Increased Toxicity of Iron-Amended Copper-Containing Bactericides to the Walnut Blight Pathogen *Xanthomonas campestris* pv. *juglandis*

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ABSTRACT

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The addition of iron to fixed copper compounds enhanced the toxicity of these materials to the walnut blight bacterium *Xanthomonas campestris* pv. *juglandis* by several mechanisms. Iron altered the physiology of the bacterium, causing it to be more sensitive to cupric ions. In the presence of iron, less free copper ions were needed to inhibit the growth of bacteria. The addition of soluble iron salts to fixed copper compounds also reduced pH and increased the amount of free copper ions; addition of iron caused the release of more free copper ions in suspensions of Kocide 101 (77% $Cu(OH)_2$) than did hydrochloric acid at the same pH. The spraying of walnut leaves with Kocide 101 amended with iron salts significantly increased the availability of free copper ions on leaf surfaces compared to the bactericide alone. Cells of copper-sensitive strains of *X. c. juglandis* did not survive on leaf surfaces treated with Kocide 101, whereas more than 25% of the cells of copper-resistant strains survived. The addition of iron (50 μg/ml) in the form of $FeCl_3 \cdot 6H_2O$ to Kocide 101 increased the concentrations of free copper ions by more than 25-fold and eliminated copper-resistant strains on leaf surfaces. In field trials, combinations of Kocide 101 or Champion (77% $CU(OH)_2$) with iron significantly reduced the incidence of bud infestation and blighted leaflets compared to non-amended treatments.

Bacterial blight of Persian (English) walnuts, *Juglans regia* L., caused by *Xanthomonas campestris* pv. *juglandis,* is the most serious aboveground disease of walnuts in California, Oregon, and other states (2,3,15,27). All varieties grown in California are susceptible. Copper-containing bactericides, such as Kocide 101 and Champion, are commonly applied to control walnut blight (4,18,19,25,28). However, more than 25 yr of copper usage has caused a selection for copper-resistant strains of *X. c. juglandis.* Most walnut orchards are infested with pathogenic copper-resistant strains (13). This partly explains why growers often achieve poor disease control even though they follow recommended bactericide application schedules. Although the efficacy of copper-containing compounds for bacterial disease control is inadequate, there are no other chemical alternatives. There is a great need to improve the formulations and/or their toxicity.

The enhancement of copper toxicity by the addition of ethylenebisdithiocarbamate fungicides (EBDCs), such as maneb and mancozeb, has been reported in the control of *X. c. vesicatoria* and *Pseudomonas syringae* pv. *tomato* (9,14). Ethylenethiram monosulfide, which can be stabilized by copper, was shown to be an antibacterial component in the EBDCs (26). In addition, EBDCs have chelating abilities that may prevent copper ions from complexing with other organic substances, and they could increase the availability of free copper ions (10).

The inhibitory effects of copper on bacteria are dependent on the concentration of free copper ions (29). Copper-containing bactericides have low water solubilities. Copper ions are mobilized from bactericide deposits by carbon dioxide in rainwater, by microbial exudates, and by solubilizing agents in exudates from plant surfaces (5). The mobilized copper ions may be in a free form or chelated by organic compounds, in which form they lose toxicity to bacteria (17). Copper-resistant strains of *X. c. juglandis* show a quantitative, rather than a qualitative resistance to copper. Increasing the toxicity and/or the availability of free copper ions still may effectively control copper-resistant strains. Accordingly, finding ways to mobilize more copper ions and to keep them in a free form would improve the efficacy of copper-based bactericides.

This paper reports on the effects of iron in enhancing the toxicity of copper to *X. c. juglandis* and the use of iron-amended fixed copper compounds to improve the effectiveness of bactericides in controlling walnut blight disease.

MATERIALS AND METHODS

Bacterial strains and media for testing toxicity of copper. Copper-sensitive and -resistant strains of *X. c. juglandis* were isolated from buds and twig cankers from different walnut cultivars grown in northern California with the Brilliant Cresyl Blue-starch semiselective medium (20). Pathogenicity was confirmed on seedlings of English walnut (cultivar Ashley) grown in a greenhouse. Walnut leaves were inoculated with bacterial suspensions (10^7 cfu/ml) by the Carborundum method (11). To test the toxicity of copper to the bacterium, serial dilutions of bacterial suspensions were spread over the surface of casitone-yeast extract (CYE)-glycerol agar medium (29) containing different concentrations of copper and/or other metal ions. Copper and iron, in the form of $CuSO_4 \cdot 5H_2O$ and $FeCl_3 \cdot 6H_2O$ respectively, were added to media. One microgram of cupric ions per milliliter of distilled water is equivalent to 61.8 μM of $CuSO_4 \cdot 5H_2O$, which consists of 25.5% copper; 1 μg of iron per milliliter is equivalent to 86.8 μM of $FeCl_3 \cdot 6H_2O$, which consists of 20.7% iron. Seeded media were incubated at 28 C. Colonies were counted after 4–6 days and were expressed as the percentage recovery of colony-forming units derived by comparing the number of colonies recovered on CYE-glycerol medium with added ions to the number recovered on CYE-glycerol medium without amendments. The experiment was repeated twice with four replicates per concentration of copper and/or other metal ions. Data were pooled for analysis.

