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An in Vitro Study on the Postinfection Activities of Copper Hydroxide and Copper Sulfate against Conidia of *Venturia inaequalis*

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The postinfection activities of copper hydroxide [$Cu(OH)_2$] and copper sulfate ($CuSO_4$) against apple scab (*Venturia inaequalis*) were evaluated in an in vitro study. Our intention was to support the aim of reducing copper application rates by appropriate timing of applications. Experiments were conducted at 20 °C with leaf disks and isolated cuticular membranes (CM) of *Malus* × *domestica* 'Gloster' and 'Elstar'. Conidia of *V. inaequalis* were used as the inoculum. In untreated controls, 7.9 and 33.2% of germinated conidia formed primary stromata 24 and 48 h after inoculation, respectively. Treatments with copper compounds were applied 24 and 48 h after inoculation, which was 16 and 40 h after infection had occurred. When working with CM and using fluorescein diacetate as a vital stain, vital and dead stromata could be distinguished. Treatment effects were assessed 72 h after inoculation by counting vital (fluorescing) primary stromata. With leaf disks, the number of stromata was counted using KOH−aniline blue fluorescence staining. $Cu(OH)_2$ and $CuSO_4$ showed postinfection activity and killed primary stromata, provided that the surface of the CM was kept wet. $Cu(OH)_2$ was more effective than $CuSO_4$ and was able to kill all primary stromata 24 h after inoculation at concentrations of 116 and 231 mg L^{-1}. When $Cu(OH)_2$ was applied at 116 mg L^{-1} to leaf disks 24 h after inoculation, the number of primary stromata did not significantly differ from the control. Results indicate different modes of action for the highly water soluble $CuSO_4$ and the slightly soluble $Cu(OH)_2$. This supports the hypothesis that spore exudates react with insoluble copper compounds and form highly toxic copper complexes. Application of $Cu(OH)_2$ to dry CM did not kill primary stromata. Hence, for $Cu(OH)_2$ to exert postinfection activity, leaves must be wet. In the field, this cannot be guaranteed and a postinfection application of $Cu(OH)_2$ cannot be recommended.

KEYWORDS: After-infection; copper fungicides; curative fungicides; cuticle; organic production

INTRODUCTION

Apple scab, caused by the fungus *Venturia inaequalis* (Cke.) Winter, is one of the most important diseases in both organic and integrated production (*1*). The use of modern synthetic fungicides allows reliable scab control in integrated production systems throughout the growing season (*2*). However, scab control is not efficient enough in organic production systems as organic guidelines do not permit the use of synthetic fungicides. Only products based on copper and sulfur are approved (*3*). Copper is an effective agent against apple scab, but copper fungicides can be phytotoxic and may cause fruit russeting during bloom and early fruit development (*1*, *4*, *5*). Furthermore, copper accumulates in the soil and is toxic to microorganisms and earthworms (*6*−*10*). This poor ecotoxicological profile conflicts with the ecological concepts of organic production.

Copper is used in the Bordeaux mixture and is one of the oldest fungicidal agents; yet, little is known about its mode of action. Research in this field (mostly with Bordeaux mixture) came to an end about half a century ago. It is believed that copper ions are able to enter the fungal spore and denature proteins and block various enzymes (*11*−*13*). Hence, water solubility of the copper compounds should be important. Pickering (*14*) proposed the theory of a cumulative mode of action. According to this theory, the spore slowly absorbs traces of copper until a toxic concentration is reached. However, the solubility of copper alone cannot explain its effectiveness as slightly soluble copper hydroxide [$Cu(OH)_2$] and copper oxychloride as well as insoluble copper oxide contained in Bordeaux mixture are also effective (*15*). There are three principal hypotheses that suggest different ways of increasing solubility

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and availability of copper in Bordeaux mixture as well as in other insoluble and slightly soluble copper compounds. Dissolution might be aided by (i) CO_2 and ammonium ions dissolved in rainwater or dew (*14*, *16*), (ii) exudates from the plant (*17*), and (iii) secretion of acids or complexing agents by the spore (*18*−*22*).

McCallan and Wilcoxon (*20*) demonstrated the presence of amino acids and malate in exudates of *Neurospora sitophila* spores. These substances were shown to dissolve copper from the deposits of Bordeaux mixture by forming soluble copper complexes with fungicidal activity (*22*). Contact between the spore and the particles of the insoluble copper compounds was essential for their effectiveness (*18*, *19*, *23*, *24*). In his review, McCallan (*23*) concluded that copper brought into solution by atmospheric components and host plant exudates might supplement the fungicidal action but was not essential.

