

Chemical Forms of Copper on Leaves in Relation to the Bactericidal Activity of Cupric Hydroxide Deposits on Plants

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ABSTRACT

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The total amount of copper, the amount of soluble but complexed copper, and the concentration of free Cu^{2+} ions on the surface of navel orange and bean leaves treated with different amounts of $\text{Cu}(\text{OH})_2$ or Bordeaux mixture were determined under field conditions. Total copper deposits decreased with time after spray application with apparent first-order kinetics with a half-life of approximately 45 and 35 days on navel orange and bean leaves, respectively. The concentration of soluble but complexed copper, however, increased for 20–30 days following spray application and comprised approximately 25 and 10% of the total copper on treated navel orange and bean leaves, respectively. The concentration of free Cu^{2+} ions on $\text{Cu}(\text{OH})_2$ -treated leaves increased with time after spray application to a maximum concentration of approximately 100 ppb after about 10–20 days following treatment. No cells of copper-sensitive strains of *Pseudomonas syringae* survived after application to

bean or navel orange leaves containing more than about 50 ppb of Cu^{2+} . In contrast, 10% or more of the cells of copper-tolerant strains survived on leaves containing up to 100 ppb of free Cu^{2+} , and nearly all of the cells of tolerant strains survived on leaves containing lesser amounts of free Cu^{2+} . Strains of *P. syringae* survived only at Cu^{2+} concentrations on leaves at or below the LC_{50} for Cu^{2+} for these strains determined in distilled water in vitro. Thus, even though very low concentrations of Cu^{2+} are present on leaves, copper-sensitive strains are killed by the concentrations of free Cu^{2+} present on leaves. However, the concentration of free Cu^{2+} on leaves is only slightly less than that tolerated by copper-tolerant strains of *P. syringae* in vitro. Soluble but complexed forms of copper, while abundant on leaves, have no significant toxicity towards strains of *P. syringae*.

Sprays of cupric hydroxide, Bordeaux mixture, and various formulations of basic copper sulfate have been widely used for the control of bacterial diseases of plants and for reducing plant frost injury caused by ice nucleation active bacteria (11,12,17,19–21). *Pseudomonas syringae* is a target for chemical control because it is an important bacterial plant pathogen and can cause frost injury to plants by contributing ice nuclei active at temperatures above -5 C (11,18,22,24). Although the efficacy of copper compounds for bacterial disease control is low and variable between locations (11,12,20), these compounds are widely used for this purpose.

Resistance to copper has been observed recently among both saprophytic and phytopathogenic bacteria (1,2,8,13,20,24,25). Some strains of *Xanthomonas campestris* pv. *vesicatoria*, *P. s. tomato*, and *P. s. syringae*, pathogens of pepper, tomato, and cherry, respectively, recently have been found to be resistant to concentrations of copper ions that are sufficient to kill sensitive strains of these species (1,8,20,25). Copper-tolerant phytopathogenic bacteria exhibit a quantitative, rather than a completely

qualitative, resistance to copper. Copper-resistant strains tolerate from 10 to 80× higher copper concentrations than do sensitive strains of the same species (1,2,8,9,15,20,23–25). Copper-tolerant strains are poorly controlled by standard applications of copper compounds in some areas (20). Many strains of *P. syringae* isolated from tree fruits in California exhibit high levels of tolerance to copper ions in culture (2,3). Copper-tolerant strains of *P. syringae* established on plant surfaces were not killed by registered rates of $\text{Cu}(\text{OH})_2$ or Bordeaux mixture in greenhouse and field trials (3). Similarly, copper-tolerant strains grew as rapidly and achieved the same population size on copper-treated plants as on nontreated plants. Copper-sensitive strains were killed under the same conditions. Growth rate or population size of copper-tolerant strains on leaves was not decreased even when the amount of copper applied on leaves was increased up to eight times the registered rate.

In the accompanying paper, we describe the direct relationship of the bactericidal effects of copper compounds in complex growth media to the concentration of free copper ions. On leaf surfaces, small quantities of copper salts are solubilized when leaves are wetted by rain or dew, but the copper ions are probably complexed with organic compounds leached from leaf surfaces (4). While

free copper ions are considered more toxic to microorganisms than complexed forms of this metal (4,6,7,13,28), neither the concentrations of free ionic copper and complexed forms of copper on copper-treated leaves nor the possible toxicity of copper complexes to bacteria are known. We found no evidence for significant toxicity of the complexes copper formed with the organic compounds found in casein hydrolysate, nor with organic acids such as citrate. Information on the concentration of ionic copper on copper-treated leaves and the possible toxicity of organic copper complexes is needed to better understand the relationship between the amount of toxic copper available on treated plants and the sensitivity of strains of *P. syringae* to such toxicants.

In the present study we investigated the chemical forms of copper on plants treated with two commonly used bactericides, $\text{Cu}(\text{OH})_2$ and Bordeaux mixture. Studies were conducted on field-grown citrus trees and bean plants where the age of the copper deposits, amount of applied copper bactericide, cumulative rainfall, and chemical type of copper compound were varied to determine the effects of each of these factors on the bactericidal forms of copper. We report here on the soluble forms (free ionic and complexed) and total amount of copper on the copper-treated leaves as well as the survival of strains of *P. syringae* differing in copper tolerance in vitro on leaves varying in amount of ionic copper.

MATERIALS AND METHODS

Design of field experiments. Mature navel orange (*Citrus sinensis* (L.) Osbeck 'Washington') trees and snap bean (*Phaseolus vulgaris* L. 'Bush Blue Lake 274') plants were used to determine the chemical forms of copper on leaves treated with copper bactericides and their relation to the differential survival of copper-tolerant and copper-sensitive bacterial strains on treated leaves. Navel orange trees (about 4 m in height), located at the University of California Lindcove Field Station near Exeter, were sprayed in January 1987 with 1) a low rate of cupric hydroxide (Kocide 101, Kocide Chemical Company, Houston, TX, 1.2 g/L of formulated product); 2) a high rate of $\text{Cu}(\text{OH})_2$ (3.6 g/L of formulated product); and 3) Bordeaux mixture ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 9.6 g/L, and $\text{Ca}(\text{OH})_2$, 9.6 g/L). The surfactant Triton CS-7 (Rohm and Haas Company, Philadelphia) was added (6.6×10^{-2} ml/L) to all sprays to facilitate coverage of leaf surfaces. All sprays were applied to runoff (about 22 L per tree) using a piston-powered sprayer. Treatments were replicated five times (one tree per replication) in a randomized complete block design. One treatment consisted of trees sprayed with $\text{Cu}(\text{OH})_2$ (1.2 g/L) that were subjected to periodic overhead sprinkling to simulate rainfall in excess of the ambient. Microsprinklers that deposited about 2 cm of water within a 4-h period were placed 2 m above trees and were activated once per week starting 1 wk following treatment of trees with $\text{Cu}(\text{OH})_2$ for a period of 21 wk. Healthy, dry, uninjured leaves were collected randomly from all trees, stored at 5 C, and used within 24 h for bioassays or chemical analyses. Bean plants were sprayed in June 1987, 6 wk after planting at a site on the University of California, Berkeley, campus. All treatments were the same as those used in trials on navel orange except that chemicals were applied using a hand-held air pressurized sprayer, the high rate of $\text{Cu}(\text{OH})_2$ used was 4.8 g/L, the 2 cm/wk of simulated additional rainfall (as distilled water) was applied using a sprinkler can held at a height of about 1.5 m above plants, and the Bordeaux mixture was not applied. All treatments were replicated four times in a randomized complete block design. Dry leaves were collected randomly from all the plants in a replication, stored at 5 C, and used within 24 h for bioassays or chemical analyses.