Measurement of free copper ions and soluble copper. Concentrations of free copper ions in media, in water suspensions, and on leaf surfaces were measured by an Orion model 94-29 cupric specific electrode (Orion Research Inc., Cambridge, MA) as described by Menkissoglu and Lindow (16). The cupric specific

electrode and the reference electrode (Orion model 90-01) were connected to an Orion model 701-pH/mV meter. The standard calibration curve was determined with solutions of Cu^{2+} (0.1–100 μg/ml) prepared from serial dilutions of a standard stock solution of 1,000 μg of $Cu(NO_3)_2$ per milliliter of double glass distilled water. A calibration curve of electrode potential and free copper concentration was linear ($r = 0.98$). A new calibration curve was prepared every day. One milliliter of sample was used, and the ionic strength of all solutions was adjusted to 1 M with a 5 M $NaNO_3$ solution, according to the manufacturer's protocol. The concentrations of free copper ions in the samples were measured by the electrode. Six replicate samples were used.

To determine whether iron interferes with the sensitivity of the electrode, the concentrations of copper ions also were measured by a plasma 40 emission spectrometer (Perkin-Elmer Corporation, Norwalk, CT) and compared with those measured by the electrode. The suspensions of Kocide 101 (77% $Cu(OH)_2$; Griffin Corporation, Valdosta, GA) amended with iron were centrifuged for 30 min at 14,000 rpm (Servall RC-20 centrifuge) to precipitate all insoluble material. The clear supernatant was transferred to clean tubes, and the concentrations of copper ions were measured by both the electrode as described below and a plasma 40 emission spectrometer with the inductively coupled plasma operated at 1.1-kw forward power, 1.6-mm flame height, 0.84–0.98 kg/cm^2 nebulized pressure, and a sample flow rate of 1.4 ml/min. The spectrometer was standardized with a stock solution of copper (10 μg/ml) in 20% nitric acid prepared by the serial dilution of a copper atomic spectral standard solution (1,000 μg/ml) (J. T. Baker Chemical Co., Philipsburg, NJ) (23).

For measurements of free copper ions on leaf surfaces, leaves of English walnut seedlings (cultivar Ashley) grown for 2–3 mo in a greenhouse were sprayed to runoff with an aqueous suspension of Kocide 101 (2.2 g/L) alone or with Kocide 101 amended with iron. Leaves were air-dried for 60 min, misted with distilled water, and incubated in a humid chamber held at near 100% relative humidity overnight. The water droplets on leaf surfaces were collected by centrifugation at 3,000 rpm for 10 min. The concentration of free copper ions in the water was measured with the cupric specific electrode and the reference electrode as described above. Three leaves were sampled for each treatment. The experiments were repeated three times, and the data were pooled for analysis.

Growth curves of bacterial cultures. To determine whether less free copper ions were needed to inhibit the growth of bacteria in the presence of iron, bacteria were cultured in CYE broth medium amended with different concentrations of copper and iron. The growth rates of bacteria were then estimated. Bacterial suspensions were diluted to 10^6 cfu/ml, and 0.1-ml aliquots of the suspensions were pipetted into test tubes containing 5 ml of CYE-glycerol broth amended with the metal ions. The cultures were placed on a rotating wheel housed in an incubator at 28 C. The turbidity was measured at different intervals at 600 nm with a spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH). The experiment was repeated twice with three replicates. The data were pooled for analysis.

Bacterial populations on copper-treated leaves. To determine whether the addition of iron to Kocide 101 enhances the toxicity of copper on leaf surfaces, the sizes of bacterial populations were determined on walnut leaves treated with Kocide 101 with and without iron. The leaves of Ashley English walnut seedlings were first sprayed to runoff with a suspension of Kocide 101 (2.2 g/L) alone or amended with iron. The plants were air-dried overnight and then sprayed with a bacterial suspension (10^7 cfu/ml in 0.01 M potassium phosphate buffer, pH 7.2) with an atomizer until the leaves were uniformly wet. Plants were maintained at 20–25 C. After 3–4 h, the leaves were removed, weighed, and submerged in 50 ml of 0.01 M potassium phosphate buffer (pH 7.2), which inactivates the bactericidal activity of copper (24). Sample containers then were sonicated for 7 min in an ultrasonic cleaner at 20 C to remove bacterial cells from the leaves (23). Serial dilutions of sonicates were plated on King's medium B agar (12). Plates were incubated at 28 C, and bacterial colonies were counted after 3–4 days. The sizes of bacterial populations per gram of leaves (fresh weight) were determined. The percentage recovery was derived by comparing the number of colonies recovered from leaves treated with copper or copper plus iron with the number recovered from leaves treated with water only. Three leaves were sampled for each treatment. The experiment was repeated three times, and the data were pooled for analysis.

