CHEMISTRY OF WEATHERING AND SOLUBILISATION OF COPPER FUNGICIDE AND THE EFFECT OF COPPER ON GERMINATION, GROWTH, METABOLISM, AND REPRODUCTION OF DOTHISTROMA PINI

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ABSTRACT

Copper fungicide (as 50% cuprous oxide W.P. formulation) applied to **Pinus radiata** D. Don needles is solubilised by complexation and oxidation processes to potentially give >30 mg cupric ion (Cu^{2+})/l in aqueous solution. Bioassays using **Dothistroma pini** Hulbary conidia showed that exposure to 20 mg Cu^{2+}/l in the presence of **P. radiata** needle aqueous exudates (which stimulate germination, and support fungal growth) for periods as short as 1.5 h was sufficient to kill the spores. Lower concentrations (10 mg/l) of Cu^{2+}, while not greatly reducing conidial germination rate, substantially reduced germ tube length and affected the hyphal anatomy, while 5 mg Cu^{2+}/l prevented production of conidia by mycelium grown **in vitro**. Low dose rates (0.1–5 mg/l) affected **D. pini** mycelium metabolism, causing a five-fold increase in secondary metabolite (mainly dothistromin) synthesis, but did not reduce conidia germination. Cu^{2+} concentrations in water films on the **P. radiata** needle surface need to be above 10 mg/l to prevent infection from taking place, or above 5 mg/l to prevent re-infections from secondary conidia.

Keywords: copper fungicide; solubilised copper; Dothistroma life-cycle; **Dothistroma pini**; **Pinus radiata**.

INTRODUCTION

Aerial application of copper (Cu) fungicide for control of Dothistroma needle blight of *Pinus radiata* has been carried out successfully for more than two decades in New Zealand. That control of the disease has been so satisfactory is due to factors such as careful assessment of the optimum time to spray (Gilmour & Noorderhaven 1973) and good operational spraying practice (Kershaw *et al.* 1982). Regardless of the particular formulation, the copper fungicides tested were effective, and persisted on *P. radiata* needles for some months (Gilmour & Nooderhaven 1973). While evidence for control of Dothistroma blight of *P. radiata* is readily apparent, there has been little work published to explain this in terms of the effect of cupric ion (Cu^{2+}) on *D. pini* conidia and mycelium, *D. pini* metabolism, and reproduction. Suggestions have been made that germinating *D. pini* conidia were highly sensitive to Cu^{2+}, and that extensive

redistribution of the fungicide spray deposits within the tree crown enabled effective contact with the fungus (Gibson *et al.* 1970). Some evidence has been presented to suggest that Cu^{2+} also reduced sporulation of the pathogen in established lesions (Gibson 1972).

While extensive solubilisation and redistribution of fungicide would be incompatible with persistence of the residue, with an even well-applied fungicide local redistribution on individual leaves has been shown to give good ion-spore contact, and hence good disease control (Hislop & Cox 1970).

To obtain water-soluble compounds from an insoluble fungicide such as cuprous oxide (Cu_2O), oxidation processes must be involved. All simple salts of copper that are soluble in water are CuII, whereas all simple cuprous salts (CuI) are insoluble.

Complex cuprous salts, such as those with anion $CuCl_3^{2-}$, although resistant to hydrolysis in aqueous solution only in the presence of excess of Cl^-, are readily oxidised by air to the cupric complex. In reported bioassays, Cu_2O has been shown to have the same slope for the dose-response curve as did Cu^{2+}, but with an LD_{50} value twice that of Cu^{2+}, implying that only half of the copper in Cu_2O was available to the fungus as Cu^{2+} (Martin *et al.* 1942).

It has also been commonly observed that Cu_2O fungicide applied to plant surfaces darkens with time (Park & Burdekin 1964) because of formation of cupric oxide (CuO). However, it has been considered that CuO is a less effective fungicide than Cu_2O in some applications (Wain & Wilkinson 1946), the Cu_2O fungicide formulation also losing fungitoxicity on prolonged storage (Hislop & Park 1962).

This paper reports studies on the chemistry of weathering and solubilisation of a typical copper fungicide (Cuprous Oxide 50% W.P.) which has been used for Dothistroma needle blight control, and the effect of Cu^{2+} on *D. pini* conidia germination, mycelial growth and secondary metabolism, and sporulation, *in vitro*.

