Factors Affecting Control of European Apple Canker by Difolatan and Basic Copper Sulfate

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ABSTRACT

Oil enhanced the toxicity of basic copper sulfate (BCS) to *Nectria galligena* conidia at copper concns between 10^0 and 10^2 μg/ml but reduced toxicity at 10^3 μg/ml as measured by germination inhibition. The effect was not observed with respect to inhibition of germ tube elongation. Difolatan was fungitoxic in vitro at 1.0 μg/ml. In field spray tests, Difolatan 4 Flowable 2.8 liters/378.5 liters (3 qt/100 gal) reduced conidial and perithecial production but BCS 2.3 kg+1.9 liters Supreme oil/378.5 liters (5 lb+2 qt/100 gal) decreased only perithecium production. Both fungicides were retained on the trees for the 12-wk leaf fall period but only Difolatan was redistributed in fungitoxic amounts. Three wk after spraying, Difolatan apparently had increased on twigs whereas it had decreased on leaves. The less retentive leaves served as a temporary reservoir for Difolatan. The copper in deposits of BCS decreased linearly with time and rainfall. Bioassays showed that Difolatan totally inhibited conidial germination during the normal infection period but inhibition on BCS-sprayed trees decreased rapidly with time.

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Additional key words: Nectria galligena, fungicides.

In California, European apple canker, caused by *Nectria galligena* Bres., was controlled by a single application of Difolatan at initiation of leaf fall (11). Basic copper sulfate (BCS) plus oil applied at initiation of leaf fall and at mid-leaf fall also provided adequate control (11). The principal mode of entrance of *N. galligena* is at leaf scars and since apple leaves abscise over a 12-wk period in California, for adequate disease control, a fungicide should have good

redistribution and retention qualities.

In the past 30 yr, studies have been made on the toxicity, deposition, retention, and redistribution of copper fungicides, principally Bordeaux mixture (2, 6, 10, 16, 18, 19, 23, 24, 25, 26, 27). Brook and Bailey (5) reported that Bordeaux misture reduced sporulation of *N. galligena*, whereas other authors have reported similar effects for benomyl and phenyl mercury chloride (2, 9).

More recently, reports dealing with toxicity, deposition, retention, and redistribution of Difolatan have been published (3, 13, 21, 22).

This paper deals with factors involved in the control of European apple canker by basic copper sulfate and Difolatan. Six factors were studied: decrease in conidial viability and germ tube elongation, suppression of conidial and/or perithecial production, and redistribution and/or tenacity of the fungicides.

MATERIALS AND METHODS.—*In vitro toxicity of BCS and Difolatan.*— Inhibition of conidial germination and germ tube elongation were used to measure toxic effects of BCS and Difolatan. Basic copper sulfate (Cities Service Company, P.O. Box 50360, Atlanta, Ga.) was incorporated into a 2.5% water agar medium at 10^{-1}, 10^{0}, 10^{2}, 10^{3} μg Cu/ml of medium. When oil (Niagara Supreme Oil, Niagara Chemical Division, FMC Corporation, P.O. Box 1589, Richmond, Calif.) was tested as an adjuvant it was added to the BCS suspension (10^{3} μg Cu/ml) at the field rate 1.9 liters (½ gal) oil/2.3 kg (5 lb) BCS and diluted serially with the BCS so the copper:oil proportion was the same at all concns of BCS. The agar medium was buffered at pH 6.5 or 7.5 with 0.08 M potassium phosphate buffer. Toxicity of Difolatan (*cis-N*-1, 1, 2, 2-tetrachloroethylthio-4-cyclohexene-1, 2-dicarboximide) (Ortho Division, Chevron Chemical Co., Richmond, California) to conidia was studied by incorporating Difolatan, in flowable form, into 2.5% water agar in logarithmic series from 10^{-3} to 10^{3} μg/ml.

Conidia (10^{6}/ml) of *N. galligena* were applied to the surface of the agar medium by means of an aerosol spray. Plates were incubated at 21 C in darkness, and germination and germ tube elongation were measured after 20-24 hr.