Besides copper, the only other option to control apple scab in organic production is sulfur. Elemental sulfur has only a weak protective activity and provides insufficient control during the primary infection season (*25*−*29*). For this reason, there is a renewed interest in lime sulfur, which gives sufficient control even if applied curatively after an infection has occurred (*25*, *30*−*32*). Unfortunately, the phytotoxicity of lime sulfur increases under wet conditions (*29*, *30*, *33*). Holb et al. (*30*) pointed out that curative schedules of lime sulfur sprays had good efficacy against apple scab, which was similar to the efficacy of copper fungicides, but it was combined with severe phytotoxicity and reduced yield.

Attempts are being made in several countries to reduce copper application rates in protective spray schedules (*5*, *34*, *35*). In addition, strategies are being developed, which help to minimize the number of spray applications. In spring, copper compounds are applied when ascospores are ready to be discharged and weather conditions favor infections (*34*, *35*). Pre- and postinfection activities of copper could help to reduce the number of spray applications. According to Szkolnik's (*36*) terminology, the postinfection activity of a fungicide is defined as the chemical activity that arrests further development of the fungus at the end of an infection period and after an infection has occurred. Hamilton (*25*) reported that Bordeaux mixture applied after the infection period gave more consistent and better scab control than certain sulfur dusts and finely divided sulfur sprays. However, there is no other report indicating that copper fungicides can have any postinfection activity.

The aim of our study was to investigate under which conditions copper can have postinfection activity. We focused on the time window available for killing conidia and primary stromata with $Cu(OH)_2$ or copper sulfate ($CuSO_4$). The importance of wetness for fungicidal activity was also investigated. With the in vitro test system that was used (*37*−*39*), the infection cycle could be observed microscopically from germination and appressoria formation to penetration of the cuticle and formation of a primary stroma. Another advantage of the test system was that the responses of conidia and primary infection structures could be evaluated directly and viability could be assessed using vital fluorescence staining. Results obtained with isolated cuticles were validated using an apple leaf disk assay.

MATERIAL AND METHODS

Chemicals. Copper(II) hydroxide [$Cu(OH)_2$, technical, Sigma-Aldrich Chemie GmbH, Steinheim, Germany] and copper(II) sulfate ($CuSO_4$, anhydrous, extra pure, Riedel-de Haën, Seelze, Germany) were used in the experiments. The water solubilities of $CuSO_4$ and $Cu(OH)_2$ are 230.5 g L^{-1} at 25 °C and 2.9 mg L^{-1} at 20 °C, respectively (*15*). Solutions and suspensions were prepared by dissolving the compounds in deionized water. Final concentrations are given in the figures. Fluorescein diacetate (FDA, Riedel-de Haën) and aniline blue (Merck, Darmstadt, Germany) were used for staining. FDA is a vital stain and an indicator for esterase activity and integrity of the cell membrane (*40*).

Production of *Venturia inaequalis* Conidia. Conidia from an in vitro culture of *V. inaequalis* were used as the inoculum. The isolate originated from the cultivar 'Elstar' and was isolated at the Biologische Bundesanstalt (Dossenheim, Germany) in 2000. The isolate was maintained on potato dextrose agar (Difco PDA, Becton Dickinson GmbH, Heidelberg, Germany). The isolate had a high germination rate (more than 80%), and its ability to infect leaves of apple seedlings has been tested repeatedly (*41*). Conidia were produced on cellophane membranes (Cellophan 235 P, Pütz GmbH und Co. Folien KG, Taunusstein, Germany) according to the method of Parker et al. (*42*). Conidia growing on the cellophane membranes were detached from their stalks by shaking them in 10 mL of deionized water. The suspension was filtered through three layers of filter cloth (Miracloth, CN Biosciences Inc., La Jolla, CA). The spore density was assessed using a Kolkwitz plankton cytometer (0.5 mL, Hydro-Bios, Kiel, Germany) and a light microscope (*43*). The titer was adjusted to 1×10^4 conidia mL^{-1}.