MATERIALS AND METHODS

Chemical Changes Occurring During Exposure of Cu_2O Fungicide to Air and Moisture

Suspensions of Cu_2O fungicide (50% metallic equivalent (m.e.) Nordox Agrochemicals Division, Oslo, Norway) in water were used at a rate of 3 g m.e./100 ml. The suspension was sprayed to run-off on to foliage of *P. radiata* rooted cuttings (6 trees, c. 50 cm height), on to 20 glass microscope slides (76 × 25 mm), and on to 20 glass microscope slides which had been coated with *P. radiata* needle epicuticular wax (Franich *et al.* 1977, 1978). The applied fungicide was allowed to dry, and the foliage and microscope slides were exposed to air, full sunlight, and rain at the Forest Research Institute, Rotorua (close to geothermal H_2S sources), and at Cpt 360, Kaingaroa Forest.

After periods of 1, 2, and 3 months samples were taken and the fungicide residue was fractionated. The samples were treated with water to remove free Cu^{2+}. The water-insoluble fraction was treated with 30% H_2O_2 (10 min) to oxidise and solubilise any CuS to $CuSO_4$. The insoluble residue from that was treated with 11 N HCl to dissolve

remaining copper oxides, as their perchloro complex. Separation of CuI and CuII complexes was carried out by paper chromatography in a CO_2 atmosphere using the solvent system diethyl ether, methanol, 11 N HCl, H_2O (10 : 6 : 1 : 3) (Paul & Janardhan 1986). The proportions of CuI and CuII were estimated from the chromatogram spots visualised with rubeanic acid. The total Cu concentration in each solution obtained above was determined by flame atomic absorption spectrometry (AAS).

Solubilisation of Cu_2O Fungicide by *P. radiata* Needle Exudates and by *D. pini* Metabolites

Pinus radiata phylloplane materials were obtained by washing 40 kg foliage for 1 min with 2 *l* distilled H_2O. The solution obtained was filtered (Whatman 1) and concentrated at 50°C using a rotary evaporator to give 1 g of a brown solid (Franich & Wells 1976).

Crude *D. pini* metabolites (principally dothistromin) were obtained by growing the fungus in 10% malt shake culture, and extracting the homogenised cultures with ethyl acetate (EtOAc). The extract was concentrated to give a red solid (Bassett *et al.* 1970).

The above mixtures (1 mg) from *P. radiata* needles or *D. pini* mycelium were shaken with Cu_2O fungicide, with CuO, and with CuS (10 mg each) in distilled water (1 ml) for 48 h. The mixtures were centrifuged, and the Cu^{2+} content of the supernatant was determined by AAS.

Dew was collected from fungicide-sprayed foliage of seedlings in the FRI nursery at Rotorua, and the Cu^{2+} content analysed by AAS. The seedlings had been sprayed at a rate of 2.24 kg Cu m.e. in 56 *l* H_2O/ha.

Toxicity of Cu^{2+} to Germinating *D. pini* Conidia

The salt used for all bioassays was $CuSO_45H_2O$.

Dothistroma pini conidia were isolated by flooding cultures grown on 10% malt-agar plates with sterile water. The suspension was centrifuged, and the supernatant discarded. The spore pellet was resuspended in sterile water, and the suspension was adjusted to a density of c. 10^4 cells/ml. On to glass microscope slides were placed conidial suspension (0.05 ml), germination stimulant (either 0.5%, + 5% aqueous malt, or 0.5%, + 5% *P. radiata* needle exudate) (0.05 ml), plus Cu^{2+} solution (0.05 ml) to give concentrations in the assay of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 40, and 50 mg/*l*. After 48 h at 18°C, the germination rate was determined microscopically. Plots of probit percentage response *v.* log Cu^{2+} concentration were made.

Conidia, isolated as above, were incubated with aqueous Cu^{2+} (20 mg/*l*) for periods of 5 min, and 1, 1.5, and 18 h. After each time interval, the suspensions were centrifuged, and the spore pellet was used as obtained, or washed with distilled water, or with 0.5% HCl, followed by distilled water. A germination test was set up as above, and the response determined after 48 h and scored as germinated, partly germinated, or non-germinated.