Collection of leaf surface water. Approximately 20 g fresh weight of leaves of each replication of each treatment was placed on a nylon screen in a dew chamber. The dew chamber consisted of a box of about 1 m on a side with walls fabricated of aluminum foil. The cubicle was placed in a cold room at 5 C. Hot water (60–65 C) was circulated in a water bath placed 50 cm under the screen. Temperature inside the chamber equilibrated at

approximately 18 C. The evaporated water condensed on the leaf surfaces due to their radiational cooling. Leaves were removed from the chamber after 9 h, a typical duration for dew at the field site, and the free water on leaf surfaces was collected by centrifugation at 1,400 g for 10 min at 5 C. No obvious injury to leaves resulted from this low-speed centrifugation. To relate the volume of leaf water extract obtained to the leaf surface area, the surface areas of replicate samples of 10 leaves were measured, the leaves were washed, and the linear relationship of leaf weight to leaf surface area was obtained.

Analytical methods. Leaf surface water collections from bean leaves were filtered through a 0.2 μm membrane filter immediately after collection to remove the particulate copper. More than 98% of the particles of $\text{Cu}(\text{OH})_2$ in Kocide 101 are larger than this diameter (Kocide Chemical Company, product information). The water collections from citrus leaves were less than those from bean leaves, so the particulate copper in leaf water collections from citrus was removed by centrifugation for 10 min at 12,800 g in an Eppendorf microcentrifuge. Dissolved copper in a filtered sample was only approximately 5% less than in a centrifuged sample of the same origin. Copper ion concentrations of collected leaf surface water were measured by a cupric ion-specific electrode (Orion model 94-29, Orion Research Inc., Boston, MA) and the recommended reference electrode (Orion model 90-02), which were connected to an Orion model 701-pH/mV meter. Five to 10 ml of sample was used, and the ionic strength of all solutions was adjusted to 1 M with a 5 M NaNO_3 solution. In the concentration range of 1 ppb to 1 $\mu\text{g}/\text{ml}$ of Cu^{2+} (pCu 7.79–4.79; $\text{pCu} = -\log_{10}[\text{Cu}^{2+}]$), serial dilutions of a standard 1,000- $\mu\text{g}/\text{ml}$ $\text{Cu}(\text{NO}_3)_2$ stock solution in distilled deionized water (Beckman Nanopure II, Beckman Instruments, Inc., Fullerton, CA) were used to prepare a standard calibration curve. Copper ion concentrations lower than 1 ng/ml were obtained with Cu^{2+} buffers with known pCu values (pCu 7–13), according to the method of Hansen (14). Calibration curves relating millivolt potential and copper ion concentration were linear ($r = 0.980$ and $r = 0.960$) for copper concentrations in the range of pCu 7.79–4.79 and pCu 7–13, respectively. A new calibration curve was prepared daily. During the measurement of electrode potentials, constant magnetic stirring with a Teflon-covered microbar was applied, and the temperature was maintained at 25 ± 1 C. All glassware and polypropylene containers used were washed with 10% HCl and rinsed with deionized distilled water. The electrode-sulfide membrane surface area was occasionally polished with polishing strips (Orion no. 94-82-01), followed by dipping the electrode in a standard $\text{Cu}(\text{NO}_3)_2$ solution for 5 min, then 0.1 M EDTA for 5 min, and rinsing carefully with distilled water. The electrode response time improved substantially after electrode surface polishing. The electrode potential response was fast (0.5–2 min) at copper ion concentrations greater than pCu 7, but decreased to 5–15 min at pCu 7–13. After measurement of ionic copper, samples were acidified with 50% HNO_3 (3 ml/L) and stored at 4 C for later analysis of total dissolved copper.

Concentrations of total dissolved copper in leaf surface water collections were measured by atomic absorption spectroscopy using a Perkin-Elmer model 2280 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) with an air-acetylene flame. Samples were analyzed directly or after dilution with acidified distilled deionized water (HNO_3 , 3 ml/L). Standard copper solutions of 0.25–10 $\mu\text{g}/\text{ml}$ of Cu^{2+} were prepared by serially diluting a 1,000 $\mu\text{g}/\text{ml}$ copper standard $\text{Cu}(\text{NO}_3)_2$ stock solution with acidified distilled deionized water. The calibration curve relating absorbance at 324.7 nm with copper concentration was linear within these limits ($r = 0.998$). The lower limit of detection using atomic absorption spectroscopy was 0.1 mg of Cu^{2+}/L .

To determine the total amount of copper deposited on the leaves, 20 leaves were selected randomly from each replicate sample. Forty 3.6-cm² leaf disks (two from each leaf) were cut randomly from the leaves with a cork borer. The disks were dried at 70 C for 24 h, weighed, and ground with a mortar and pestle. For each sample, two 0.1-g subsamples were weighed, transferred

to Pyrex screw-cap tubes, and digested with 2 ml of concentrated HNO_3 for 24 h at 75 C in a heating block. All glassware was washed with 10% HCl and rinsed with glass-distilled, deionized water before use. The digested samples were diluted to 10 ml with distilled deionized water, transferred into polypropylene centrifuge tubes, and centrifuged for 1 h at 12,000 g to precipitate all insoluble material. The clear supernatant was transferred into clean test tubes and the copper concentration measured by atomic absorption spectroscopy as described above. Standard solutions were prepared by diluting a 1-mg/ml $\text{Cu}(\text{NO}_3)_2$ stock solution with 20% HNO_3 . Blank samples containing only reagents were analyzed to check for copper contaminants in the reagents and on the glassware on every occasion. All estimates of copper content of test samples were corrected for the copper detected in blank control samples (less than approximately 1% of the measured amount of copper in all test samples).