Inoculation of Cuticular Membranes (CMs). Astomatous adaxial CMs were isolated enzymatically (*41*) from healthy and just fully expanded leaves of field-grown trees of *Malus* × *domestica* Borkh. 'Gloster' and 'Elstar' (*38*). With the morphological outer surface facing up, ten CM disks with a diameter of 2 cm were floated on deionized water in a Petri dish (**Figure 1A**). Prior to the respective treatment, a 5 μL drop of the conidia suspension containing approximately 50 conidia was pipetted onto the center of each CM disk. Petri dishes were sealed with Parafilm (American National Can, Chicago, IL) and incubated in a growth cabinet in darkness at 20 °C. Sealing of the Petri dishes resulted in 100% relative humidity, which prevented droplet drying. The respective incubation times are given below.

Inoculation of Leaf Disks. For leaf disk experiments, the second fully unfolded leaves from extension shoots of greenhouse grown 'Elstar' trees, grafted on rootstock M9, were collected. Leaves were surface sterilized by shaking for 90 s in potassium hypochlorite (0.75%). After they were rinsed twice in sterile deionized H_2O and dried with soft tissue paper, disks 8 mm in diameter were punched out. Leaf disks were placed on water agar (1%) in Petri dishes with their upper surfaces facing up (10 per dish). Prior to the respective treatment, a 10 μL drop of the conidial suspension containing approximately 100 conidia was pipetted onto the center of each leaf disk. Petri dishes were sealed with Parafilm, incubated in a growth cabinet at 20 °C, and illuminated at 40 $\mu mol^{-2}\ s^{-1}$ PAR for 16 h per day. Sealing of the Petri dishes resulted in 100% relative humidity. The respective incubation times are given below.

Experimental Design. The in vitro test system is a highly standardized procedure (*38*). Conditions for each Petri dish in the growth cabinet as well as conditions for each CM in a Petri dish were identical, and there were no uncontrolled variables that might have affected the results. Hence, a specific experimental design to evaluate such random effects was not necessary. Each CM in a Petri dish was regarded as a true replicate, and two independent experiments, each consisting of 10 replicates (CM) per treatment, were conducted, resulting in 20 observations per treatment. The same applied to the leaf disk experiments.

In each experiment, one Petri dish containing 10 CMs or 10 leaf disks was not treated with chemicals and served as the control. With these untreated controls, the number of primary stromata was evaluated 24, 48, and 72 h after inoculation, as described below. In addition, one Petri dish with six untreated CM was included in each experiment to estimate the percentage of germinated conidia 24 h after inoculation. Only conidia that had formed at least one germ tube and one appressorium were counted as having germinated (**Figure 1B**). On each CM or leaf disk, all conidia or fluorescing primary stromata were counted. Experiments were evaluated using a Zeiss Axioplan2 microscope equipped with a Zeiss AxioCam digital camera and AxioVision 3.1 software (Carl Zeiss, Göttingen, Germany).