Suppression of conidial and perithecial production.— This experiment was done in a Nectria-affected apple orchard in Sebastopol, California, during October, 1971-January, 1972. Twenty-five randomly selected, 1-year-old Red Delicious apple twigs with cankers were placed in 10-cm funnels in the orchard. The funnels, three per treatment, were tied to a steel stake and connected to a plastic jug by a flexible plastic tubing. Conidial counts were made on five different dates after rainfall and all counts per treatment combined. Estimation of the number of conidia in collected rainwater was made with a haemacytometer. Fungicides (concentrations given in results) were sprayed on the twigs until "run off" with a DeVilbiss atomizer after the first rain in October.

The twigs used for the conidial suppression study began to produce perithecia by mid-December and these were mostly mature on the unsprayed twigs by 6 January 1972. At this time they were brought into the laboratory and individual perithecia were counted in a 1.7-cm swath in the periphery of the cankered area to ascertain the effect of the fungicides. Seventy-five twigs per treatment were observed.

Tenacity of fungicides.— Sample twigs with residues of both fungicides were obtained from a European canker control trial during October-December, 1971. A spray consisting of 2.3 kg (5 lb) BCS plus 1.9 liters (½ gal) Niagara Supreme Oil/378.5 liters (100 gal) water, 3,740 liters/hectare (400 gal/acre) was applied by hand-sprayer on Red Delicious apple trees in October, 1971. Difolatan 4 Flowable (2.8 liters) (3 qt)/378.5 liters (100 gal) water, 3740 liters/hectare (400 gal/acre), was applied using an air-blast sprayer. The trees had not been sprayed with either of these materials during the preceding year. Samples of current season twigs were taken on four dates after rainfall and leaf samples were taken periodically after rainy periods until all leaves had fallen.

Copper was extracted from twig and leaf surfaces in 0.1 N HNO_3. Twig or leaf samples were agitated on a shaker at 24 C for 1.0 h and the extraction fluid was then filtered through Whatman #1 filter paper. Based on preliminary experiments in the laboratory, it was assumed that essentially all surface copper would be recovered. Copper concn was determined with an atomic absorption spectrophotometer.

Difolatan concn on twigs and leaves was determined by a modification of Kilgore and White's (17) method. The plant parts were placed on a shaker for 0.5 hr at 24 C in double-distilled benzene. The benzene was then filtered through Whatman 2V fluted filter paper containing 5 g anhydrous Na_2SO_4. The extraction liquid was frozen and kept in darkness until used. Assay was made using a gas-liquid chromatograph with an electron capture detector. The column used was 5% DC-200 on Chromosorb G (60/80 mesh, acid-washed, and DCMS-treated).

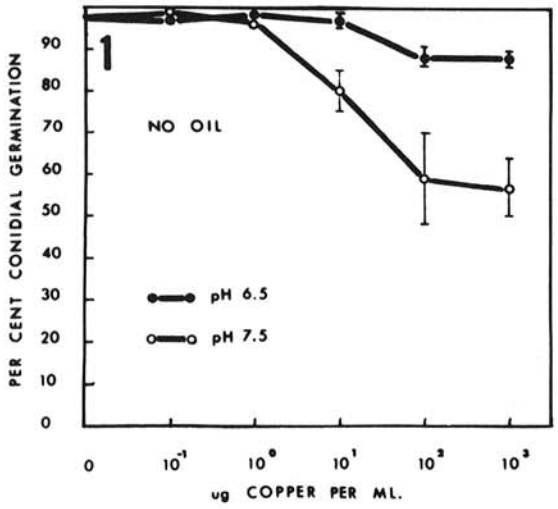
Representative twigs were also bioassayed for fungicide using conidia of *N. galligena* and Neely's (21) cellophane method.