Field experiments. The efficacy of copper and copper-plus-iron compounds in the reduction of *X. c. juglandis* infestation of buds and infection of leaflets and nutlets was investigated in two commercial walnut orchards in Butte and Tehama counties in California. Treatments consisted of Kocide 101 and Champion (77% $Cu(OH)_2$) at a concentration of 2.2 g/L, either alone or amended with iron. Iron was added as $FeCl_3 \cdot 6H_2O$ or Fe_2O_3. The latter contains 70% iron. Although both Kocide 101 and Champion are cupric hydroxide, they differ somewhat because of their formulations (proprietary information). Since both are commonly used in walnut orchards, it was of interest to test both products.

English walnut trees (cultivar Ashley) were sprayed with a handgun sprayer to runoff 4–6 times at intervals of 7–10 days. Treatments were applied to single tree plots arranged in a completely randomized design; there were eight replicates. To detect the incidence of infestation of buds by *X. c. juglandis,* 10- to 15-cm twig segments were collected at random from branches, and single buds were aseptically excised from the twigs with a sterile razor blade. Each excised bud was macerated in 5 ml of 0.01 M potassium phosphate buffer (pH 7). Serial dilutions of the macerate were made and spread onto blue-starch semiselective medium. Plates were incubated for 4–6 days at 28 C, and colonies of *X. c. juglandis* were counted. Incidences of bud infestation in different treatments were compared. To evaluate disease incidence, at least 150 leaflets and 80 nutlets were sampled randomly from each replicate and evaluated for blight symptoms.

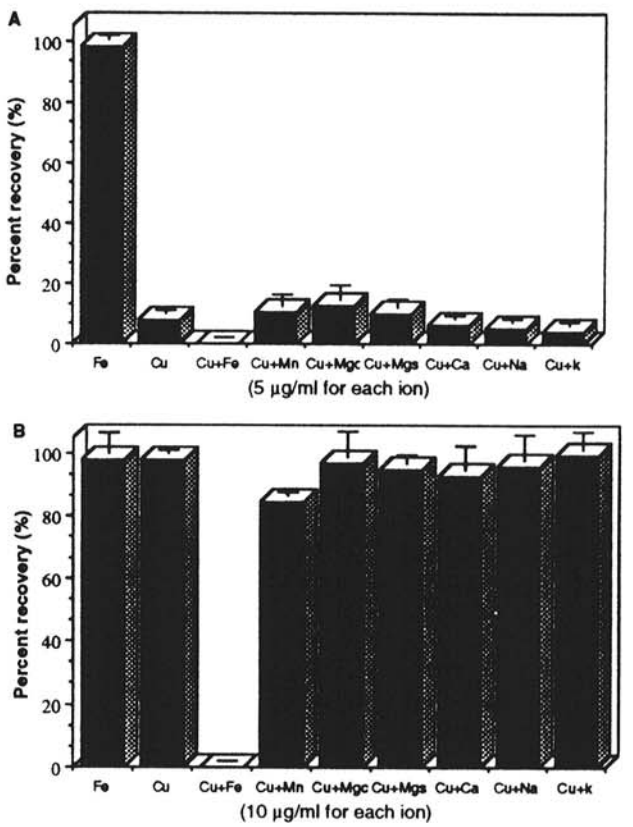

Fig. 1. A, Recovery of *Xanthomonas campestris* pv. *juglandis* copper-sensitive strain NF1 and **B,** recovery of copper-resistant strain C1 on casitone-yeast extract–glycerol agar medium containing three different metal ions. The experiment was performed twice with four replicates. The value given is the mean of eight subsamples. (Fe was from $FeCl_3 \cdot 6H_2O$; Cu, $CuSO_4 \cdot 5H_2O$; Mn, $MnSO_4$; Mgc, $MgCl_2$; Mgs, $MgSO_4$; Ca, $CaCl_2$; Na, NaCl; and K, KCl.)