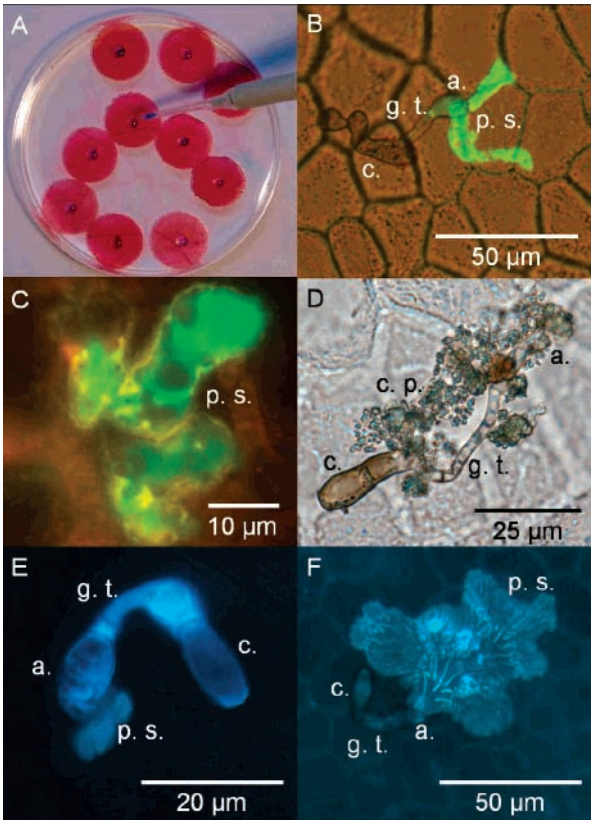

Figure 1. (**A**) Application of conidia suspension to enzymatically isolated CMs of *Malus* × *domestica* 'Gloster' floating on deionized water. CMs and conidia suspensions were stained for better visualization. (**B**) Germinated conidium 72 h after inoculation on 'Gloster' CM. CM has been penetrated, and a primary stroma (p.s.) has formed. (**C**) Primary stroma (p.s.) 72 h after inoculation on 'Gloster' CM. (**B**,**C**) Primary stromata were stained with FDA. (**D**) Conidium (c.) of *V. inaequalis* with germ tube (g.t.) and appressorium (a.) 48 h after inoculation on 'Gloster' CM. $Cu(OH)_2$ at 116 mg L^{-1} was applied 24 h after inoculation and rinsed 15 min later. $Cu(OH)_2$ particles (c.p.) adhere to the spore. (**E**,**F**) Conidia (c.) with germ tubes (g.t.) and appressoria (a.) on 'Elstar' leaf disks 24 (**E**) and 72 h (**F**) after inoculation. CMs have been penetrated, and primary stromata (p.s.) have formed. Fungal structures were visualized using KOH–aniline blue staining technique.

Treatments (Wet Incubation). Suspensions of $Cu(OH)_2$ and $CuSO_4$ at the respective concentrations (**Figures 2** and **3**) were applied 24 or 48 h after inoculation of the CM. Excess water was removed from the inoculum by careful blotting with soft tissue paper. After that, 5 μL droplets of the respective treatment suspension were pipetted onto the same spot. After an incubation period of 15 min, water and dissolved chemicals were removed by rinsing three times with 5 μL droplets of deionized water and intermittent blotting. This washing procedure did not remove all undissolved solid particles of $Cu(OH)_2$. After washing, a droplet of deionized water was applied to the treated area and the CMs were once again incubated in sealed Petri dishes until 72 h after inoculation, as described above. To assess the influence of a prolonged incubation time for $CuSO_4$ treatments, the rinsing process was delayed for 24 h. After application of the treatment, the Petri dishes were sealed and put back into the growth cabinet until rinsing. The controls were assessed at the time of application 24 or 48 h after inoculation, respectively.

On leaf disks, $Cu(OH)_2$ was applied in the same fashion 24 h after inoculation. $Cu(OH)_2$ was applied at the minimum effective concentration evaluated in the previous experiment (**Figure 4**). Untreated controls were assessed 24 and 72 h after inoculation, and treatment effects were assessed 72 h after inoculation.

Treatments (Dry Incubation). $Cu(OH)_2$ was applied 48 h after inoculation as described above. It was applied at the minimum effective concentration evaluated under wet incubation conditions (**Figure 5**). Instead of rinsing with water, the treatment suspensions on the CM surfaces were dried within 1 h using a fan. After drying, the Petri dishes were sealed and incubated until 72 h after inoculation as described above.

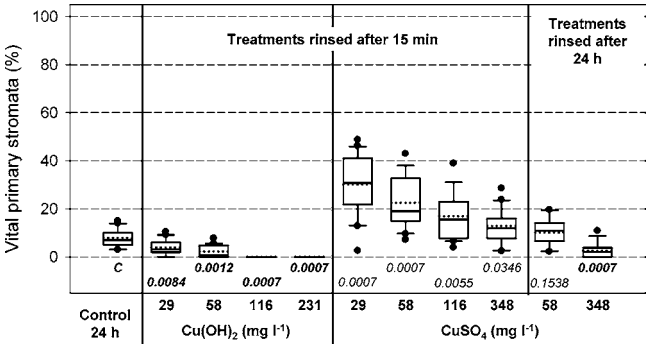

Figure 2. Effects of $Cu(OH)_2$ and $CuSO_4$ at various concentrations on percentage of vital primary stromata of *V. inaequalis* on 'Gloster' CM. Treatments were applied 24 h after inoculation and rinsed off 15 min and 24 h later, respectively. The number of vital primary stromata was assessed 72 h after inoculation using FDA as a vital stain. The percentage of vital primary stromata is given relative to the number of germinated spores in the untreated controls. Treatments were compared with the control, which was assessed 24 h after inoculation. The control and each treatment comprised 20 replicates. Adjusted *p* values are given in italics. Bold *p* values indicate that the percentage of vital primary stromata was significantly reduced as compared to the control ($p < 0.05$). Pairwise comparisons were conducted using the Wilcoxon rank sum test. *P* values were adjusted for carrying out multiple comparisons with the step-up procedure according to Hochberg. Data are presented as box plots. The dotted line shows the arithmetic mean, and the solid line shows the median. The boxes contain 50% of the data. The whiskers show the 10th and the 90th percentiles. Dots below and above the whiskers mark the outliers.