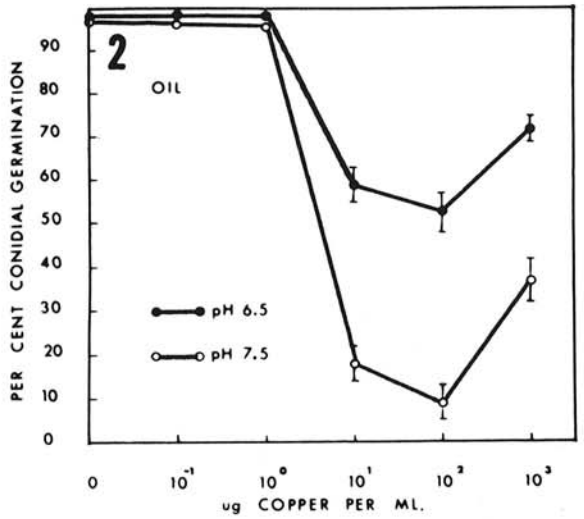
Redistribution of fungicides.— Redistribution of both fungicides was studied by observing twig surfaces that had been covered at time of spraying and by catching drip water after rains. The trees, type of twigs, and fungicide treatments were the same as used in the tenacity study.

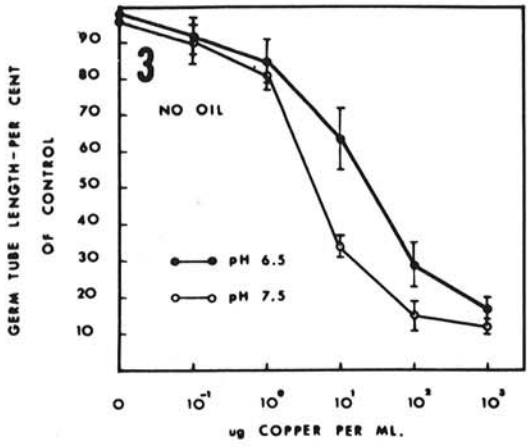
Randomly selected twig areas were covered with waterproof vinyl plastic tape (Scotch Brand #88, 3M Co., St. Paul, Minn.) before spraying. After specific periods the tape was removed and the twig exposed to normal weather conditions. The unsprayed areas were then checked for the appropriate fungicides using the chemical and bioassays previously described.

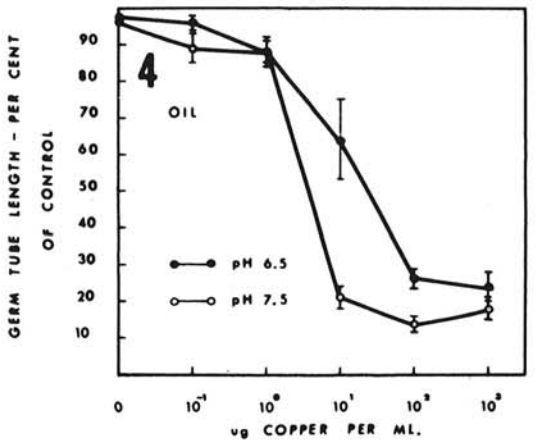
Drip water within the tree canopy was caught in metal cans for Difolatan and plastic jugs for BCS. The water was assayed for Difolatan by benzene extraction as previously described; whereas, for copper, the drip water was adjusted to 0.1 N HNO_3 by addition of concentrated HNO_3, filtered, and read directly on the atomic absorption spectrophotometer.

RESULTS.—*In vitro toxicity study.*— Comparison of the effect of BCS, with and without oil, on conidia (Fig. 1, 2) indicates that, without oil, BCS is less effective in inhibiting germination. Between 10^{0} and 10^{2} μg/ml copper and oil there is an enhancement of BCS inhibition of germination. The increase in germination (Fig. 2) at 10^{3} μg/ml may be due to oil blocking spore penetration of BCS at this concn since it is 10 times more concentrated than 10^{2} μg/ml copper. Interestingly, oil does not affect toxicity to