RESULTS

Toxicity tests in CYE agar medium. The addition of $FeCl_3 \cdot 6H_2O$ to CYE-glycerol agar medium containing $CuSO_4 \cdot 5H_2O$ enhanced the toxicity of copper to walnut blight bacteria (Fig. 1). The recovery of both copper-sensitive and -resistant strains of *X. c. juglandis* on the medium containing copper was much lower than that on unamended media and was eliminated when iron was added to the medium. $CuSO_4$ at a concentration of 10 μg/ml did not inhibit growth of copper-resistant strain C1, but the combination of copper at a concentration of 10 μg/ml and iron at 10 μg/ml completely inhibited growth. Other metal ions, such as magnesium, manganese, calcium, sodium, and potassium, did not affect the toxicity of copper to *X. c. juglandis.*

Effect of iron on the measurement of free copper ions with a cupric specific electrode. The addition of $FeCl_3 \cdot 6H_2O$ (up to 200 μg of iron per milliliter) to aqueous suspensions of Kocide 101 did not alter the estimate of free copper ions with a cupric specific electrode (Table 1). Concentrations of free copper ions in water measured with the cupric specific electrode were not different from those measured with the inductively coupled plasma 40 emission spectrometer.

Growth curves and concentrations of free copper ions in broth medium. The addition of $FeCl_3 \cdot 6H_2O$ to CYE-glycerol broth decreased the pH and increased the concentrations of free copper ions (Table 2). Addition of iron (10 μg/ml) to media containing added copper (10 μg/ml) decreased pH values by almost one unit and increased the concentration of free copper ions by nearly 40-fold. Additionally, growth rate studies in which bacterial growth in media containing added copper (30 μg/ml) was compared with growth in media containing both added copper and iron (10 μg/ml) indicated that in the presence of iron, less free copper ions were needed to inhibit the growth of the copper-resistant strain of *X. c. juglandis* (Fig. 2). The copper-resistant *X. c. juglandis* strain C5 grew well after a long lag phase in media containing added copper ions (30 μg/ml) even though the medium had a low pH value (5.42) and a high content of free copper ions (430.50 ng/ml) (Table 2). However, the strain did not grow in media containing both added copper and iron (10 μg/ml), which had a higher pH value (5.65) and a concentration of free copper ions (39.80 ng/ml) 10 times lower than the media containing only added copper.

Concentrations of free copper ions in Kocide 101 suspensions and on walnut leaf surfaces. The addition of iron to aqueous suspensions of Kocide 101, as in CYE-glycerol broth, lowered pH values and increased the concentrations of free copper ions (Table 3). The concentration of free copper ions in an alkaline Kocide 101 suspension (pH approximately 7.5–8.0) was low, always less than 0.05 μg/ml. Addition of iron (20 μg/ml) to the suspension decreased the pH by almost 1.8 units and increased the concentration of free copper ions to over 25 μg/ml. Moreover, iron caused the release of more free copper ions in Kocide 101 suspensions than did hydrochloric acid at the same pH values (Fig. 3). At a pH near 6, the addition of iron caused the release of twice as many free copper ions as did hydrochloric acid.

On walnut leaf surfaces, more free copper ions were released from deposited Kocide 101 than were released in aqueous suspensions (Table 3). The increase in free copper ions was caused in part by the fact that the pH on leaf surfaces was lower (pH 6.7). The addition of iron to Kocide 101 also caused a decrease

TABLE 1. Concentrations of dissolved and free copper ions and pH values of Kocide 101 suspensions amended with $FeCl_3 \cdot 6H_2O$

Added iron (μg/ml)	Dissolved copper[y] (μg/ml)	Free copper[z] (μg/ml)	pH
0.0	0.08	0.02	7.8
20.0	22.60	20.99	6.0
50.0	68.50	66.99	5.9
100.0	145.30	152.41	5.6
200.0	287.50	321.47	5.4

[y] A Kocide 101 suspension (2.2 g/L) amended with $FeCl_3 \cdot 6H_2O$ was centrifuged at 14,000 rpm for 30 min, and the concentrations of dissolved copper in the supernatants were measured with an inductively coupled plasma 40 emission spectrometer.

[z] Concentrations of free copper ions in a Kocide 101 suspension (2.2 g/L) amended with $FeCl_3 \cdot 6H_2O$ were measured directly with a cupric specific electrode.

TABLE 2. pH values and concentrations of free copper ions in casitone-yeast extract–glycerol broth containing different concentrations of added copper and iron[y]

Concentration of added iron (μg/ml)	Concentration of added copper (μg/ml)					
	10		20		30	
	pH	Cu^{2+} (ng/ml)	pH	Cu^{2+} (ng/ml)	pH	Cu^{2+} (ng/ml)
0	6.51	1.05	5.78	50.50	5.42	430.50[z]
1	6.39	1.48	5.80	60.30	5.38	474.20
5	6.10	6.28	5.50	169.04	5.21	750.00
10	5.65	39.80[z]	5.19	433.50	4.98	1,261.83

[y] Each value is a mean of six replicate samples from two experiments. Copper and iron were added as $CuSO_4 \cdot 5H_2O$ and $FeCl_3 \cdot 6H_2O$, respectively. Copper ion concentration was measured with a cupric specific electrode.