Uptake of Cu^{2+} by *D. pini* Cells

A *D. pini* cell suspension was obtained as a mixture of short mycelial fragments and conidia by pressing fungal material through a fine stainless steel sieve. The suspension (405 mg dry weight) was incubated at 18°C for 19 h with aqueous Cu^{2+} (20 mg/*l*). The suspension was centrifuged and washed with distilled water (3×), and the solid was hydrolysed using 4 N HCl (20 ml) at 110°C for 4 h. The filtered hydrolysate was diluted to 100 ml, and analysed by AAS for Cu content.

Effect of Cu^{2+} on Growth, Metabolism, and Sporulation of *D. pini in vitro*

Dothistroma pini was cultured at 18°C on 0.5% malt agar including Cu^{2+} at 0.1, 0.2, 0.5, 1, 2, 5, 10, 15, 20, and 30 mg/*l*, together with control cultures. After 2 and 4 weeks' incubation, the flasks were flooded with distilled water (50 ml), agitated gently for 10 min, and a spore count was made using a Hemacytometer.

Liquid shake cultures of *D. pini* were grown in 10% malt medium, and in the presence of a range of Cu^{2+} concentrations, as above, at 18°C for 7 days. The cultures were homogenised, and extracted with equal volumes of EtOAc to recover anthraquinone metabolites, principally dothistromin. The organic layer was separated by filtration through phase-separating paper (Whatman PS-1), and the absorbance measured at 485 nm. The mycelium suspension was dried at 80°C *in vacuo* and weighed.

RESULTS

Chemical Changes Occurring During Exposure of Cu_2O Fungicide to Air and Moisture

On glass microscope slides, and in the presence of geothermal sulphur sources (FRI, Rotorua), Cu_2O oxide fungicide underwent oxidation and reaction with H_2S and air to form CuS and $CuSO_4$. The total residue also diminished over the 3-month period. The results, expressed as a percentage of original Cu m.e. applied, are summarised in Table 1.

TABLE 1–Weathering of Cu_2O films on glass microscope slides kept at FRI (geothermal environment). Mean Cu m.e. residues as a percentage of initial application.

Component in residue	1 month	2 months	3 months
$CuSO_4$	5	5	5
CuS	15	25	25
$CuO + Cu_2O$	60	45	25
Total residue	80	75	55

Pinus radiata needle wax had no significant effect on the weathering of Cu_2O fungicide, as the composition of the residue from wax-covered and uncoated glass slides after 1 month of exposure was similar (Table 2). The Cu oxides residue contained

TABLE 2–Composition (%) of Cu m.e. residues on glass and on microscope slides coated with *P. radiata* wax after 1 month of exposure in a geothermal environment

Component	Glass	Needle wax
$CuSO_4$	6	5
CuS	24	14
$CuO + Cu_2O$	70	81

c. 1 : 1 ratio of CuI : CuII as estimated by paper chromatography of the perchloro derivatives.

In the absence of geothermal sulphur gases in the air (Cpt 360, Kaingaroa Forest), the Cu_2O fungicide residue underwent very little chemical change over 3 months. The residue contained c. 10% CuO as estimated from paper chromatograms of the perchloro derivatives of the Cu oxides.

On *P. radiata* foliage in the presence of geothermal sulphur gases, Cu_2O fungicide weathered to give higher concentrations of water-soluble Cu than obtained from the glass microscope slide experiments. The composition of the residue was essentially the same for each month of harvesting (Table 3). In the absence of geothermal sulphur gases, the composition of the residue on *P. radiata* foliage was similar.

TABLE 3–Composition of Cu m.e. residues on *P. radiata* foliage after 1 month in a geothermal environment

Component	Percentage in residue
$CuSO_4$	20
CuS	5
$CuO + Cu_2O$	72
Cu fixed on needle	3

Solubilisation of Cu_2O Fungicide by *P. radiata* Needle Exudates and by *D. pini* Metabolites

Cu was brought into aqueous solution when Cu_2O fungicide was shaken with water, and with *P. radiata* needle exudates, and with *D. pini* metabolites. In particular, *P. radiata* needle exudates increased the dissolution of Cu_2O, CuO, and CuS by one or two orders of magnitude (Table 4).