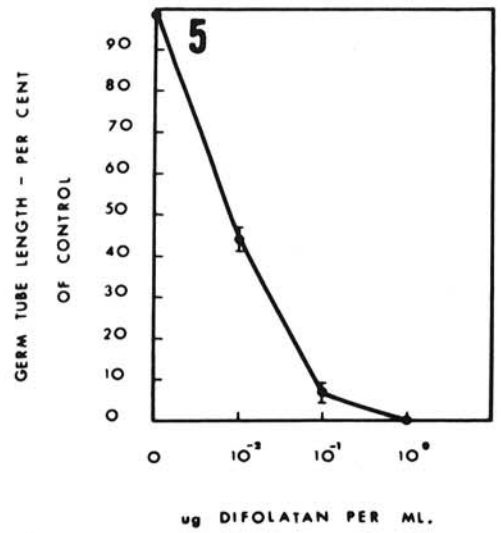


Fig. 1-5. 1) Effect of basic copper sulfate on germination of conidia of *Nectria galligena* at pH 6.5 and 7.5 in agar medium. Each point is the mean of five replicates. Vertical lines in all graphs represent ± SE. **2)** The same as Fig. 1 but with oil adjuvant added to the medium. **3)** Effect of basic copper sulfate on germ tube elongation of conidia of *N. galligena* at pH 6.5 and 7.5 in agar medium. Each point is the mean of five replicates. **4)** The same as Fig. 3 but with oil adjuvant added to the medium. **5)** Effect of Difolatan in agar medium on germ tube elongation of *N. galligena* conidia. Each point is the mean of six replicates.

germ tube elongation (Fig. 3, 4). Also the oil control, at field rate, did not inhibit germination or germ tube elongation.

The toxicity of BCS in the medium buffered to pH 7.5 was higher than in the one at pH 6.5.

Difolatan was extremely toxic to conidia and inhibited germination completely at 1 μg/ml and above, but allowed 100% germination at 10^{-1} μg/ml. Conidial germination at 5×10^{-1} μg/ml reached 27% in 72 h. Effect on germ tube elongation was great at low concns; e.g., at 10^{-1} μg/ml germ tube length was reduced ca. 93% below that of the control (Fig. 5).

Suppression of conidial and perithecial production.— Under the conditions of the experiment, Difolatan inhibited production of conidia and perithecia significantly (Table 1). Maturation of perithecia seemed to be delayed by Difolatan since perithecial initials were commonly observed but did not mature on twigs sprayed with Difolatan.

Although there was a consistent reduction of conidia due to treatment with BCS and oil, it was not statistically significant, nonetheless the copper-oil treatment did reduce perithecial production significantly (Table 1). Many more mature sporulating perithecia were produced on twigs treated with BCS and oil than with Difolatan. In both treatments, where mature perithecia were produced, the ascospores were viable.

Retention of fungicides.— The copper deposit on leaf and twig surfaces decreased linearly with time and rainfall (Fig. 6, 7, 8). Trees used as controls in the spray trial, had no measurable copper on leaves or twigs. Bioassay of twig surfaces (Fig. 9) indicated that the major effect on spores was inhibition of germ tube elongation for a longer period than inhibition of germination. An initial deposit of 30.2 μg/cm^2 of copper on twigs reduced germ tube lengths by ca. 93%, whereas conidial germination was decreased 82%. Control twigs bioassayed immediately after spraying permitted 99% conidial germination. Leaves retained less initial spray deposit (17.9 μg/cm^2 on leaves and 30.2 μg/cm^2 on twigs) than did twigs. This may be due to the fact that both sides of the leaf were used in calculation of copper/cm^2 and/or the much greater pubescence of twigs possibly allowed greater initial deposit.

Retention of Difolatan on twigs (Fig. 10) did not fit any simple theoretical consideration. Initial deposits on sprayed leaves and twigs did not differ greatly, although leaves generally had more. Border trees (Fig. 10) which were assayed for fungicide to check for spray drift had measurable amounts of Difolatan. Fungicide levels on twigs taken on 11 November, indicated a possible increase in fungicide over the initial deposit. Various observations correlate with this seemingly paradoxical phenomenon. The apparent increase is correlated with a period of slowly increasing rainfall (Fig. 8) thereby enhancing the likelihood of fungicide redistribution. Also, current-year twigs are very pubescent and possibly retain more fungicide than do leaves. It is possible that leaves, which have a much greater surface area than twigs, and which to some extent may protect twigs from the viscissitudes of weather, serve as a reservoir of Difolatan while attached to the tree. Observations on defolitation indicated that the period of principal leaf fall was between November 11 and December 23, precisely when Difolatan remaining on twigs showed its linear decrease. Although based on circumstantial evidence, the explanation is quite plausible. Indeed, even twigs of the border trees (Fig. 10) contaminated by Difolatan drift showed this apparent increase over initial deposit. Bioassay of sprayed twigs showed that a single Difolatan application was sufficient to completely inhibit conidial germination throughout the normal infection period during November and December 1971.