[z] Comparison of data in Figure 2 with those in this table revealed that blight bacteria grew well in media containing 30 μg of added copper ions per milliliter and a low pH, whereas no growth occurred in media with only 10 μg of added copper per milliliter and a higher pH when iron was added.

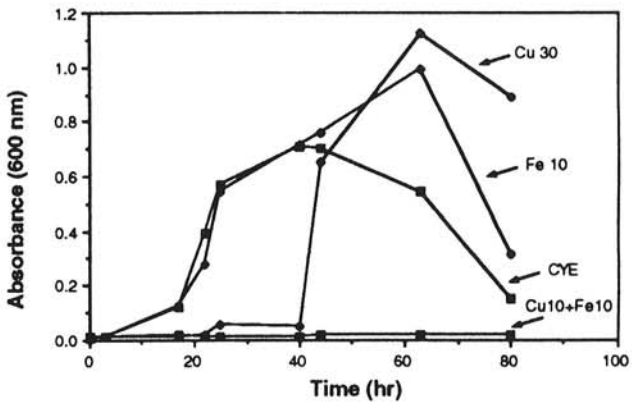

Fig. 2. Growth of *Xanthomonas campestris* pv. *juglandis* copper-resistant strain C5 in casitone-yeast extract (CYE)-glycerol broth medium containing different concentrations of copper and iron. Cu30 = CYE medium containing 30 μg of added Cu^{2+} per milliliter supplied as $CuSO_4 \cdot 5H_2O$; Fe10 = CYE medium containing 10 μg of added Fe^{3+} per milliliter supplied as $FeCl_3 \cdot 6H_2O$; CYE = CYE medium without amendment; Cu10+Fe10 = CYE medium containing 10 μg each of added Cu^{2+} and Fe^{3+} per milliliter. Each value represents the mean of six replicates.

TABLE 3. pH values and concentrations of free copper ions in Kocide 101 suspensions and on walnut leaf surfaces sprayed with Kocide 101 amended with different concentrations of iron[y]

Concentration of added iron (μg/ml)	Water suspensions		Leaf surfaces[z]	
	pH	Cu^{2+} (μg/ml)	pH	Cu^{2+} (μg/ml)
0	7.9	0.03	6.7 ± 0.1	0.79 ± 0.3
20	6.1	25.80	6.5 ± 0.2	4.20 ± 1.0
40	6.0	60.40	6.4 ± 0.1	13.50 ± 3.5
50	5.9	80.70	6.2 ± 0.2	22.60 ± 5.1
80	5.8	101.86	5.9 ± 0.3	28.50 ± 5.1

[y] Concentrations of free copper ions in suspensions of Kocide 101 were measured with a specific cupric electrode. Iron was added in the form of $FeCl_3 \cdot 6H_2O$.

[z] The experiment was repeated three times. Each replicate had three subsamples. Each value is a mean of nine subsamples expressed as the mean ± standard error.

in pH and an increase in concentrations of free copper ions on leaf surfaces, but the changes were not as great as those in aqueous suspensions.

Bacterial population on walnut leaf surfaces. The copper-resistant strain C1 of *X. c. juglandis* survived much better on walnut leaves treated with Kocide 101 than did the copper-sensitive strain NF1 (Fig. 4). Cells of the copper-sensitive strain were not recovered on leaves treated with Kocide 101, whereas about 35% of the cells of copper-resistant strains survived. Recovery of the copper-resistant strain on treated leaves decreased significantly ($P < 0.05$) when the concentrations of iron were increased (Fig. 4). The most effective treatment was Kocide 101 supplemented with iron at a concentration of 50 μg/ml. This eliminated the copper-resistant strain on leaf surfaces.

Field experiments. Kocide 101 with or without iron applied to trees significantly ($P < 0.05$) reduced the incidence of bud colonization compared with the treatments of iron alone and no treatment (Table 4). However, Kocide 101 plus iron was significantly better than Kocide 101 alone. In the first survey (before rain), there were no significant differences between Kocide 101 and Kocide 101 with iron. In the second survey (after 7.6 cm of rain), the incidences of bud infestation on trees treated with Kocide 101 with and without iron were 6.9 and 23.3%, respectively, and Kocide 101 with iron was significantly ($P < 0.05$) better than Kocide 101 alone.

