Effects of Two Systemic Fungicides on Ultrastructure of Haustoria of the Oat Crown Rust Fungus

M. D. Simons

Cooperative investigation of the North Central Region, Agricultural Research Service, U.S. Department of Agriculture: Project No. 1752, and Journal Series Paper No. J-7848 of the Iowa Agriculture and Home Economics Experiment Station, Ames.

Research Plant Pathologist, North Central Region, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

All agricultural chemicals recommended for use in this report have been registered by the Environmental Protection Agency. They should be applied in accordance with the directions on the manufacturer's label as registered under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended by the Federal Environmental Pesticide Control Act.

Accepted for publication 16 October 1974.

ABSTRACT

Oxycarboxin and benomyl were applied in water suspension to soil in which were growing seedling oat *(Avena sativa)* plants. They previously had been infected with crown rust *(Puccinia coronata* var. *avenae).* Infected leaves were fixed and embedded in epoxy resin for electron microscopy 12 and 24 hours after soil treatment. Mitochondria and nuclei of haustoria of the fungus, fixed 12 hours after treatment with oxycarboxin, were clearly recognizable, with membranes ranging from nearly normal to obviously deteriorating. The interior structure of these organelles, and of the cell cytoplasm was severely damaged. Haustoria fixed 24 hours after treatment with oxycarboxin were difficult to fix and section and had greatly-enlarged, empty mitochondria. The plasmalemma and cell wall were generally intact. Haustoria from plants fixed 12 hours after treatment with benomyl showed no gross effects of treatment. However, 24 hours after treatment, nuclear membranes and plasmalemmae were severely damaged or missing, mitochondria were greatly enlarged, and cytoplasm was deteriorated. The ultrastructural effects of the two fungicides were shown to correlate, respectively, with physiological studies on the different modes of action of the two fungicides.

Phytopathology 65:388-392

Additional key words: fine structure.

In 1966, Von Schmeling and Kulka (11) reported that the oxathiin compound later named oxycarboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide-4,4-dioxide) would control bean rust (caused by *Uromyces phaseoli).* It could be applied by foliar application, as a seed treatment, or by application to the soil in which the host plants were growing. The chemical was readily transported in the xylem of the host, with no sign of injury to the host. They also mentioned control of wheat leaf rust (caused by *Puccinia recondita*), but gave no details. Since that time, many reports have confirmed the efficacy of oxycarboxin as a systemic fungicide to control the rusts, including crown rust (caused by *Puccinia coronata)* of ryegrass (4).

In 1968, Hardison (5) reported control of stripe rust of bluegrass (caused by *Puccinia striiformis*) with methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (later named benomyl) applied to soil in which the plants were growing. However, further study of benomyl has focused on fungi other than the rusts. The rapidly growing

literature on both oxycarboxin and benomyl has been reviewed recently by Erwin (2).

Despite much research on oxycarboxin and benomyl, there has been little investigation of the effect of these two fungicides on the ultrastructure of fungi. Such study could describe the rate of movement of the fungicides in host plants, indicate possible sites of action, and facilitate comparisons of different fungicides. Reports of work in this area include that by Lyr et al. (6), who found that carboxin (and presumably oxycarboxin) damaged the mitochondrial system and the vacuolar membranes of the fungus *Rhodotorula mucilagenosa* within 2 hours after treatment. Mitochondria were swollen and irregular in shape. However, plasmalemma, nuclear envelope, and endoplasmic reticulum were not affected. Richmond and Pring (9) treated germinating conidia of *Botrytis fabae* with benomyl, and noted that the usual pattern, in which organelles and endoplasmic reticulum were oriented toward the hyphal tip, was disorganized. The endoplasmic reticulum in treated hyphae appeared as short, broken segments rather than as the typical multiple strands. Mitochondria and lipid bodies generally were not affected, although some mitochondria had looped cristae. Wall thickness was not affected.

This study was undertaken to determine the effects of oxycarboxin and benomyl on haustoria of the crown rust fungus *(Puccinia coronata* Cda. var. *avenae* Fraser & Led.) in oat *(Avena sativa* L.) leaves. Such information should aid understanding of how these two systemic fungicides affect the ultrastructure of fungi sensitive to them.