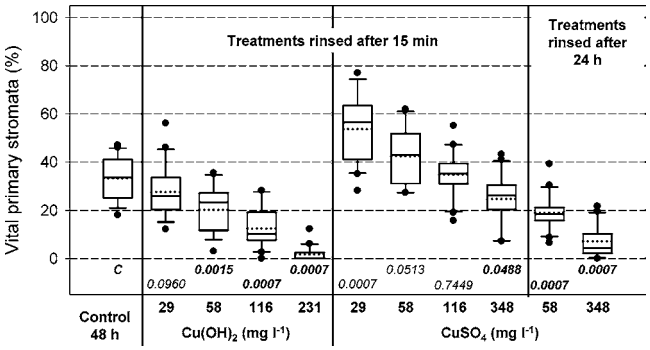

Figure 3. Effects of $Cu(OH)_2$ and $CuSO_4$ at various concentrations on percentage of vital primary stromata of *V. inaequalis* on 'Gloster' CM. Treatments were applied 48 h after inoculation and rinsed 15 min and 24 h later, respectively. The number of vital primary stromata was assessed 72 h after inoculation using FDA as the vital stain. The percentage of vital primary stromata is given relative to the number of germinated spores in the untreated controls. Treatments were compared with the control, which was assessed 24 h after inoculation. The control and each treatment comprised 20 replicates. Adjusted *p* values are given in italics. Bold *p* values indicate that the percentage of vital primary stromata was significantly reduced as compared to the control ($p < 0.05$; for statistics, see **Figure 2**). Data are presented as box plots (for details, see **Figure 2**).

Microscopic Evaluation. For counting the primary stromata associated with CMs, each CM was pulled onto a cover slip with tweezers and 20 μL of FDA solution was added in such a way as to allow it to seep between the CM and the cover slip. FDA solution was prepared according to the method of Hamel et al. (*45*). Care was taken to prevent

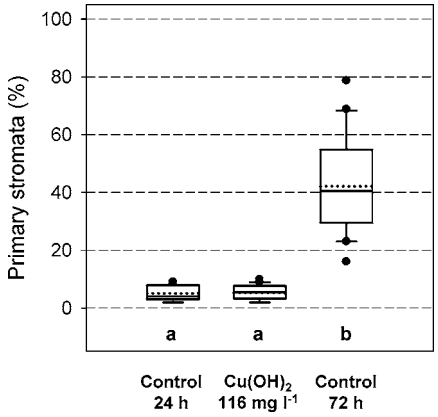

Figure 4. Effect of $Cu(OH)_2$ at 116 mg L^{-1} on percentage of primary stromata of *V. inaequalis* on 'Elstar' leaf disks. Treatments were applied 48 h after inoculation and rinsed off 15 min later. The number of primary stromata was assessed 72 h after inoculation using KOH–aniline blue staining technique. Controls were assessed 24 and 72 h after inoculation. The controls and the treatments consisted of 20 replicates each. All-pair comparisons were conducted. Treatments marked with the same letter do not differ significantly ($p < 0.05$; for details of statistics, see **Figure 2**).

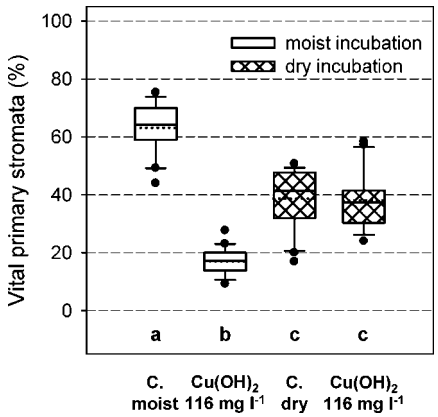

Figure 5. Effect of $Cu(OH)_2$ at 116 mg L^{-1} on percentage of primary stromata of *V. inaequalis* on 'Gloster' CM. Treatments were applied 48 h after inoculation and rinsed 15 min later (wet incubation) or dried within 1 h using a fan (dry incubation), respectively. The number of vital primary stromata was assessed 72 h after inoculation using FDA as the vital stain. The percentage of vital primary stromata is given relative to the number of germinated in the untreated controls. Each treatment and the respective controls (C.) comprise 20 replicates. All-pair comparisons were conducted. Treatments marked with the same letter do not differ significantly ($p < 0.05$; for statistics, see **Figure 2**).

contact of FDA solutions with the outer surface of the CM carrying the inoculum. The water drop on the outer surface of the CM was blotted off with a piece of tissue paper, and a drop of immersion oil was added. The cover slip was turned upside down and was placed on a microscopic glass slide. After this operation, the morphological inner surface of the CM faced the microscope objective and the immersion oil prevented FDA solution from reaching the germinated conidia on the outer surface of the CM. After 10 min, the inner surface of the CM with the fluorescent stromata could be examined under UV excitation using the Zeiss filter set no. 9 (BP 450−490 nm, LP 520 nm). As FDA is a vital stain, only vital stromata exhibiting fluorescence were visible and were counted.