Champion, either with or without iron, significantly reduced the incidence of leaflet infection of walnut as compared to the control (Table 5). Although there were no significant differences in efficacy between Champion and Champion amended with iron early in the season, the incidence of leaflet infection on trees treated with Champion plus iron was significantly ($P < 0.05$) lower than Champion alone by the end of the blight season (4.4 and 12.2%, respectively) (Table 5). Champion, either with or without iron, also significantly reduced the incidence of walnut nutlet infection compared with the control (Table 6). However, no significant differences were found between Champion and Champion plus iron. In these experiments, insoluble ferric oxide was used to replace half of the concentrations of ferric chloride for the purpose of increasing persistence. Phytotoxicity was noted on trees treated with Champion plus both ferric chloride and ferric oxide, although there was no difference in the efficacy between these two treatments.

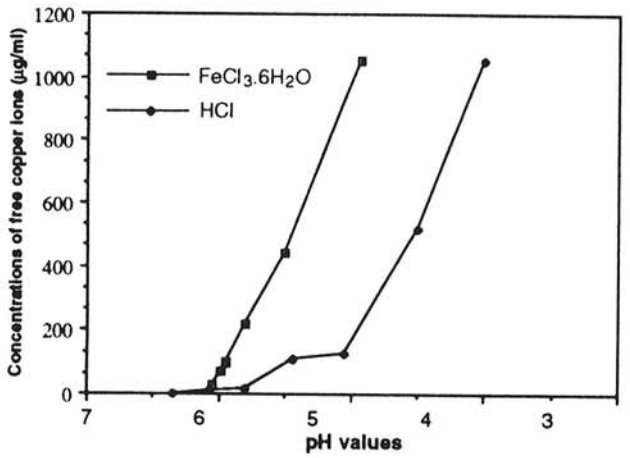

Fig. 3. Relationship between concentration of free copper ions and pH values of Kocide 101 suspensions (2.2 g/L) amended with $FeCl_3 \cdot 6H_2O$ or HCl.

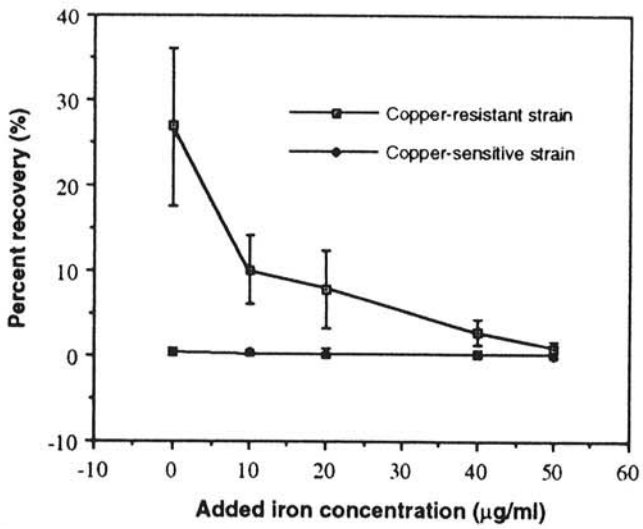

Fig. 4. Percentage recovery of the copper-resistant strain C1 and copper-sensitive strain NF1 of *Xanthomonas campestris* pv. *juglandis* on walnut leaf surfaces treated with Kocide 101 suspensions (2.2 g/L) amended with different concentrations of iron added as $FeCl_3 \cdot 6H_2O$. The experiment was performed three times. Each replicate had three subsamples. The value given is the mean of nine subsamples and expressed as the mean ± standard error.

DISCUSSION

The addition of ferric chloride to copper-containing bactericides enhanced the toxicity of these compounds to *X. c. juglandis*. The effect was caused by iron and not by chloride, since the addition of other metals in a chloride form had no effect. This enhancement is attributed to at least two mechanisms. Iron

TABLE 4. Effects of different treatments on the incidence of colonization of English walnut buds by *Xanthomonas campestris* pv. *juglandis*

Treatment[x]	Colonization incidence[y,z]	
	Before rain	After rain
Kocide 101	10.7 ab	23.3 a
Kocide 101 + iron (50 μg/ml)	3.3 a	6.0 b
Iron (50 μg/ml)	17.9 bc	68.7 c
Control	28.3 c	75.3 c

[x] Kocide 101 was applied at the rate of 2.2 g/L. Iron was added as $FeCl_3 \cdot 6H_2O$. All treatments were applied four times to the cultivar Ashley, beginning on 16 April 1990, at intervals of 7–10 days. Samples were first collected on 21 May 1990 and again on 4 June 1990 after 7.6 cm of rain.

[y] Values are the means for each treatment replicated eight times in a completely randomized design at two different experimental sites. Each treatment (175–200 buds) was assayed for the presence of *X. c. juglandis*.