Dew collected 2 days after *P. radiata* seedlings had been sprayed with Cu fungicide contained 30 mg Cu^{2+}/l.

Toxicity of Cu^{2+} to Germinating *D. pini* Conidia

The dose-response data for toxicity of Cu^{2+} ion to germinating *D. pini* conidia showed dependence on the strength and type of the germination stimulant used in the assay. The plots of probit percentage response *v.* log Cu^{2+} concentration are shown

TABLE 4–Concentration (mg/*l*) of Cu^{2+} in water, water plus *P. radiata* needle exudates, and water plus *D. pini* metabolites (mainly dothistromin)

Cu source	H_2O	H_2O + *D. pini* metabolites	H_2O+ *P. radiata* exudates
Cu_2O	3	16	350
CuO	0.1	12	130
CuS	10	24	100

in Fig. 1, for 0.5% and 5.0% germination stimulant concentrations. At both concentrations, *P. radiata* needle exudate appeared to promote germination more than did malt extract (the usual growth medium employed in *D. pini* culture). LD_{50} values were 0.5% malt, 12 mg/*l*; 0.5% needle exudate, 23 mg/*l*; 5% malt, 40 mg/*l*; 5% needle exudate, 48 mg/*l*.

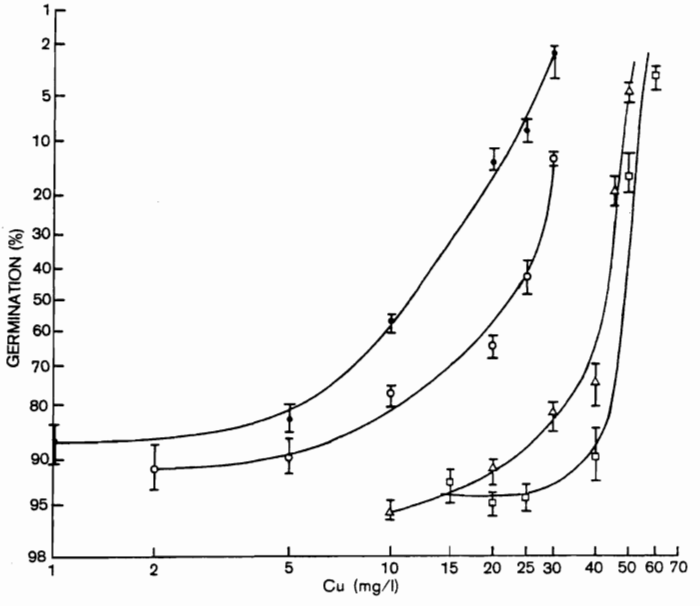

FIG. 1—Plot of probit percentage germination of **D. pini** conidia in presence of Cu^{2+} and 0.5 (●) and 5% (Δ) malt, or aqueous needle exudate 0.5 (○) and 5% (□). Bars ± S.D.

Uptake of Cu^{2+} by *D. pini* Cells

Five minutes contact of *D. pini* spores with Cu^{2+} (20 mg/*l*) was insufficient for toxicity, but after 1.5 h germination was inhibited. After contact with Cu^{2+} (20 mg/*l*) for 18 h, *D. pini* conidia had accumulated 1728 μg/g dry weight of tissue (c. 0.17%).

Effect of Cu^{2+} on *D. pini* Growth, Metabolism, and Reproduction *in vitro*

During growth of *D. pini* in the presence of Cu^{2+} in the concentration range of 0.5 to 5 mg/*l*, it was observed that the cultures appeared dark purple-brown in colour. The coloured material was extractable with EtOAc after acidification of the cultures with 0.5% HCl, and was shown to be a mixture of dothistromin and other *D. pini* secondary metabolites by UV-visible absorption spectrometry and thin-layer chromatopography (Gallagher & Hodges 1972). Growth of *D. pini* was substantially reduced in the presence of >5 mg Cu^{2+}/*l*. The effect of Cu^{2+} concentration on *D. pini* mycelial weight produced in culture, and on production of secondary metabolites (absorbance at 485 nm) is indicated in Fig. 2.