Fungal structures on leaf disks were visualized by using the KOH−aniline blue fluorescence staining technique described by Hood and Shew (*46*) and modified by Ortega et al. (*47*). Leaf disks were examined under UV excitation using the Zeiss filter set no. 2 (G 365 nm, LP 420 nm). With this staining technique, it was not possible to distinguish between vital and dead stromata.

Treatment effects on CM, and leaf disks were assessed 72 h after inoculation by counting the number of fluorescing stromata. If one conidium had formed more than one appressorium and penetrated the CM more than once, only one primary stroma was counted. The number of fluorescing primary stroma is given as the percentage of the average number of germinated conidia of the respective germination control. For the experiments with CM, results are given as percentages of vital primary stromata. For the leaf disk experiments, results are given as percentages of primary stromata.

Data Analysis. For data analysis, the statistical computer application package SAS, version 8.01 (SAS Institute Inc., Cary, NC), was used. Two sample Wilcoxon rank sum tests were performed using the "npar1way" procedure. Probability (p) values were adjusted for multiplicity with the step-up procedure according to Hochberg using the "multtest" procedure. The error level was $\alpha = 0.05$. Data are presented as box plots (SigmaPlot 8.02, SPSS Inc., Chicago, IL).

RESULTS

Effects of $Cu(OH)_2$ and $CuSO_4$ on Vitality of Primary Stromata in CM under Wet Conditions. On the surfaces of untreated 'Gloster' CMs, more than 80% of the conidia germinated and formed at least one appressorium 24 h after inoculation. In 7.9 [standard deviation (SD) ± 3.7%] and 33.2% (SD ± 8.7%) of the controls, vital primary stromata had formed 24 and 48 h after inoculation, respectively (**Figures 2** and **3**). After 72 h, 63.2% (SD ± 8.6%) of germinated conidia had penetrated the CM and formed vital primary stromata (**Figures 1B**,**C** and **5**). These primary stromata were located at the point of contact between deionized water and the inner surface of the CM. Treatment with $Cu(OH)_2$ (116 mg L^{-1}) for 15 min and subsequent washing with water did not remove all solid particles of $Cu(OH)_2$. Particles preferentially adhered to conidia, germ tubes, and appressoria, while the CM appeared relatively clean (**Figure 1D**).

When treatments were applied 24 h after inoculation and rinsed off 15 min later, application of $Cu(OH)_2$ at 116 and 231 mg L^{-1} killed all existing primary stromata and prevented the formation of new ones (**Figure 2**). Treatments with $CuSO_4$ 24 h after inoculation were less effective, as some vital primary stromata developed even at a concentration of 348 mg L^{-1} (**Figure 2**). Extending the incubation from 15 min to 24 h led to a further reduction of vital stromata.

When treatments were applied 48 h after inoculation and rinsed off 15 min later, $Cu(OH)_2$ at 58, 116, and 231 mg L^{-1} significantly reduced the percentage of vital primary stromata as compared to the control (**Figure 3**). Only the treatment with $CuSO_4$ at 348 mg L^{-1} for 15 min reduced the percentage of vital primary stromata significantly. Extending the exposure to $CuSO_4$ to 24 h resulted in an additional reduction in the percentage of vital stromata at concentrations of 58 and 348 mg L^{-1}. Neither $Cu(OH)_2$ nor $CuSO_4$ was able to reduce the percentage of vital primary stromata to zero if applied 48 h after inoculation.

Effect of $Cu(OH)_2$ on Development of Primary Stromata on Leaf Disks under Wet Conditions. Boiling the leaf disks in KOH removed ungerminated conidia from the surface of the leaf disks. Therefore, the germination rate was evaluated on 'Elstar' CM; it was more than 80%. On 'Elstar' leaf disks, 5.1% (SD ± 2.5%) of primary stromata developed within 24 h after inoculation (**Figures 1E** and **4**). Primary stromata that formed in the outer epidermal wall of leaf disks (**Figure 1 E**,**F**) resembled those formed at the CM/water interface (**Figure 1C**), but the former ones appeared more structured.