MATERIALS AND METHODS.—Seedlings of the crown rust-susceptible oat cultivar Markton were inoculated with urediospores of race 290. They were held in a growth chamber for about 5 days or until infection was shown clearly by flecking. Then an excess of oxycarboxin or benomyl was applied in water suspension to the soil to control the fungus. Infected leaves were sampled at 12- and 24-hour intervals after application of the fungicides. For controls, infected leaves from untreated plants were sampled 24 hours after the time of fungicide treatment.

To fix small leaf disks cut from infected areas, I modified the simultaneous glutaraldehyde-osmium technique of Franke et al. (3). The disks were placed in a mixture (1:1, v/v) of 2% glutaraldehyde and 1% OsO_4 buffered with 0.05 M sodium cacodylate at pH 6.8, at 4 C for 30 minutes. Then they were rinsed three times in cold buffer and given a 30-minute treatment in 2% buffered OsO_4 at 4 C. The material was dehydrated in a graded ethanol-propylene oxide series, and embedded in Epon 812. Sections were cut with a diamond knife, mounted (unsupported) on copper grids having 79 or 158 openings per cm (200- or 400-mesh), stained with uranyl acetate in methanol (10), and examined in a Hitachi HU-11C electron microscope.

RESULTS.—*Macroscopic evidence of fungicide effects.*—Further development of the fungus in plants treated with oxycarboxin appeared totally suppressed. Leaves examined a few days after treatment generally showed less flecking than they had at the time of treatment.

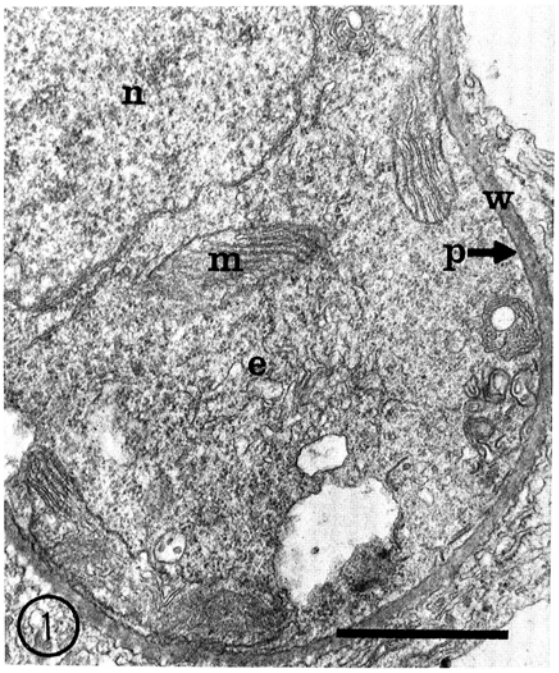
The effect of benomyl was less dramatic. The fungus developed small uredia, but these usually failed to break through the epidermis of the host. Their formation was slower than on control plants, and they were smaller and dull brown, rather than the usual bright orange-yellow.

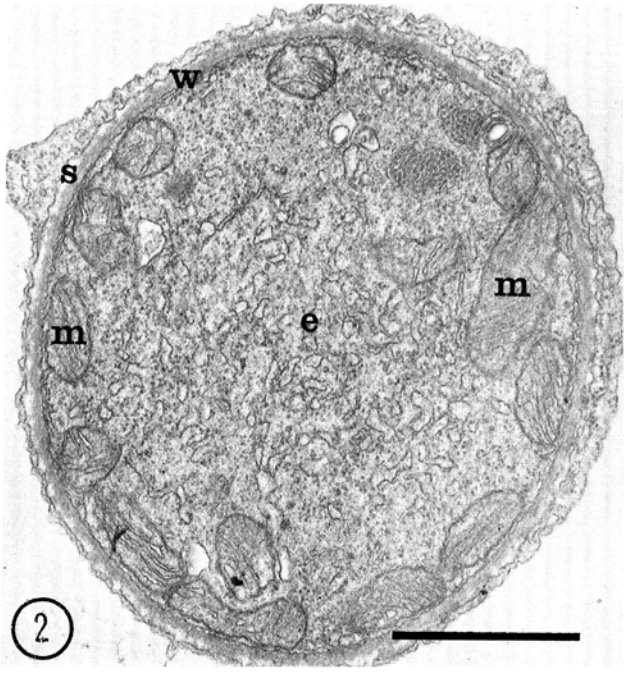
Development of haustoria on control plants.—The appearance of haustoria varied somewhat in different host cells of control plants and sometimes even within the same host cell. It was, therefore, necessary to examine the range and types of variation occurring in haustoria in leaves of control plants to establish a standard by which to judge the effects of fungicides on the fungus in the treated plants. Similarly, it was also necessary to examine a reasonable number of haustoria in treated plants to be sure of drawing valid conclusions on the effects of the fungicides. For both control and treated material, the micrographs generally represent typical or average haustoria. Ultrastructural details typical of the haustoria of rust fungi are evident in the micrographs of haustoria in the control plants (Fig. 1, 2, 3).