TABLE 1. Effect of Difolatan and basic copper sulfate+oil on conidial and perithecial production

Fungicide	Mean no. of conidia $\times 10^4$/ml water[a]	Mean no. of perithecia per canker[b]
Difolatan 2.9 liters/378.5 liters (0.75 gal/100 gal) water	1.88	3.57
Basic copper sulfate 2.3 kg.+1.9 liters oil/378.5 liters (5 lb+ 0.5 gal oil/100 gal) water	3.18	12.64
Control (unsprayed)	3.80	20.63

[a] LSD ($P=0.05$) $=1.50\times10^4$ conidia/ml.
[b] LSD ($P=0.001$) $=10.1$ perithecia/canker.
LSD ($P=0.01$) $=7.9$ perithecia/canker.

Difolatan levels on border trees were also inhibitory to germination in varying degrees.

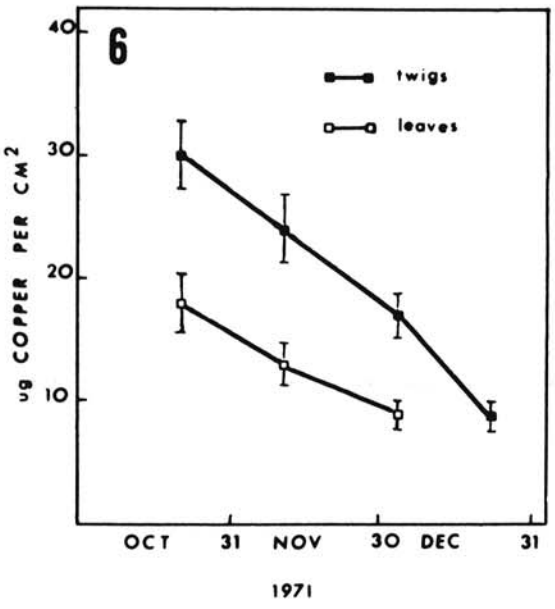
Redistribution of fungicides.— Table 2 demonstrates that regardless of rainfall incidence during the two periods, redistribution was observed. The toxicant resulting from an application of BCS and oil did not redistribute in large quantities onto bare twig surfaces. Instead, it was continually removed from the trees as shown by Fig. 6. Open containers placed within the tree canopy accumulated rainwater over a 3-wk period and these contained varying amounts of copper ranging from 2-23 μg/ml of water.

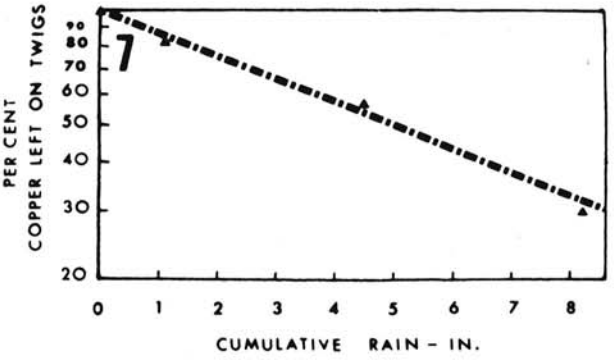
Difolatan, in contrast to BCS, was highly mobile and redistributed onto unsprayed twig areas in large quantities (Table 3). It is difficult to explain the large (59.9 μg/cm^2) quantity on areas of redistribution, since it was higher than the 38 μg/cm^2 observed on sprayed twigs on that date (Fig. 10). Previously taped areas may have been more adhesive and therefore more fungicide could have stuck to them. But laboratory tests did not support this hypothesis nor does the deposit of redistributed Difolatan in the second observation period.