Treatment with $Cu(OH)_2$ at 116 mg L^{-1} for 15 min prevented development of additional primary stromata; their percentage

did not differ significantly from the 24 h control (**Figure 4**). In the 72 h control, additional stromata had formed in 42.1% of germinated conidia (SD ± 17.1%).

Effect of $Cu(OH)_2$ on Vital Primary Stromata in CM under Dry Conditions. In the control, 63.2% (SD ± 8.6%) of germinated conidia formed vital primary stromata within 72 h, provided that the inoculum was kept wet (**Figure 5**). Treating the inoculum with $Cu(OH)_2$ 48 h after inoculation and maintaining the wet surface of the inoculum reduced the percentage of vital primary stromata to about 17.0% (SD ± 4.7%). When the surfaces of CM were allowed to dry 48 h after inoculation and dry incubation was extended for another 24 h, the percentage of vital primary stromata was 38.7% (SD ± 10.5%; **Figure 5**). Dry incubation of inoculum treated with $Cu(OH)_2$ resulted in 38.0% (SD ± 9.3%) vital primary stromata and was not able to significantly reduce the percentage of vital primary stromata as compared with the dry control.

DISCUSSION

Effects of $Cu(OH)_2$ and $CuSO_4$ on CM under Wet Conditions. According to Szkolnik's definition of postinfection activity (*36*), $Cu(OH)_2$ and $CuSO_4$ showed such activity against *V. inaequalis* under the conditions of our in vitro test system, provided that the surfaces of CM and leaf disks were kept wet. Using CM and FDA as a vital stain, it could be shown that already established primary stromata were killed by treatments with $Cu(OH)_2$ and $CuSO_4$ (**Figures 2** and **3**). At 20 °C, about 8 (conidia) or 6 h (ascospores) is required for infection by *V. inaequalis* to occur (*48*). All primary stromata were killed when $Cu(OH)_2$ was applied at concentrations of 116 and 231 mg L^{-1} 16 h after infection. At these concentrations, a reasonably good and significant reduction was obtained even 40 h after infection.

$CuSO_4$ was less effective than $Cu(OH)_2$, and treatments were not able to kill all primary stromata (**Figures 2** and **3**). Results were slightly better with rinsing after 24 h as compared with rinsing after 15 min. $CuSO_4$ is highly water soluble, and the entire dose was dissolved, whereas with $Cu(OH)_2$ very few copper ions are in solution. A complete removal of dissolved $CuSO_4$ probably occurred during rinsing. Furthermore, an incubation period of 15 min was not sufficient to reduce the percentage of vital primary stromata to zero. Even with rinsing being delayed by 24 h, an effect comparable with that of $Cu(OH)_2$ was not obtained.

The copper contents of $Cu(OH)_2$ and $CuSO_4$ are 65 and 40 wt %, respectively (*15*). $Cu(OH)_2$ has a water solubility of 2.9 mg L^{-1}. Hence, a concentration of approximately 1.9 mg L^{-1} copper ions was present at all concentrations tested. As the water solubility of $CuSO_4$ is 230.5 g L^{-1}, $CuSO_4$ was completely in solution at all concentrations tested. Hence, copper ion concentrations ranged from a minimum of 12 mg L^{-1} (at 29.0 mg $CuSO_4$ L^{-1}) to a maximum of 139 mg L^{-1} (at 348 mg $CuSO_4$ L^{-1}). $Cu(OH)_2$ at 116 mg L^{-1} killed all primary stromata 24 h after inoculation; of the 75 mg Cu L^{-1} applied, only 1.9 mg L^{-1} was present as dissolved ions. $CuSO_4$ did not kill all primary stromata at the much higher concentration of 348 mg L^{-1}. This indicates that the effectiveness of $Cu(OH)_2$ cannot be explained by the concentration of dissolved copper ions. Our data support the hypothesis that spore exudates form toxic copper complexes (*18*−*22*). These copper complexes have to be more toxic than dissolved copper ions. Martin et al. (*21*) examined dosage response curves for various inorganic and organic copper compounds. Copper malate, which was also found in spore exudates, was found to be the most toxic copper complex. It was much more toxic than Cu^{2+} ions. The contact between the spore and the residues of the applied copper compounds is regarded as another important factor for their fungicidal effects (*18*, *19*, *24*). As shown in **Figure 1D**, $Cu(OH)_2$ particles adhered to the spore, germ tube, and appressorium and were not removed by the rinsing process.