Bacterial cultures. Rifampicin-resistant, copper-tolerant strains A1513R, A1489, A1444, and B728a of *P. syringae* isolated from almond trees and bean plants, with LC_{50} for Cu^{2+} in distilled water of 183.8, 157.0, 138.0, and 18.1 ng/ml, respectively, were used in bioassays of copper toxicity on leaves. Rifampicin-resistant, copper-sensitive *P. syringae* A1487, A1539, A1555, A1563, and A1423 isolated from almond trees, with LC_{50} for Cu^{2+} in distilled water of 6.8, 3.1, 5.1, 4.2, and 3.7 ng/ml, respectively, also were used. The origin and other characteristics of these strains have been described previously (3). All strains were stored in 15% glycerol at -80 C. Cultures were grown first on King's medium B (16) containing 100 $\mu\text{g}/\text{ml}$ rifampicin (KBR) and then transferred onto copper-amended casitone-yeast extract-glycerol agar (CYE) (28). Copper-sensitive strains were grown for 24 h at 30 C on CYE medium containing 1 $\mu\text{g}/\text{ml}$ of added Cu^{2+} (as $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$) and then were transferred onto CYE medium containing 10 $\mu\text{g}/\text{ml}$ of added Cu^{2+} . Copper-tolerant strains were initially grown on CYE medium amended with 10 $\mu\text{g}/\text{ml}$ of Cu^{2+} and then transferred to CYE amended with 50 $\mu\text{g}/\text{ml}$ of Cu^{2+} . Suspensions of cells (2×10^5 cells per milliliter) in sterile distilled water were prepared from fresh cultures and used for tests of bacterial survival on leaf surfaces.

Bacterial survival on copper-treated leaves. The survival of strains of *P. syringae* was determined on excised navel orange and bean leaves. Six leaves from each replicate leaf sample to be uniformly moistened were placed for 1 h in the same dew chamber used for the collection of leaf surface water extract. Each moist leaf was placed on a wet filter paper in a 9-cm glass petri dish. Five 10- μl droplets of a bacterial suspension were placed on each moist leaf and incubated at 21 C for 2 h. Each leaf was then immersed in a test tube containing 10 ml of sterile distilled water and mixed. One hundred microliters of the solution was spread uniformly on the surface of KBR medium containing 100 $\mu\text{g}/\text{ml}$ of cycloheximide and 50 $\mu\text{g}/\text{ml}$ of benomyl (Benlate, Dupont Chemical Co., Wilmington, DE) and incubated at 21 C. The number of colonies was counted on each plate after 3-5 days. The number of bacterial cells that survived on the copper-treated leaves was expressed as the percentage of the cells that survived exposure on leaves from control plants that were not treated with copper compounds.

Statistical methods. Statistical computations were made by using software provided by Statistical Analysis Systems (release 5.16; SAS Institute Inc., Cary, NC). The SAS means procedure was used to perform analysis of variance on cell survival and chemical deposits.

RESULTS

Weather conditions during field experiments. During the field experiments on navel orange trees from January to June 1987, rainfall totaling 34 mm occurred during the period from 10 to 30 days following spray application of copper compounds. Rainfall totaling 60 mm also occurred between 30 and 60 days following chemical treatment. No rainfall occurred 60 days or more after treatment. No rainfall occurred during the 40 days after bean plants were sprayed with copper compounds. The mean daily

air temperatures during the months of January to June at Exeter, CA, were 6.1, 10.5, 13, 15.6, 18.3, and 22.2 C, respectively. The mean air temperature at Berkeley, CA, during experimentation on bean plants was about 18.5 C.

Chemical forms of copper on leaves. Both total soluble and free ionic copper were determined in the surface water collected from copper-treated leaves. Free water that was deposited on leaves in the dew chamber approximated the amount of natural dew that formed on the leaves in the field. An equilibrium condition presumably existed between the amount of applied copper that is solubilized and the amount of leaf exudates in this moisture.

The solubilized copper that was measured in leaf surface water included all of the soluble forms of copper, including inorganically or organically complexed as well as free ionic copper. Dissolved copper concentrations in the leaf surface water, determined by atomic absorption spectroscopy, were 1,000-10,000 times higher than the free ionic copper concentrations (Figs. 1 and 2). For this reason, estimates of soluble copper are presented without correction for the amount of ionic copper measured in the samples. The amount of dissolved copper on either navel orange trees or bean plants gradually increased with time after the application of a copper spray, irrespective of the amount of copper applied or whether plants received supplemental precipitation (Figs. 1 and 2). The amount of dissolved copper increased during the first month after the spraying of citrus leaves and then decreased gradually on leaves treated with a low rate of copper and on leaves from sprinkled trees. The decrease in amount of soluble copper 1 mo after spraying was most pronounced on trees treated with the high rate of $\text{Cu}(\text{OH})_2$ (Fig. 1). The amount of dissolved copper on bean leaves increased for about 15-20 days after treatment with $\text{Cu}(\text{OH})_2$ and then decreased slightly and remained constant for at least the next 20 days (Fig. 2).

The amount of dissolved copper in the leaf surface water on navel orange represented up to 25% of the total amount of copper deposited on the leaf surface (Fig. 1), while it was less than about 10% of the total copper on bean plants (Fig 2). The amount of dissolved copper on both bean and navel orange treated with a low rate of $\text{Cu}(\text{OH})_2$ was similar whether the plants were given supplementary precipitation or not, but was always lower than that on plants treated with a high rate of $\text{Cu}(\text{OH})_2$ (Figs. 1 and 2). The amount of dissolved copper on navel orange leaves treated with Bordeaux mixture was less than that on leaves from trees receiving any other copper treatment (Fig. 1).

The concentration of soluble ionic copper in the leaf surface water of copper-treated navel orange and bean leaves was very low, always less than 0.1 $\mu\text{g}/\text{ml}$ ($\text{pCu} > 5.5$) (Figs. 1 and 2). The ionic copper is expressed in pCu units (the negative logarithm of the molar concentration of cupric ions) because of the low concentrations and the wide range of concentrations observed on leaves. Only a small fraction, less than about 0.1%, of the dissolved copper on leaves was in an ionic form. The concentration of ionic copper on leaves of both navel orange and bean tended to decrease slowly with time 10 or more days following treatment with copper compounds. Ionic copper concentrations were lower on navel orange and bean leaves that received supplemental precipitation, but concentrations were not increased substantially by increasing the amount of applied $\text{Cu}(\text{OH})_2$ (Figs. 1 and 2). The ionic copper concentration on Bordeaux-treated navel orange leaves was considerably lower than on leaves treated with $\text{Cu}(\text{OH})_2$ (Fig. 1).