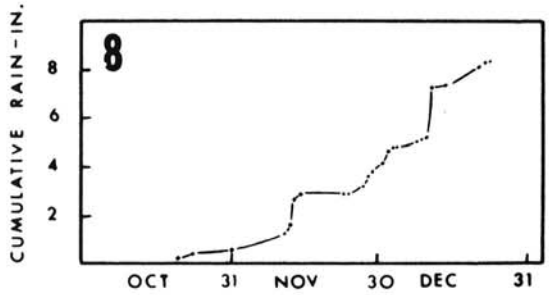
During the second observation period much less Difolatan remained on unsprayed areas than during the first period. This was probably due to the decrease of available Difolatan from heavy leaf fall and removal of the fungicide by heavier and more frequent rains (Fig. 8). This period also coincides with the period of rapid decrease of Difolatan on sprayed twig areas (Fig. 10).

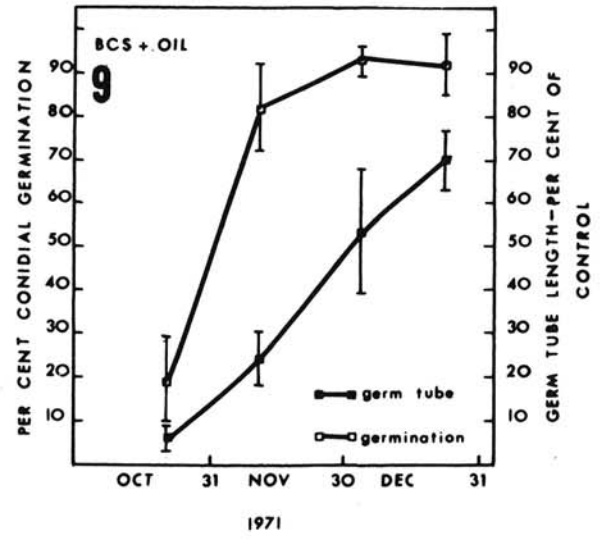
Drip water from sprayed trees contained 1-14 μg/ml of Difolatan when water was accumulated over a 3-wk period in open containers. This is at best a semiquantitative measure since Difolatan was possibly decomposed to some extent during this period and the assay method used would not detect it, whereas in the copper assay, copper in any form would be detected.

Bioassay with conidia placed on areas of redistribution indicated a large reduction in germinability (Table 3).









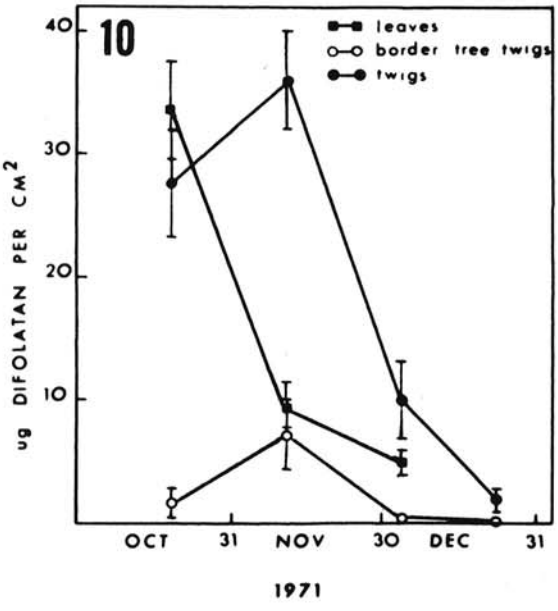


Fig. 6-10. 6) Retention of copper on twigs and leaves of Red Delicious apple trees between 21 October and 23 December, 1971. Values are means of four or five replicates. Control trees had no measurable copper. Vertical lines in all graphs represent ±SE. **7)** Linear relationship of the logarithm of per cent copper remaining on twigs between October 21 and December 23, 1971, versus cumulative rainfall. **8)** Cumulative rainfall between October 19 and December 23, 1971. Data from an official U.S. Weather Station, Graton, California, ca. 1 mile from test orchard. **9)** Bioassay of twig surfaces used in copper retention study (Fig. 6) indicating conidial germination and germ tube growth between October 21 and December 23, 1971. **10)** Retention of Difolatan on twigs and leaves of Red Delicious apple between October 19 and December 23, 1971. Values are means of four or five replicates. Border trees of the spray plot were contaminated with drift from the airblast sprayer.