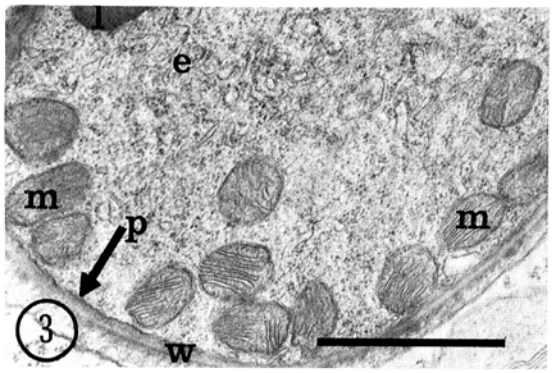
Preliminary sampling showed little, if any, difference in haustoria of control plants that had been fixed at the 12- and 24-hour intervals used for sampling from the fungicide-treated material. Therefore, haustoria in untreated leaves fixed 24 hours after the time of fungicide treatment were considered satisfactory controls for both the 12- and 24-hour fungicide treatments.

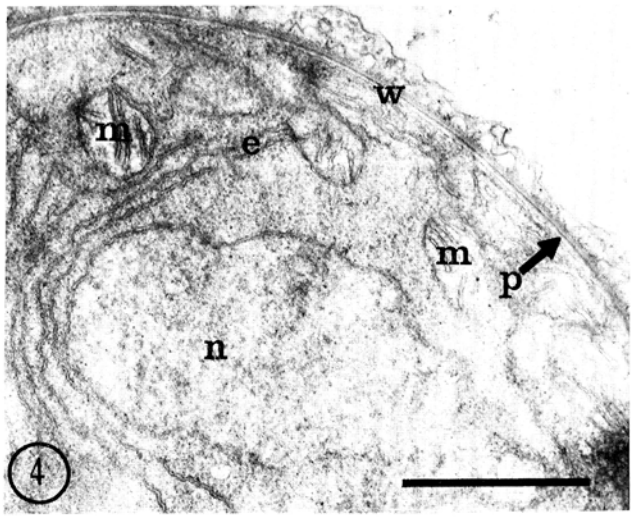
Haustoria treated with oxycarboxin.—Oxycarboxin strikingly affected haustoria 12 hours after application of the fungicide to the soil (Fig. 4, 5). Compared with the control, the overall ultrastructure of haustoria in the treated group was disorganized and had deteriorated. Endoplasmic reticulum was almost absent in some haustoria. However, mitochondria and nuclei were clearly recognizable, because the membranes of these organelles generally were still intact. The condition of the membranes ranged from seemingly near normal to thickened and deteriorating. On the other hand, the contents of these organelles differed sharply and uniformly from those in the controls: the treated organelles looked empty or at least did not clearly differentiate in background from the cytoplasm of the cell. The cytoplasm had lost most of the detailed structure characteristic of the control at this stage of development. Mitochondria and haustoria varied in size in untreated material. Nevertheless, there was a strong indication that both mitochondria and haustoria in the treated material were significantly larger than in the controls.