Total amount of copper on treated leaves decreased with time after spray treatment (Figs. 1 and 2). Amount of copper detected on plants shortly after treatment with copper compounds was approximately proportional to the concentration of copper in spray mixtures. For example, the total amount of copper detected on navel orange leaves 10 days after they were treated with the low rate of $\text{Cu}(\text{OH})_2$ was about 30% that on leaves treated with a rate of $\text{Cu}(\text{OH})_2$ that was 3 \times higher (Fig. 1). A similar dosage-dependent recovery of copper from treated bean leaves also was observed (Fig. 2). The rate of loss of total copper deposits on navel orange leaves decreased with increasing time after spray treatment (Fig. 1). One-half of the total amount of copper

remaining on both citrus and bean leaves was lost approximately every 45 days, irrespective of the amount of copper compound initially deposited and whether the leaves received supplemental precipitation or not. Approximately 50% of the $\text{Cu}(\text{OH})_2$ initially deposited on bean leaves was removed from leaves within about 35 days of spray treatment irrespective of the application of artificial precipitation (Fig. 2).

Bacterial survival on copper-treated leaves. Copper-tolerant strains of *P. syringae* survived much better on leaves receiving a given copper treatment than did copper-sensitive strains. No cells of copper-sensitive strains and generally less than 50% of the cells of copper-tolerant strains survived after placement on navel orange or bean leaves within about 10 days of copper treatment (Figs. 3 and 4). The fraction of cells of all strains that survived on copper-treated leaves increased with time after spray

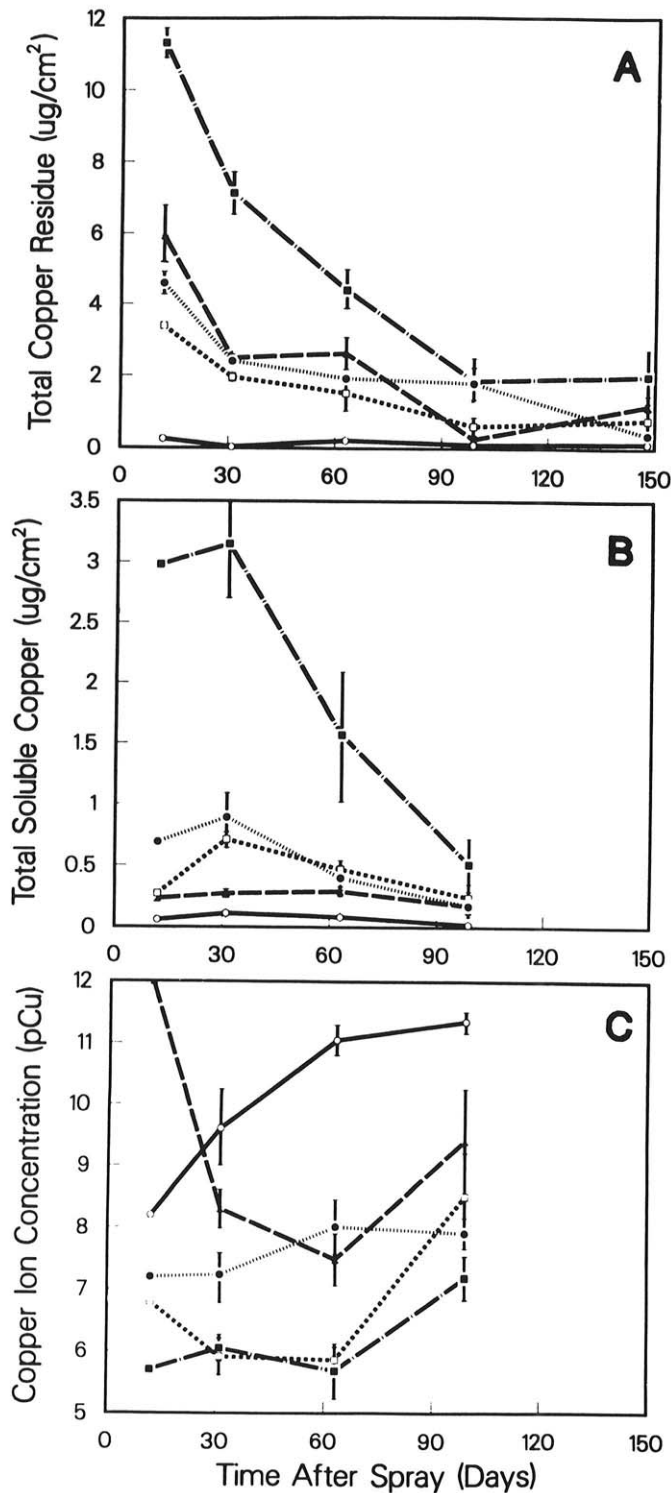


Fig. 1. A, Total amount of copper; B, amount of dissolved copper; and C, concentration of free Cu^{2+} on copper-treated navel orange leaves as a function of time after spray application of 1.2 g/L of $\text{Cu}(\text{OH})_2$ (□ and ●), 3.6 g/L of $\text{Cu}(\text{OH})_2$ (■), Bordeaux mixture (▲), and on trees sprayed with surfactant alone (○). Some trees also received supplemental application of precipitation (●). The vertical bars represent the standard error of mean copper concentrations.