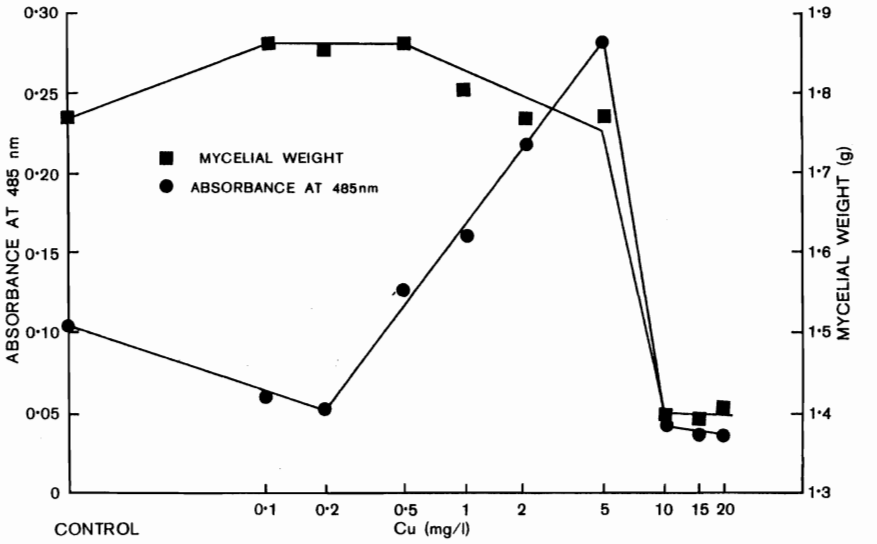

FIG. 2—Effect of Cu^{2+} concentration on **D. pini** mycelial growth, and on production of **D. pini** secondary metabolites (absorbance at 485 nm).

The effect of Cu^{2+} on sporulation of *D. pini* in culture is shown in Fig. 3. After 2 weeks' incubation little effect on conidial production was observed for Cu^{2+} doses up to 0.5 mg/*l*. After 4 weeks, when the spore count for the control cultures had doubled, Cu^{2+} at concentrations greater than 0.1 mg/*l* effectively reduced sporulation. Very few conidia were produced by *D. pini* growing in the presence of Cu^{2+} at >5 mg/*l* concentration.

Cu^{2+} also affected the germ tube length and anatomy of *D. pini* (Fig. 4). Conidia and germ tubes displayed a nodular appearance (arrowed) in the presence of 1–5 mg Cu^{2+}/*l* (Fig. 4b) compared with the controls (Fig. 4a). While 10 mg Cu^{2+}/*l* had little effect on conidial germination rate, germ tube length was reduced and the emergent germ tube wall often appeared ruptured, with possible leakage of cell contents (Fig. 4c). In the presence of 30 mg Cu^{2+}/*l*, germination was almost completely inhibited, although the spores appeared to be fully imbibed (Fig. 4d).