Effect of $Cu(OH)_2$ on Leaf Disks under Wet Conditions. The minimum effective concentration of $Cu(OH)_2$ of 116 mg L^{-1} (**Figure 2**) was also tested on apple leaf disks. $Cu(OH)_2$ applied 24 h after inoculation stopped the formation of new primary stromata (**Figure 4**). No significant difference could be observed between the $Cu(OH)_2$ treatment and the control. Furthermore, the percentage of primary stromata found 72 h after inoculation was considerably reduced as compared with the control. Although a distinction between vital and dead stromata was impossible, it is likely that the $Cu(OH)_2$ treatment killed all primary stromata as they had not increased in size and frequency as compared to the stromata of the control.

Effect of $Cu(OH)_2$ on CM under Dry Conditions. When the surfaces of CM were dried after application of the treatments, no significant difference between the $Cu(OH)_2$ treatment and the control was observed (**Figure 5**). This indicates that $Cu(OH)_2$ at 116 mg L^{-1} was not able to kill primary stromata at the CM/water interface when the surface of the CM with the inoculum was dry. Formation of additional primary stromata was stopped when surfaces were dry. The percentage of vital primary stromata was significantly reduced as compared with the control under wet conditions but also significantly higher as compared with the $Cu(OH)_2$ treatment under wet conditions.

These results suggest that $Cu(OH)_2$ is toxic only if its particles in contact with spores, germ tubes, and appressoria are covered by a water film. During wet incubation, the spore apparently secretes exudates that form copper complexes. These copper complexes eventually kill the spore. The longer the incubation period, the higher the probability that toxic concentrations inside the spore are reached and kill primary stromata. We did not measure the minimum duration for wet incubation. A duration of 24 h was sufficient, but it is likely that the minimum incubation period needed to kill stromata depends on the number of copper-containing particles associated with spores, germ tubes, and appressoria on the outer surface of the cuticle. Existing primary stromata located in the cell wall of leaf disks and at the CM/water interface were killed, even though they had no contact with the particles. Apparently, the copper complexes are translocated from spores, germ tubes, and appressoria through the infection pegs to the stromata. This translocation requires that copper is mobile and not bound ionically to negative charges in the fungus. The greater toxicity of copper complexes as compared to copper ions may thus be linked to their ease of translocation. This would require that complex-forming compounds were not exudated when $CuSO_4$ was applied.

Impact on Scab Control under Field Conditions. We have shown that $Cu(OH)_2$ affects apple scab for at least 24 h after infection, provided that leaf surfaces are wet after the infection has occurred. In the study of Hamilton (*25*), Bordeaux mixture reduced the number of lesions by between 51 and 14% as compared with the control, depending on the incubation temperature and the time of application after an infection period. The study was conducted under controlled conditions in a wet chamber and in a greenhouse. The lack of wind and the high relative humidity in the wet chamber and greenhouse may have prevented leaf surfaces from drying quickly. This could be the reason a postinfection activity was found. The postinfection activity of Bordeaux mixture has never been critically tested

under field conditions, possibly due to the effectiveness of lime sulfur and other compounds.

Our results also provide evidence that fungal structures on the CM are more sensitive to copper than structures that have already penetrated the CM. Low concentrations of $Cu(OH)_2$ and $CuSO_4$ of 29 and 58 mg L^{-1}, respectively, applied 48 h after inoculation, did not significantly reduce the number of vital primary stromata but stopped the formation of new ones (**Figure 3**). Hence, conidia, germ tubes, and appressoria on the upper surface of the CM must have been killed. This indicates that with strategies where copper is not applied until spores are present a greater effectiveness will be obtained when leaves remain wet for a longer time. Furthermore, the results suggest that copper application rates could be reduced using $Cu(OH)_2$ or even less water soluble or insoluble copper compounds due to the higher effectiveness of the formed copper complexes. The use of these compounds might also reduce phytotoxic and ecotoxicological side effects because the amount of copper ions in solution, which can easily run off during rain or dew, would be lower.

ABBREVIATIONS USED

CM, cuticular membrane(s); $Cu(OH)_2$, copper hydroxide; $CuSO_4$, copper sulfate; FDA, fluorescein diacetate; SD, standard deviation.

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