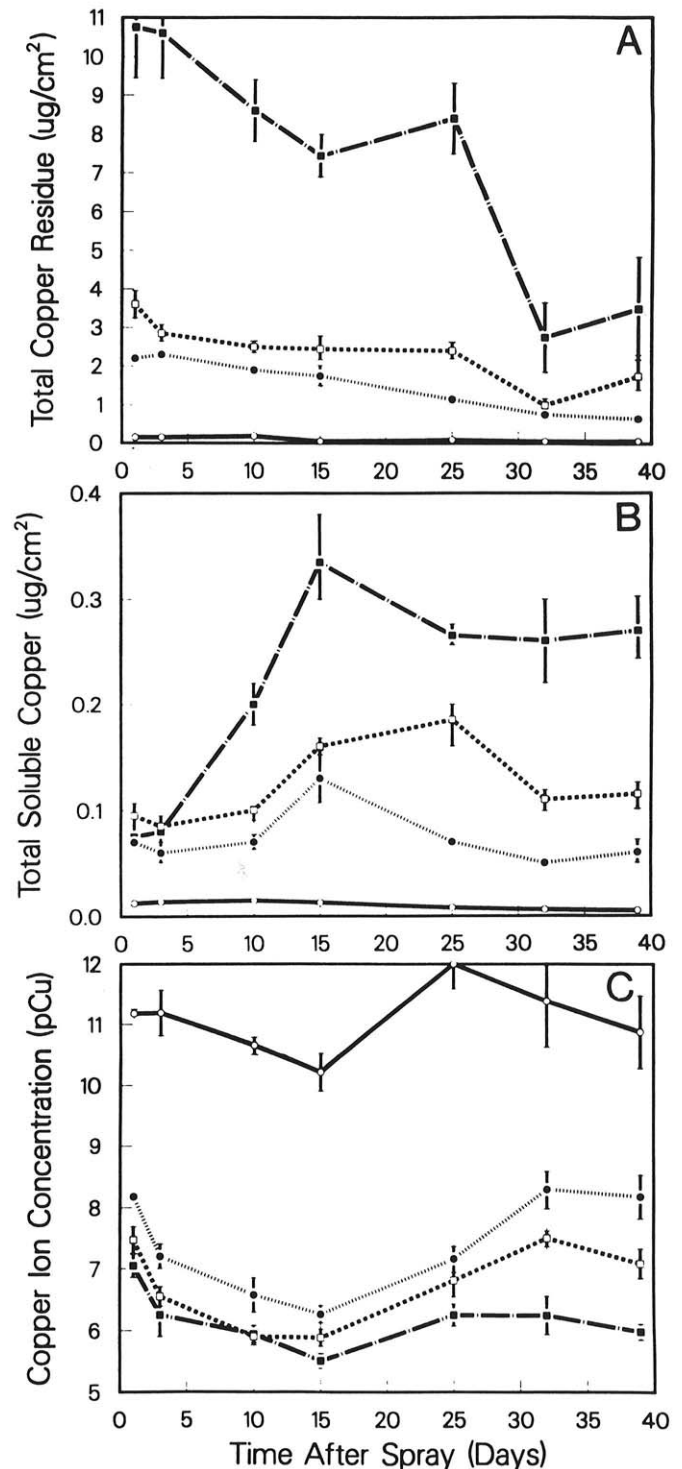


Fig. 2. A, Total amount of copper; B, soluble but complexed; or C, free Cu^{2+} ions on bean leaves treated with 1.2 g/L of $\text{Cu}(\text{OH})_2$ and subjected to normal weather conditions in the field (□) or sprinkled with distilled water at the rate of 2 cm per week (●), sprayed with $\text{Cu}(\text{OH})_2$ at a rate of 4.8 g/L (■) or left unsprayed (control) (○), as a function of time after spray application of copper compounds. The vertical bars represent the standard error of mean copper concentrations.

treatment (Figs. 3 and 4). Only 3 mo after a single application of either a low or high rate of $\text{Cu}(\text{OH})_2$ to navel orange leaves did more than 10% of the cells of copper-sensitive strains of *P. syringae* survive (Fig. 3). In contrast, more than 50% of the cells of copper-tolerant strains survived on trees treated with a low rate of $\text{Cu}(\text{OH})_2$ within about 30 days, while more than 10% of the cells of copper-tolerant strains survived during this period on trees treated with a high rate of $\text{Cu}(\text{OH})_2$ (Fig. 3). Fewer

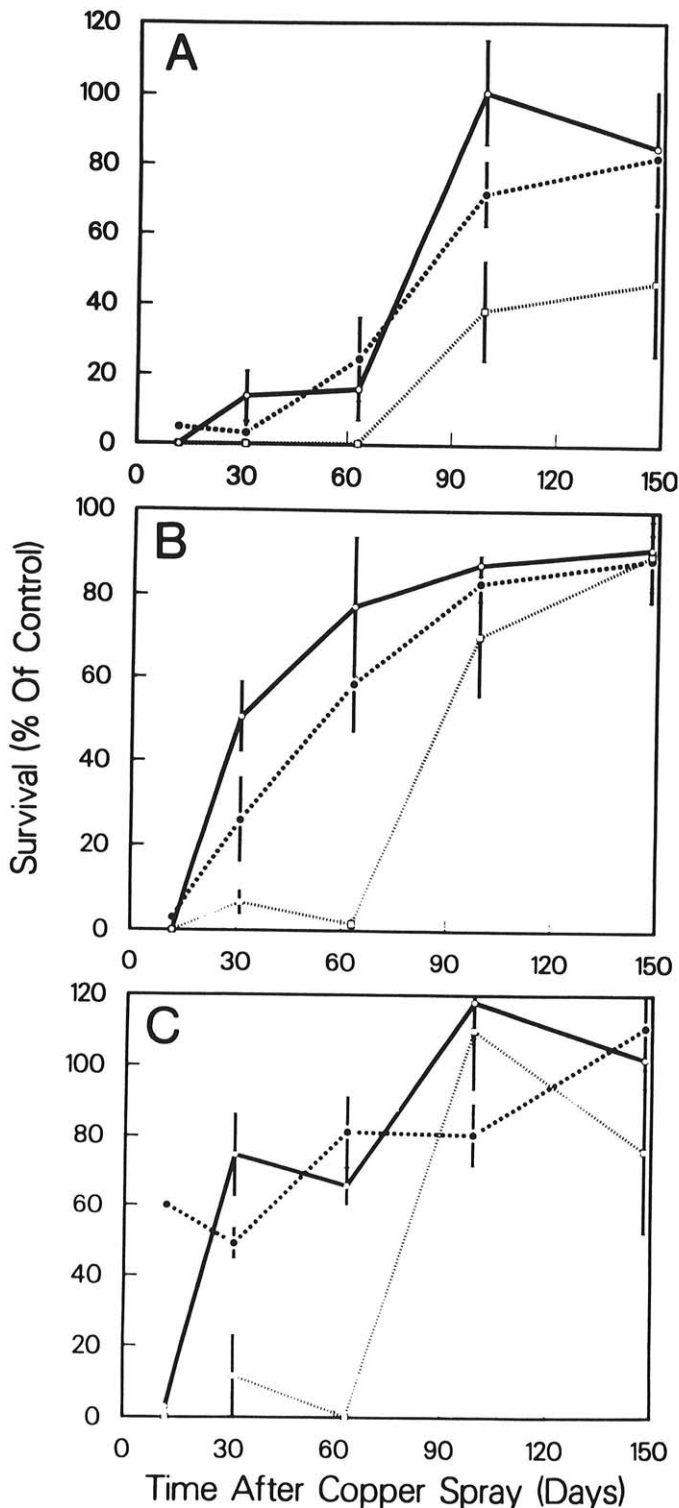


Fig. 3. Survival of cells of copper-tolerant strains Al513R (○) and Al489 (●), and copper-sensitive strain Al487 (□) of *Pseudomonas syringae* on copper-treated navel orange leaves as a function of time after spray application of A, 3.6 g/L of $\text{Cu}(\text{OH})_2$, or B and C, 1.2 g/L of $\text{Cu}(\text{OH})_2$ to trees, or C, application of 2 cm of supplemental precipitation per week. The vertical bars represent the standard error of mean cell survival.

than 50% of the cells of copper-sensitive strains survived when placed on navel orange leaves even 5 mo following application of a high rate of $\text{Cu}(\text{OH})_2$ (Fig. 3A). Over 10% of the cells of copper-tolerant strains survived exposure to bean leaves that were treated with a high dosage of $\text{Cu}(\text{OH})_2$, while few cells of copper-sensitive strains survived on these leaves, even 40 days after treatment (Fig. 4). In contrast, the majority of cells of copper-tolerant strains survived on such plants 40 days or more after treatment with $\text{Cu}(\text{OH})_2$ (Fig. 4). Application of supplemental precipitation increased the survival of strains on both citrus and bean leaves (Figs. 3 and 4). Both copper-tolerant and copper-sensitive strains survived slightly better on leaves that received supplemental precipitation compared with leaves subjected to normal weather conditions in the field.