[z] Means within a column followed by the same letter are not significantly different ($P = 0.05$); least significant difference = 13.8 (before rain) and 16.1 (after rain).

TABLE 5. Effects of different treatments on the incidence of walnut leaflet infection by *Xanthomonas campestris* pv. *juglandis*

Treatment[w,x]	Disease incidence[y,z]	
	First survey	Second survey
Champion	9.5 a	12.2 a
Champion + $FeCl_3$	6.6 a	4.4 b
Champion + $FeCl_3$ + Fe_2O_3	5.6 a	5.5 b
Control	19.7 b	27.4 c

[w] Champion was applied at the rate of 2.2 g/L. Iron was added as $FeCl_3 \cdot 6H_2O$ or Fe_2O_3. Champion + $FeCl_3$: iron (100 μg/ml) as $FeCl_3 \cdot 6H_2O$ was added. Champion + $FeCl_3$ + Fe_2O_3: iron (50 μg/ml) as $FeCl_3 \cdot 6H_2O$ and Fe_2O_3 (total 100 μg of iron per milliliter) was added.

[x] All treatments were applied to the cultivar Ashley, beginning on 16 March 1991, six times at intervals of 10–14 days. Samples were first collected on 18 April 1991 and again on 10 June 1991.

[y] Values are the means for each treatment replicated eight times in a completely randomized design. Each replicate (175–200 leaflets) was assayed for infection by *X. c. juglandis*.

[z] Means within a column followed by the same letter are not significantly different ($P = 0.05$); least significant difference = 4.6 (first survey) and 6.0 (second survey).

apparently has a direct physiological effect on *X. c. juglandis,* causing it to be more susceptible to the toxic effect of the cupric ion. This was shown by the finding that copper-resistant *X. c. juglandis* could grow in media containing copper ions (30 μg/ml) but not in media amended with both copper and iron ions (10 μg/ml). Thus, when exposed to iron, less copper ions were needed to inhibit growth. Iron also has been reported to enhance the efficacy of copper in controlling *Anabaena* spp. (8). In this case, iron prevents the production of a siderophore elaborated by the algae. The siderophores chelate copper in estuaries, thus reducing the amount of free copper ions available to kill algae. The siderophore apparently has sites that bind both copper and iron. It is not uncommon for proteins to have sites that can bind these two ions. For example, copper in ovotransferrin can be replaced by iron (7). In *X. c. juglandis,* the enhancement of copper sensitivity by iron is most likely caused by the fact that iron competes for copper-binding sites in copper-binding proteins. Thus, in the presence of iron, copper is no longer bound and enters the cytoplasm. It has been suggested that sequestering by copper-binding proteins is the copper-resistance mechanism in copper-resistant strains of *P. syringae* pv. *tomato* (6).

In the second mechanism by which soluble iron, such as $FeCl_3 \cdot 6H_2O$, enhances the toxicity of copper-containing compounds, iron causes the release of more free copper ions by lowering the pH and by cation exchange. The lowering of the pH is a result of the fact that the ferric ion has a greater affinity for the hydroxide group than does the copper ion. At neutral pH, iron is precipitated as the oxyhydroxide polymer, the analytical composition of which is FeOOH. The amount of soluble ferric ions in water can be estimated by the solubility product of ferric hydroxide, which is approximately 10^{-38} (22). In contrast, the solubility product of cupric hydroxide is 10^{-19}. Accordingly, in aqueous mixtures of copper and iron, ferric ions bind to the hydroxide group of water and decrease pH, thereby causing more free copper ions to become available. We also speculated that iron might mediate cation exchange, since iron released more free copper ions in Kocide 101 suspension than did hydrochloric acid at the same pH.

Copper bactericides are not nearly as effective against bacterial pathogens as would be expected, considering the toxicity of free copper ions. There are several possible reasons for this. First, copper bactericides have low water solubility. Water-soluble copper ions are mobilized from these compounds by the solubilizing effects of environmental factors and by complexation with microbial exudates and leaf leachates (5). Since soluble complexed forms of copper have no significant toxicity towards bacteria (17), the toxicity of copper to bacteria, which is affected by pH, is related to the concentration of free copper ions (29). Although enough free copper ions were released from Kocide 101 on leaf surfaces (because of the natural acidic environments of plant leaves) to kill copper-sensitive strains of *X. c. juglandis,* copper-resistant strains were not killed. Thus, the occurrence of copper-resistant bacteria is undoubtedly another factor accounting for the low efficacy of copper compounds in disease control. However, the addition of iron to Kocide 101 resulted in total elimination of *X. c. juglandis* on leaves. This was caused in part by the increase of free copper ions on leaf surfaces, as shown by experiments measuring the amount of free copper ions. Aside from lowering the pH, iron also may function in a cation exchange system on leaf surfaces by replacing copper in copper-binding sites of organic chelators, thereby releasing more copper ions.