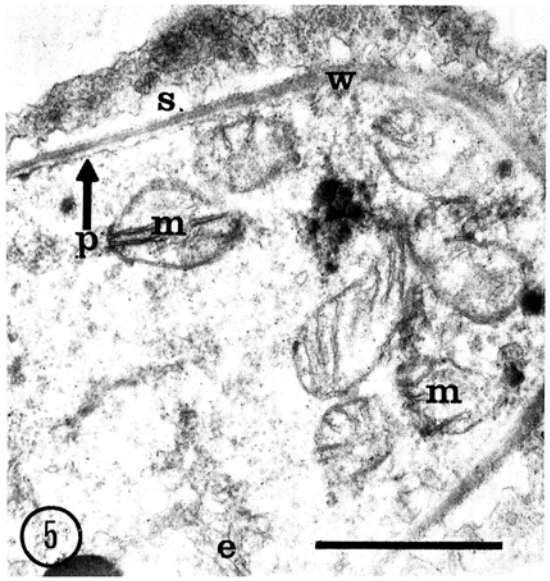
Material sampled 24 hours after treatment with oxycarboxin was difficult to fix and section satisfactorily, and haustoria recognizable in the microscope were relatively rare. Those that were found (Fig. 6) had almost totally deteriorated. Enlarged mitochondrial remnants occupied most of the volume of the haustoria. These remnants were almost empty, but in some, the original double membrane, and in others the remnants of a few cristae could be seen. The space between the mitochondria was filled with a relatively dense, granular material, in which some membranes (probably endoplasmic reticulum) could be seen. The granular material seemed to be filling the mitochondria in places where there were breaks in the mitochondrial membrane. The plasmalemma and cell wall were generally intact in these haustoria.