Quantitative relationship between ionic Cu^{2+} concentrations on leaves, in vitro copper sensitivity, and survival of bacteria on leaves. A large number of strains of *P. syringae* differing in LC_{50} for Cu^{2+} in distilled water in vitro were placed on navel orange or bean leaves having different concentrations of free Cu^{2+} because of different ages of $\text{Cu}(\text{OH})_2$ deposits, application of supplemental precipitation, or difference in amount of $\text{Cu}(\text{OH})_2$ initially applied. The cells of a given strain survived on leaves only at Cu^{2+} concentrations at or below the LC_{50} for the strain (Figs. 5 and 6). When the free Cu^{2+} ion concentration in leaf surface moisture was greater than the LC_{50} for a strain determined in vitro, few if any of the cells of that strain survived for even 1 h on copper-treated navel orange or bean leaves (Figs. 5 and 6). Conversely, when the Cu^{2+} concentration measured on a leaf was less than the LC_{50} for a strain, at least 10% of the cells of that strain survived. The fraction of cells of a given strain that survived exposure to $\text{Cu}(\text{OH})_2$ -treated leaves progressively increased as the concentration of Cu^{2+} ions on leaves decreased to a lower fraction of the LC_{50} for that strain as determined in vitro (Figs. 5 and 6); when the free Cu^{2+} ion on leaves was tenfold less than the LC_{50} for that strain, about 80% of the cells of that strain survived exposure to the leaves.

The maximum concentration of free Cu^{2+} measured on leaves (approximately pCu 5.6) was only slightly less than the in vitro-measured LC_{50} for the several copper-tolerant strains of *P. syringae* that were tested (pCu 5.3–5.6). In contrast, the maximum Cu^{2+} concentration on leaves was over 30 times higher than the LC_{50} for most copper-sensitive strains tested (approximately pCu 7.0) (Figs. 1, 2, 5, and 6).

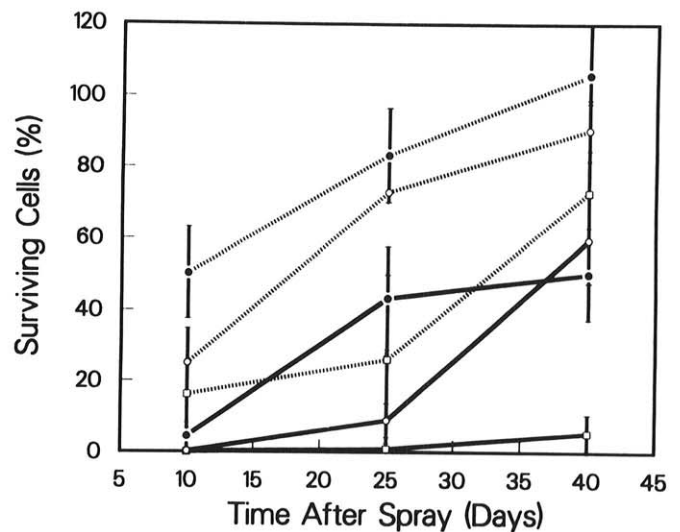


Fig. 4. Survival of cells of copper-tolerant strain Al513R (dashed lines) and copper-sensitive strain Al487 (solid lines) of *Pseudomonas syringae* on leaves of bean as a function of time after spray application of 4.8 g/L of $\text{Cu}(\text{OH})_2$ (□) or 1.2 g/L of $\text{Cu}(\text{OH})_2$ (○ and ●) to plants, or application of 2 cm of supplemental precipitation each week (●). The vertical bars represent the standard error of mean cell survival.

DISCUSSION

This study represents the first attempt to relate the chemical forms of copper that are present on leaves treated with copper-containing bactericides with the bactericidal activity of these compounds. While other studies have described the dynamics of copper residues on treated trees (22), no attempt was made to ascertain the various chemical forms in which copper might exist. We undertook this study to ascertain whether the sole chemical form of copper that is toxic to bacteria while on copper-treated leaves is the ionic form, as we had found in vitro (21). While previous studies have addressed the solubilization of copper from insoluble deposits of Bordeaux mixture and other insoluble copper salts (5), no attempt was made to determine whether the soluble form of copper was toxic to bacteria. We have found no evidence that complexed forms of copper are toxic to bacteria in vitro (21). Therefore, precise measurement of the content of uncomplexed Cu^{2+} on leaf surfaces was undertaken to understand the bactericidal effects of application of insoluble copper salts to leaf surfaces.

Removal of total copper deposits from both navel orange and bean leaves under different environmental conditions in California was independent of the quantity of precipitation that impinged on the leaves. Removal of copper from leaves appeared to proceed with approximate first-order kinetics; that is, the amount of copper lost per unit time was proportional to the amount of copper remaining to be removed from the leaf. The first-order loss of copper with time from leaves would result in the quantity of copper remaining on leaves with time to asymptotically approach zero, as was most obviously observed in the case of copper-treated navel orange leaves (Fig. 1). An approximate half-life for the residency of total copper on treated navel orange leaves was approximately 45 days, while it was only about 35 to 40 days

on copper-treated bean leaves (Figs. 1 and 2). The similar apparent half-life of copper on treated navel orange and bean leaves was remarkable since the environmental conditions to which those leaves were exposed in the field differed dramatically. No rainfall impinged on treated bean leaves, while navel orange leaves received light rainfall on several occasions. The apparent half-life of 45 days of copper residues on treated navel orange leaves also was apparently similar both when rainfall did occur, as well as two or more months after treatment when rainfall in the field plot area ceased (Figs. 1 and 2). Application of supplemental precipitation to both navel orange and bean leaves did not substantially increase the rate of loss of copper residues from these leaves (Figs. 1 and 2). Supplemental rainfall was applied as droplets that fell only 1–2 m before striking treated leaves. Natural rainfall events at the field site consisted of light rainfall. Water drops striking leaves at low velocity may not be an efficient mechanism by which copper residues can be removed from leaves. Our rainfall events might differ substantially from vigorous rain showers that can occur elsewhere in the United States, such as in Michigan, where rainfall was strongly associated with the removal of copper deposits from treated cherry leaves (22). Because copper deposits were removed nearly as rapidly on leaves that never received precipitation, other factors such as wind or leaf abrasion may be more important in the removal of copper deposits in such environments.