TABLE 6. Effects of different treatments on the incidence of walnut nutlet infection by *Xanthomonas campestris* pv. *juglandis*

Treatment[w,x]	Infection[y,z] (%)
Champion	12.3 a
Champion + $FeCl_3$	5.6 a
Champion + $FeCl_3$ + Fe_2O_3	10.2 a
Control	34.6 b

[w] Champion was applied at the rate of 2.2 g/L. Iron was added as $FeCl_3 \cdot 6H_2O$ or Fe_2O_3. Champion + $FeCl_3$: iron (100 μg/ml) as $FeCl_3 \cdot 6H_2O$ was added. Champion + $FeCl_3$ + Fe_2O_3: iron (50 μg/ml) as $FeCl_3 \cdot 6H_2O$ and Fe_2O_3 (total 100 μg of Fe^{3+} per milliliter) was added.

[x] All treatments were applied to the cultivar Ashley, beginning on 16 March 1991, six times at intervals of 10–14 days. Nutlets were evaluated on 10 June 1991.

[y] Values are the means for each treatment replicated eight times in a completely randomized design. Eighty nutlets for each replicate were assayed for infection by *X. c. juglandis.*

[z] Means within a column followed by the same letter are not significantly different ($P = 0.05$); least significant difference = 9.48.

The efficacies of copper-iron compounds in disease control was higher than that of copper compounds alone in all field tests conducted. Kocide 101 amended with iron was significantly better than Kocide 101 alone in reducing the incidence of bud infestations by blight bacteria. This mixture was also significantly better in reducing the severity of leaf infections. These findings have important implications for the control of nut blight, which is the most important aspect of the disease. Buds serve as a principal source of blight bacteria for infection of nuts. Buds become infested with blight bacteria throughout the season, and the bacteria overwinter in these sites (21). Thus, preventing the infestation of buds should reduce the primary inoculum for nut blight the following season. Reducing the infection of leaves also is important, especially because infected leaves provide a source of inoculum for the infestation of buds and maturing nuts.

The effect of copper-iron mixtures in reducing blight of nuts has not been significantly better than copper compounds alone to date. Although such mixtures always reduced disease incidence, the variation among replicates is so great that statistical significance has not been obtained. Moreover, it is suspected that the action of copper compounds on nuts is different from that on buds and leaves. Whereas copper compounds are very effective in controlling blight of leaves, they have never demonstrated such effectiveness on nuts. This is likely because of the hydrophobicity of the nutlets, which prevents the desirable amount of coverage by applied copper compounds. In addition, there may not be an even amount of redistribution of copper ions on nuts such as occurs on leaves during wet infection periods. Furthermore, it is likely that the nutlets are already colonized by blight bacteria before the application of copper compounds. Thus, even though the addition of iron to copper compounds results in more free copper ions, this may not be enough to eradicate populations already established on nuts. Established populations of *P. syringae* were controlled much less effectively than inoculated cells on copper-treated bean leaves (1). Wetting agents and other such adjuvants may be important in improving the efficacy of the copper compounds in controlling nutlet infection.

Although the addition of ferric chloride to fixed copper compounds increases the concentration of free copper ions, phytotoxicity has not been observed in the field. However, phytotoxicity occurred when the insoluble ferric oxide was combined with ferric chloride. The reasons for this are unknown but probably have something to do with the long-term release of iron ions that interact with the fixed coppers. This surprising result indicates that much work yet has to be done to find the best formulation that will result in the greatest kill of bacteria over an extended period of time while at the same time not harming tender walnut tissues. The effect of iron in increasing the efficacy of copper compounds offers a new advance in the use of the age-old copper compounds and should lead to greatly improved control of bacteria such as *X. c. juglandis.* However, this will greatly depend on both the ecology of copper-resistant bacteria and the efficacy of new formulations in eradicating established populations.

NOTE ADDED IN PROOF

Since the acceptance of the manuscript, additional research revealed that the addition of iron to fixed copper compounds such as Kocide 101 caused an increase in the aggregation and size of the particles in the suspension. This was reduced by the addition of $MgSO_4$ H_2O (250 μg/ml) and the surfactant CS-7

(1.9 ml/3.78 L of suspension) (Rohm and Haas, Philadelphia, PA). The new formulation was 35% more effective than Kocide 101 in reducing the incidence of nut blight (untreated control, 83%; Kocide 101, 30.5%; new formulation, 19.7%; pooled data from two field plots). The difference was significant at $P < 0.01$.

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