DISCUSSION.— Results of in vitro toxicity studies are difficult to compare due to wide variation in experimental procedures. High concns of copper are generally needed for toxicity to spores (20) and results reported herein also indicated this. Tröger (29) working with copper sulfate toxicity to conidia of *Calonectria decemcellulare* Brick. found that copper uptake was more rapid at pH 8.5-10 than at pH 6.5-7.5. Results in this paper show an increase in toxicity at pH 7.5 compared to 6.5 in buffered medium but the mechanism is not clear. Several investigators (14, 24) have found that adhesives decrease toxicity of copper compounds by preventing entrance of the toxicant, or by flocculating the copper into larger, less toxic particles. This occurred in studies presented herein but only at a copper concn of 10^3 μg/ml. It is interesting to observe that at 10^0-10^2 μg/ml oil actually increased the fungitoxicity of BCS. It is generally thought that mineral oils are nontoxic to spores (7, 8) although oils alone have been useful in controlling such diseases as citrus greasy spot (12) and Sigatoka disease of banana (8). In both cases the authors felt that the effect was not on the pathogen per se but was due to a modification in host susceptibility. The fact that toxicity of copper fungicides is influenced by the host surface (1, 16) makes the value of in vitro studies quite relative. Field results cannot be equated directly to the in vitro studies. However, it seems that the control obtained with BCS and oil is probably more related to the reduction of germ tube elongation than to the decrease in germinability of *N. galligena* conidia. Neely (21) found that Difolatan in vitro was extremely toxic to conidia of *Monilinia fructicola* (Wint.) Honey and that 1.0 μg/ml provided 99% inhibition of germination. In studies with *Verticillium albo-atrum* Reinke and Berth. and *Ceratocystis ulmi* (Buism.) C. Moreau, an ED_{99} value of 0.2 μg/ml was obtained (22). Our studies show that *N. galligena* conidia are as sensitive to Difolatan as those of *M. fructicola*.

Brook and Bailey (5) noted that control of European apple canker with 10-8-100 Bordeaux mixture was associated with suppression of perithecial production and death of existing ones. However, no supporting data were presented. Corke et al. (9) found that a copper-phenyl mercury chloride combination retarded conidial production of *N. galligena* under field conditions. The number of germinable conidia were reduced by Bordeaux powder, according to Bennett (2), whereas benomyl reduced the number of sporodochia but didn't affect conidial germinability in the field. Results presented in this paper indicate that BCS plus oil and Difolatan reduced perithecial production of *N. galligena*, but only Difolatan significantly decreased the number of conidia produced. It is not known whether the latter effect is due to a reduction in size or number of sporodochia, number of conidia/sporodochium or a combination of these.

Burchfield and Goenaga (6) observed that the relation between the amount of "rain" applied to leaves and the logarithm of tenacity was linear for freshly prepared 10-10-100 Bordeaux mixture. However, a rapid initial loss was followed by a slower erosion rate with aged Bordeaux mixture or particulate coppers. The copper from "insoluble" copper salts appears to dissolve to form a saturated solution in rain, and the amount of copper removed is linearly proportional to the amount of rain and quite independent of initial deposit or intensity of rainfall, according to Courshee (10). Somers and Thomas (27), on the other hand, showed that low initial deposit increased the retention of copper fungicides. Our results, obtained with BCS plus oil, differ somewhat from those reported by both Courshee (10) and Burchfield and Goenaga (6). We observed a linear loss with time but the loss did not appear to be directly proportional to the amount of rainfall. The initial sloughing-off phase (6) reported for cuprous oxide was not observed in this study. This might have been due to increased tenacity of BCS applied with the oil adjuvant.