Plant species and weather conditions greatly affected the percentage of total copper that existed in a solubilized form on treated plants. More than 10 times higher concentrations of solubilized copper were measured on navel orange leaves treated with similar amounts of $\text{Cu}(\text{OH})_2$ as bean leaves, even though treated citrus leaves were subjected to natural rainfall events that could have removed some solubilized copper (Figs. 1 and 2). Solubilization of copper on leaves is probably due to complexation with organic compounds leached from leaves (5), facilitating the dissociation

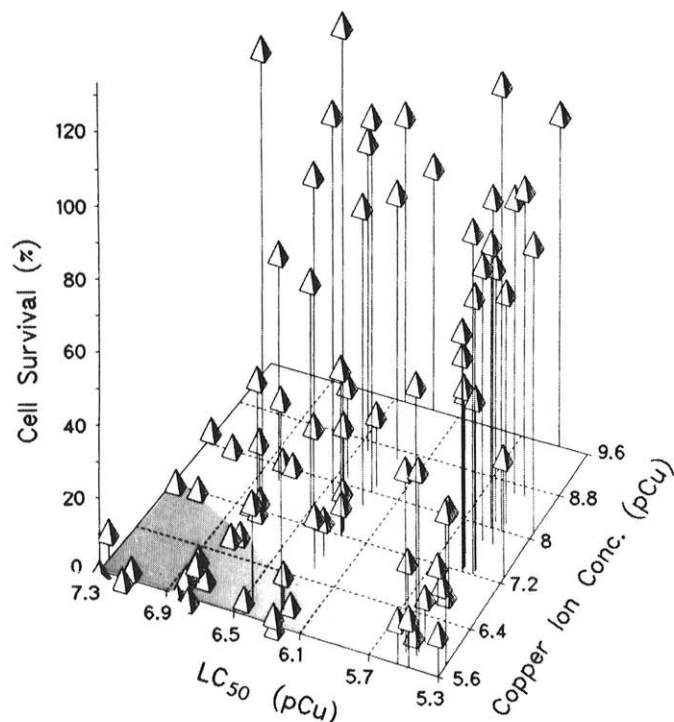


Fig. 5. Relationship between survival of strains of *Pseudomonas syringae* having different tolerances of Cu^{2+} ions in vitro (LC_{50} (expressed as pCu) ($\text{pCu} = -\log[\text{Cu}^{2+}]$) on navel orange leaves having different concentrations of free Cu^{2+} ions (expressed as pCu). Bacterial strains were applied to leaves that had varying amounts of copper ions due to different age of copper deposit and amount of $\text{Cu}(\text{OH})_2$ applied, as well as to unsprayed leaves. The number of viable cells of each strain recovered from copper-treated leaves was expressed as the percentage of the number recovered from unsprayed leaves. The shaded area represents the event arena in which the measured free Cu^{2+} concentration on leaves is more than the estimated LC_{50} in vitro for a given strain.

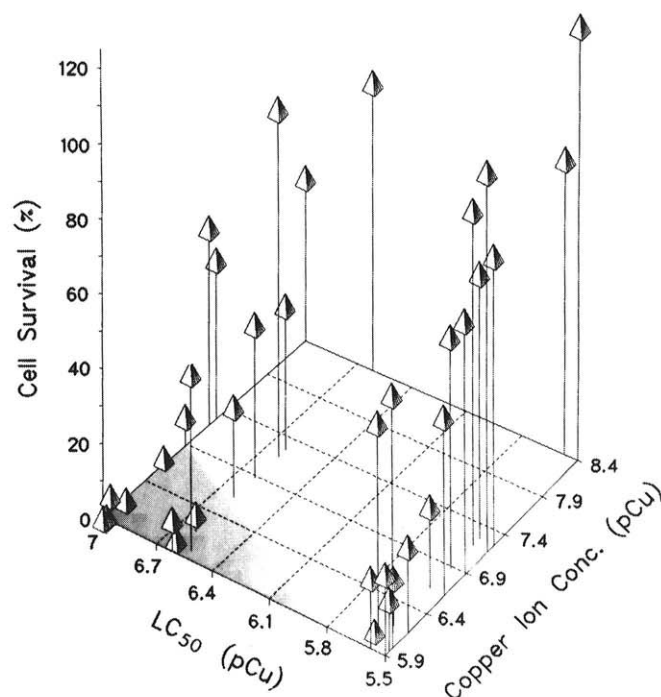


Fig. 6. Relationship between survival of strains of *Pseudomonas syringae* having different tolerance of Cu^{2+} ions in vitro (LC_{50} (expressed as pCu) ($\text{pCu} = -\log[\text{Cu}^{2+}]$) on bean leaves having different concentrations of Cu^{2+} ions (expressed as pCu). Bacterial strains were applied to leaves that had varying amounts of free copper ions due to differences in age of copper deposit, amount of $\text{Cu}(\text{OH})_2$ applied, and presence of supplemental precipitation as well as to unsprayed leaves. The number of viable cells was expressed as the percentage of the number recovered from inoculated unsprayed leaves. The shaded area represents the event arena in which the measured free Cu^{2+} ions on bean leaves exceeded the estimated LC_{50} in vitro for a given strain.

of the relatively insoluble copper salts by removal of free copper ion from the vicinity of the particulate copper. A previous study had indicated that copper-solubilizing agents were present even in successive water washings of leaves (5), indicating that leaf leachates that act as solubilizing agents are probably released continuously. The accumulation of solubilizing agents on leaves by leaching might account for the progressive increase in the total amount of solubilized copper observed in our studies (Figs. 1 and 2).

Navel orange and bean leaf surfaces appear to differ in the amount of complexing agents that they contain. There does not appear to be an excess of complexing agent relative to copper deposits on copper-treated bean leaves, unlike on navel orange leaves. Increasing the amount of $\text{Cu}(\text{OH})_2$ applied to bean leaves 4 \times did not increase the amount of measured soluble copper 4 \times ; instead, soluble copper increased only ≤ 2 (Fig. 2). In contrast, the amount of soluble copper present on copper-treated navel orange leaves was a rather constant percentage of the total amount of $\text{Cu}(\text{OH})_2$ applied to the leaf (Fig. 1). When the amount of $\text{Cu}(\text{OH})_2$ applied to navel orange leaves was increased by 3 \times , the amount of measured soluble copper also increased approximately 3 \times (Fig. 1). This also is consistent with the observation that a higher percentage of the total amount of copper applied to navel orange leaves was solubilized relative to that on bean leaves. Thus, processes such as diffusion of copper, rather than the amount of complexing agent present, may limit the amount of copper that is solubilized by leachates on the surface of navel orange leaves.

