure of cells to copper may be sufficiently low that all cells of copper-tolerant strains could survive to express induced levels of copper tolerance, and sensitive strains in as many as 10% of all of the microsites on a leaf could escape death.

The application of manganese-zinc ethylene bis-dithiocarbamate in mixtures with $Cu(OH)_2$ either as a protectant or an eradicant was effective in the control of copper-tolerant strains of *P. syringae* in both greenhouse and field trials. The toxicity of ethylene bis-dithiocarbamate (EBDC) compounds appears to be associated with ability of the dithiocarbamate anion to chelate copper and transport the cation to a copper-susceptible site within the cell (37). In addition, EBDC has been reported to alter the permeability of the cell membrane, resulting in the disruption of cellular transport mechanisms (46). Thus, the synergistic effect between the copper compounds and EBDC in controlling copper-resistant strains is most likely attributable to the circumvention of resistance mechanisms to allow lethal amounts of cupric ion into the bacterial cell. While this material shows promise in the control of copper-tolerant strains of *P. syringae*, it is no longer registered for use on tree fruits.

The large number of copper-tolerant strains of *P. syringae* present in agricultural areas where copper bactericides have been used is probably responsible for the incomplete control of bacteria on crops with copper compounds in such areas. It is unlikely that simply increasing the amounts of copper bactericides alone will lead to effective control of such strains. Further studies designed to measure the concentrations of free cupric ions on leaves should determine whether or not copper-tolerant strains of *P. syringae* can be controlled by copper-containing bactericides under any environmental conditions.

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