TABLE 2. Redistribution of basic copper sulfate onto unsprayed areas of twigs during October to December 1971

Observation	Redistribution period	
	21 October-11 November	11 November-4 December
μg Cu/cm² of twig[a]	2.39±0.43[b]	1.69±0.39
% conidial germination on area of redistribution	92.0	98.0
Rainfall, mm (inches)	20.8 (0.82 in)	92.5 (3.64 in)

[a] Mean of four to five replicates/period.
[b] ±SE.

TABLE 3. Redistribution of Difolatan onto unsprayed areas of twigs during October to December 1971

Observation	Redistribution period	
	19 October-11 November	11 November-4 December
μg Difolatan/cm² of twig[a]	59.9±14.90[b]	2.1±0.97
% conidial germination on area of redistribution	0.0	5.0
Rainfall, mm (inches)	28.9 (1.14 in)	92.5 (3.64 in)

[a] Mean of four or five replicates/period.
[b] ±SE.

Various authors have noted the resistance of Difolatan to weathering. Neely (22) found that Difolatan still was fungistatic to *M. fructicola*, *V. albo-atrum* and *C. ulmi* after 60 cm of simulated rain. Difolatan persisted longer on leaf surfaces than 14 other fungicides and average persistence on 12 hosts was 5-6 wk (21). Gilpatrick et al. (13) stated that one heavy Difolatan spray controlled apple scab, thus replacing five weekly sprays of Captan 50 WP. They attributed this success to tenacity and redistribution of the fungicide. Blazquez (3), however, found that Difolatan disappeared in 7 days on tomato leaves. This might have been due to the low concn used. In our studies fungitoxic amounts of Difolatan were observed throughout the 12-wk period that infection normally occurs in California. Sprayed twigs bioassayed in late December permitted no conidial germination.

The best explanation for the increase in Difolatan on twigs 3 wk after spraying is that the leaves, which retain Difolatan less than twigs, serve as a fungicide reservoir while still on the tree. Although quantitative data are lacking for leaf abscission on the sprayed trees, quantitative observations in a nearby orchard of the same variety showed that more than 60% of the leaves were present until

November 11, and then decreased rapidly thereafter. Observations in the sprayed orchard agreed with these data.

Redistribution has been recognized as an important factor in fungicide effectiveness in recent years. Interest in low volume sprays and aerial application will accentuate its importance (4). Hamilton et al. (15) noted that a definite balance between retention and redistribution is essential, and observed this with Fermate and sulfur. They also found that oil stickers did not prevent movement of sulfur. Rich (23) also found that Bordeaux mixture redistributed and stuck onto areas which had not received initial sprays. Hislop (16) checked for copper in drip water from apple trees and found that Bordeaux mixture and copper sulfate as measured by the presence of metallic copper, moved readily in rainwater. His results, although variable, indicated that copper fungicides were somehow detoxified in rainwater.

Interestingly, Hislop (16) noted that copper sprays on apple trees during low autumn rains would probably be important in controlling *N. galligena* due to redistribution. This does not agree with the redistribution data reported herein.

Gilpatrick et al. (13) attributed success in controlling apple scab to Difolatan's retentiveness and movement to unsprayed flower parts when applied at green tip to ½-in green flower stage. Results presented here show the importance of redistribution in helping to achieve control with Difolatan. It was retained at a high level on the tree and was redistributed from leaves to twigs during the infection period. Results with BCS plus oil seem less amenable to indicating a redistribution role in European canker control. It seems more probable that the effectiveness of copper is due to its reduction of the inoculum potential by decreasing germinability of conidia and/or rate of germ tube elongation. Although BCS and oil diminish perithecial production, the ascosporic stage is relatively unimportant in infection in California. However, in areas such as Northern Ireland where ascospores are thought to be major infective propagules, this effect presumably would be of great value (28).

The fact that BCS plus oil at low concn is not very toxic to conidia, does not redistribute well onto twigs, and does not significantly reduce conidial production, indicates why it may be necessary to make two applications of this material during leaf fall in order to achieve adequate control. Furthermore, it may be postulated that one Difolatan spray not only would reduce conidial germination to almost nothing for the entire infection period and decrease conidial and perithecial production significantly, but also would redistribute onto unsprayed leaf scars and probably protect them from infection.

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