Only a very small quantity of noncomplexed ionic Cu^{2+} was measured on copper-treated navel orange and bean leaves. The quantity of free Cu^{2+} generally increased with increasing quantities of solubilized copper on treated leaves, but was present as a very small fraction of the total solubilized copper. A substantially higher fraction of the total soluble copper on bean leaves existed in an ionic form compared with navel orange leaves. For example, if we assumed that 1 ml of water from dew or precipitation would accumulate on leaves having a total surface area of 100 cm^2 , then approximately 10 $\mu\text{g}/\text{ml}$ of soluble copper would be present, as opposed to only 0.1 $\mu\text{g}/\text{ml}$ of free ionic copper (Fig. 2). Therefore, only approximately 1% of the total soluble copper on bean leaf surfaces exists in a free ionic state. By this reasoning, only approximately 0.1% of the soluble copper on navel orange leaves exists as free Cu^{2+} ions (Fig. 1). While numerous organic compounds have been found in leachates from different plants (10,26,27), quantitative evaluation of leaf surface leachates from neither bean nor navel orange has been performed. It is, however, likely that these two plants differ in the amount and composition of organic compounds that might act as complexing agents. For example, citrus leaves might be expected to contain rather high concentrations of different salts of citric acid. Sodium citrate is a very efficient chelator of Cu^{2+} . Higher amounts of compounds such as citrate on navel orange leaves could easily account for the higher total amount of solubilized copper as well as the lower fraction of ionic copper that exists in a noncomplexed state.

There is no obvious relationship between the total amount of copper deposited on leaves and the amount of free Cu^{2+} ions measured on navel orange or bean leaves. The concentrations of ionic Cu^{2+} on bean leaves that were treated with either high or low doses of $\text{Cu}(\text{OH})_2$ were very similar (Figs. 1 and 2). The amount of free Cu^{2+} on leaf surfaces will be largely determined by the equilibrium constants of the complexes and leaf surface pH, and not by the quantity of insoluble copper salts that are present. It is noteworthy that the concentration of free copper ions on both navel orange and bean leaves were remarkably similar. It was surprising to observe that the maximum concentration of ionic Cu^{2+} occurred on $\text{Cu}(\text{OH})_2$ -treated bean and citrus leaves several days after treatment (Figs. 1 and 2). It is possible that the dissociation of copper ions from the relatively few particles of insoluble copper salts that are deposited on leaves is the limiting factor in the accumulation of free Cu^{2+} on leaf surfaces. Complexation of Cu^{2+} ions at the surface of insoluble $\text{Cu}(\text{OH})_2$ or other insoluble salt deposits on leaf surfaces likely facilitate its dissociation.

Subsequent disassociation of Cu^{2+} from organic complexes in equilibrium with leaf surface pH and in competition with alternative complexing agents may determine the ultimate concentration of free Cu^{2+} on leaf surfaces.

The survival of strains of *P. syringae* was closely predicted by the measured concentration of ionic Cu^{2+} on leaves and the LC_{50} for Cu^{2+} ions measured in vitro. Strains that do not exhibit tolerance of copper are extremely sensitive to the presence of Cu^{2+} ions in vitro, exhibiting an LC_{50} for Cu^{2+} ions of less than about 5 ng/ml (3). While copper-tolerant strains can survive in the presence of substantially higher quantities of Cu^{2+} , they exhibit LC_{50} for Cu^{2+} in vitro of less than 100–200 ng/ml. The total amount of copper and the amount of soluble but complexed copper on $\text{Cu}(\text{OH})_2$ -treated leaves is greatly in excess of the LC_{50} even of copper-tolerant strains. However, the concentration of free Cu^{2+} present on sprayed bean and citrus leaves is extremely low, being only about tenfold higher than the LC_{50} of sensitive strains (Figs. 1 and 2). Therefore, under the conditions of our field experiments, the concentration of free Cu^{2+} within about 20 days of the treatment of leaves with $\text{Cu}(\text{OH})_2$ was sufficiently high that few if any copper-sensitive strains survived after being placed on treated leaves. However, as the concentration of free copper ions decreased by 20–40 days after spraying, insufficient ionic copper remained to kill all sensitive strains (Figs. 1, 2, and 4). It is noteworthy that the maximum concentration of free Cu^{2+} measured on $\text{Cu}(\text{OH})_2$ -treated navel orange or bean leaves (pCu 5.6) is very similar to the measured LC_{50} of Cu^{2+} to copper-tolerant strains in vitro. At these maximum Cu^{2+} concentrations on treated leaves, some, but not all, copper-tolerant strains were killed. Apparently, strains of *P. syringae* have not evolved more tolerance to free Cu^{2+} than has been needed to maintain at least some members of the population in the event of their exposure to maximum doses of $\text{Cu}(\text{OH})_2$ on treated plants. This suggests that there may be an ecological cost to the expression of copper tolerance among such strains. The relative fitness of copper-tolerant and copper-sensitive strains in the absence of strong selection in the presence of $\text{Cu}(\text{OH})_2$ has not been demonstrated, but is crucial to a better understanding of the likelihood of continued dispersal and retention of tolerant strains on crops where copper bactericides are only occasionally used for disease or frost control.

Complexed forms of copper that occur on $\text{Cu}(\text{OH})_2$ -treated leaves apparently have little if any toxicity to strains of *P. syringae*. Since complexed forms of copper exist as 100 and 1,000 \times excesses on bean and navel orange leaves, respectively, then much less than 50% of the cells of a given strain of *P. syringae* would be expected to survive if even a small percentage of such complexed forms are toxic to the strains. In fact, the fraction of cells that survived at a given Cu^{2+} concentration was very close to that predicted from measurements of the toxicity of Cu^{2+} in distilled water solutions in vitro (Figs. 5 and 6).

Procedures other than increasing the amount of $\text{Cu}(\text{OH})_2$ applied to plants will be necessary to control copper-tolerant strains of *P. syringae*. Increasing the concentration of $\text{Cu}(\text{OH})_2$ applied to plants to three times the recommended rate did not appreciably increase the concentration of free Cu^{2+} on leaves, nor did it decrease appreciably the fraction of copper-tolerant strains that survive application to $\text{Cu}(\text{OH})_2$ -treated plants. Since free Cu^{2+} appears to be the only toxic form of copper on $\text{Cu}(\text{OH})_2$ -treated plants, and since only a very small percentage of the solubilized copper on plants exists as a free ion, measures designed to control copper-tolerant bacteria might profitably address factors that affect the complexation of copper on leaf surfaces. For example, if it did not cause phytotoxicity, the pH of leaf surfaces could be reduced sufficiently to increase the dissociation of Cu^{2+} from insoluble $\text{Cu}(\text{OH})_2$ deposits and to affect the equilibrium that exists between complexed and free Cu^{2+} in aqueous solutions on leaves.

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