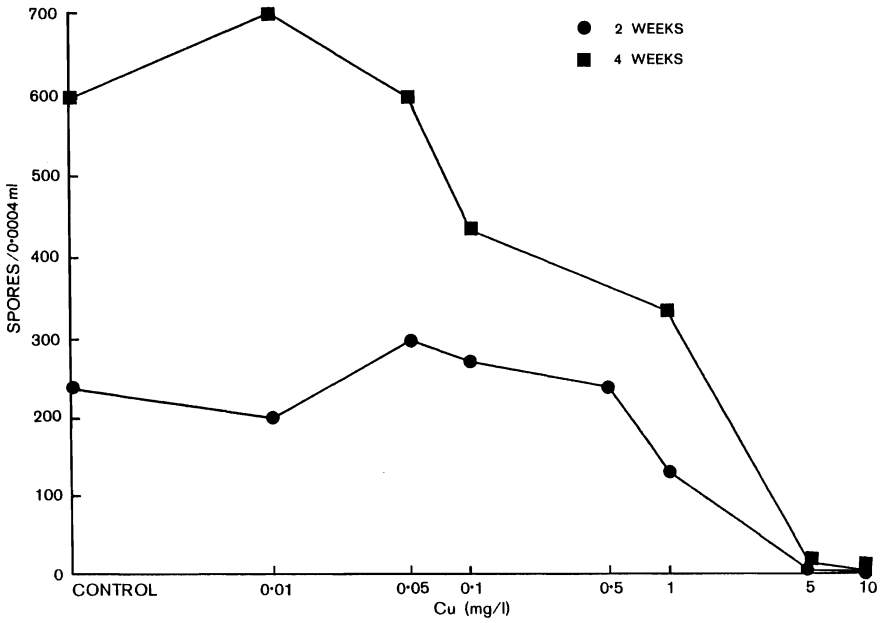

FIG. 3—Inhibition of conidia production of **D. pini** in presence of Cu^{2+}.

DISCUSSION

Cu_2O fungicide applied to glass surfaces underwent weathering chemical changes to CuO and, in the presence of geothermal sulphur gases in the air, reaction to give CuS and $CuSO_4$.

On *P. radiata* needles, Cu_2O fungicide underwent different weathering processes, giving more (water-soluble) Cu^{2+} in the residue, suggesting that compounds at the foliage surface were effecting changes in the applied fungicide. This was shown by reacting Cu_2O fungicide with the needle exudate material composed of carbohydrates, amino acids, organic acids, and protein (Franich & Wells 1976) to provide a solution containing several hundred ppm Cu^{2+} probably complexed as amino acid and carboxylic acid chelates and salts. The mixture of metabolites produced by *D. pini* could also be effective in contributing to solubilisation of the copper residue on the needle. Therefore, solubilisation and oxidation processes can take place on the pine needle surface to transform insoluble Cu_2O into Cu^{2+}, or complex Cu^{2+} compounds, and Cu_2O undergoes greater solubilisation than does CuO (Table 4).

That the dose-response relationship for toxicity of Cu^{2+} to *D. pini* was dependent on the concentration and type of germination stimulant used suggests that some free Cu^{2+} may be complexed to compounds in the media, and made unavailable to the germinating conidia. It is known that complexing Cu^{2+} with hydroxy acids, dibasic acids, or amino acids can affect its fungitoxicity (Martin *et al.* 1942), and that a Cu^{2+}

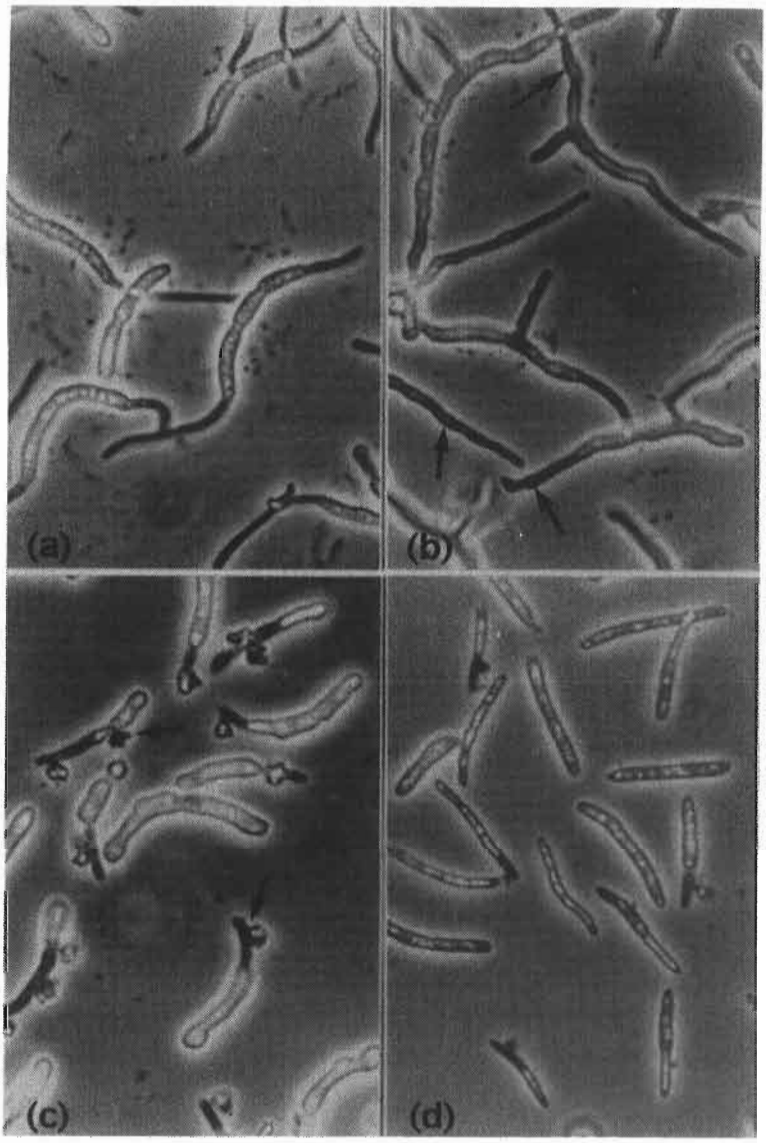

FIG. 4—(a) Germinating **D. pini** conidia in 0.5% malt medium.
(b) Same as (a), with addition of 5 mg Cu^{2+}/l.
(c) As for (a), with 10 mg Cu^{2+}/l.
(d) As for (a), with 30 mg Cu^{2+}/l.

complex may be more able to penetrate the bipolar cell membrane of the fungal spore than can free Cu^{2+} (Miller 1967). Ion exchange processes between the fungal cell wall and Cu^{2+} complexes have also been postulated (Somers 1963). The probit response *v.* log Cu^{2+} concentration relationships obtained were not linear but concave, of the type

described by McCallan (1948), and suggest more than one type of interaction of Cu^{2+} with *D. pini* conidia. A minimum of 1.5 h contact time was needed for Cu^{2+} to be toxic to germinating *D. pini* conidia. After 18 h exposure, the spores had accumulated c. 0.17% of their tissue dry weight as Cu, similar to the uptake of Cu by *Neurospora crassa* Shelear & Dodge (Somers 1963). While concentrations of c. 30 mg Cu^{2+}/l on the pine needle surface could reduce the *D. pini* conidia germination rate to non-pathogenic levels, such concentrations may not be necessary, since 10 mg Cu^{2+}/l reduced germ tube length and growth to a point that could reduce the number of stomatal penetrations by hyphae. Cu^{2+} concentrations as low as 5 mg/*l* may effectively reduce the production of secondary conidia by pine needle surface mycelia, a factor considered to be important in the pathogenesis of *P. radiata* by *D. pini* (Gadgil 1967).

At low concentrations, Cu^{2+} had an effect on *D. pini* secondary metabolism. It was apparent that Cu^{2+} in the range 1–5 mg/*l* perturbed *D. pini* cells in some way which resulted in an increase in secondary metabolite (mainly dothistromin) biosynthesis. Similar effects on *D. pini* have been found with natural fungistatic compounds, such as hydroxydehydroabietic acids found on the *P. radiata* needle surface (Franich *et al.* 1983). Low concentrations of Cu^{2+} also caused changes in the cell wall of the *D. pini* conidia, and emerging germ tubes. An increase in production of dothistromin, known to be toxic to *P. radiata* needles (Shain & Franich 1981) caused by low Cu^{2+} concentrations acting on *D. pini* may be potentially damaging. This is considered unlikely since dothistromin forms a brown water-insoluble complex with Cu^{2+}, which would probably make the toxin unavailable to the needle tissue. However, dothistromin could also remove some of the available Cu^{2+} on the needle surface by chelation.

CONCLUSIONS

Cu_2O fungicide can react with aqueous exudates on *P. radiata* needles and, to a lesser extent, with *D. pini* metabolites, to form free or complexed Cu^{2+} in aqueous solution at concentrations sufficient to inhibit the germination of *D. pini* conidia. The interaction of Cu_2O fungicide and geothermal H_2S to produce CuS and subsequently $CuSO_4$ can contribute to solubilising the fungicide. Low Cu^{2+} concentrations effectively reduce germ-tube growth and inhibit the production of secondary conidia, as well as stimulate dothistromin biosynthesis. It is the combination of solubilisation and redistribution of copper as Cu^{2+}, or its complexes, and their ready uptake by *D. pini* conidia which can explain the good control of Dothistroma needle blight by copper fungicides.

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