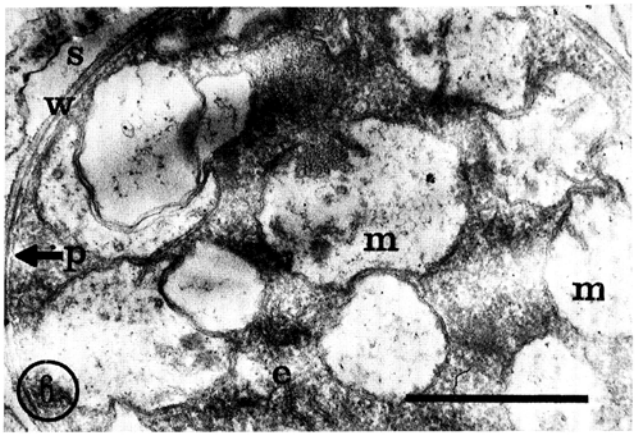

n
w
m
p
e
1


w
s
m
m
e
2


e
m
p
m
3
w


w
m
e
m
p
n
4


s
w
p
m
m
5
e


s
w
p
m
m
6
e

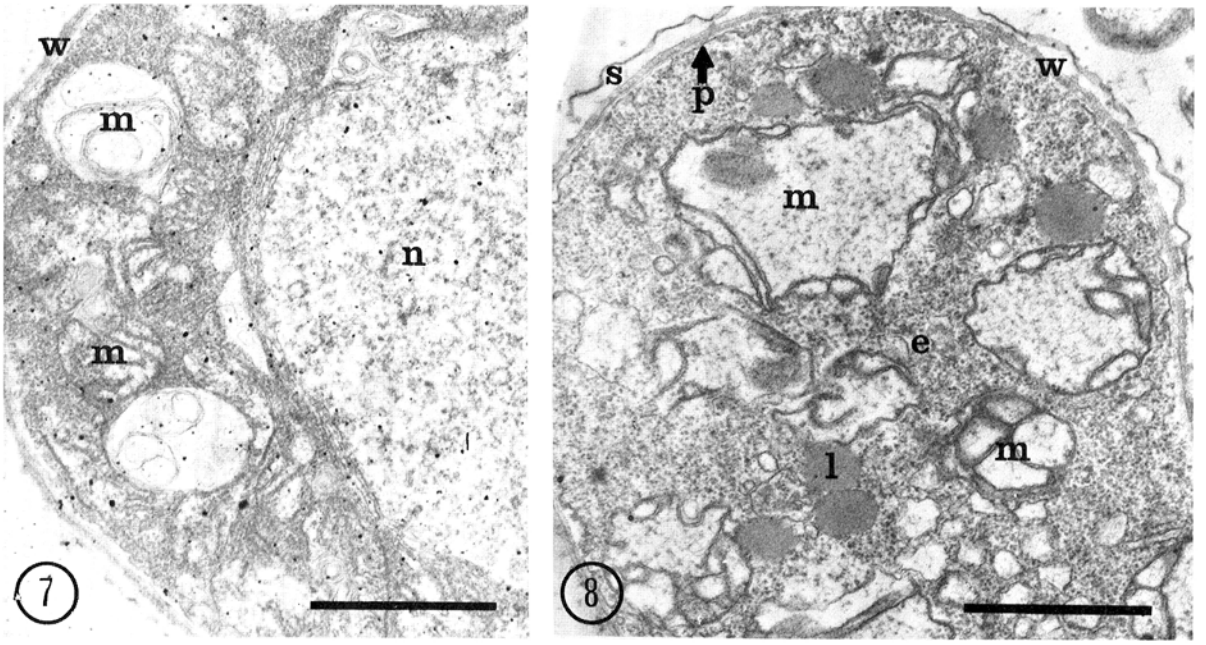


Fig. 7-8. The scale bars represent 1.0 μm. Legend: e = endoplasmic reticulum; m = mitochondrion; n = nucleus; w = haustorium wall; l = lipid body; p = plasmalemma; s = sheath. Haustoria fixed 24 hours after application of benomyl to the soil around roots of host plants (× 24,200, and 24,800, respectively).

Haustoria treated with benomyl.—Haustoria sampled 12 hours after treatment with benomyl did not differ greatly in ultrastructure from the controls. Such differences as might have existed were difficult to verify because the condition of the haustoria in the controls varied. However, 24 hours after treatment haustoria showed severe ultrastructural damage (Fig. 7, 8). In some, the basic membranous structure of most of the mitochondria was intact, but the membranes were greatly deteriorated. In fact, in certain haustoria the two membranes of each mitochondrion often were indistinguishable. In other haustoria, mitochondria were almost unrecognizable, being greatly enlarged and almost empty. The cristae of these enlarged mitochondria often formed peculiar, looped configurations inside the mitochondrion, similar to those Richmond and Pring (9) observed in *Botrytis fabae* that had been treated with benomyl.

Nuclei were severely affected. The nuclear membrane was often at least partly missing; and, in other cases, fragments of this membrane were difficult to distinguish from nearby endoplasmic reticulum.

In the controls, the plasmalemma usually could be seen mostly intact around the periphery of the haustorium. In material fixed 24 hours after teatment with benomyl, the plasmalemma appeared only in segments. The walls of the haustoria in the treated material were intact, in the sense that they showed no breaks, and thus were maintaining the basic structural integrity of the haustorium. They differed markedly from haustorial walls in the controls because they were delimited quite sharply, particularly at their outer surface. Also, they showed some internal, laminar structure in contrast to the almost amorphous structure of the controls. As with oxycarboxin-treated material, the haustoria appeared to have swollen significantly. The stretching of the haustorial walls involved in this swelling may be related to their altered appearance.

DISCUSSION.—Rather than other fungal structures, haustoria were chosen for study partly because of their relative uniformity and the ease with which they can be fixed and sectioned. More important, they represent the most intimate and direct point of contact between the fungus and its host. Because fungicides applied to the soil must travel through the host to reach the fungus, the effects of the fungicide on the fungus might be seen most easily where host and fungus have their greatest contact.

The continued, although greatly reduced, macroscopic development of the fungus after treatment with benomyl is difficult to reconcile with the heavily damaged

Fig. 1-6. The scale bars represent 1.0 μm. Legend: e = endoplasmic reticulum; m = mitochondrion; n = nucleus; w = haustorium wall; l = lipid body; p = plasmalemma; s = sheath. **1-3)** Haustoria of the crown rust fungus from untreated oat leaves (× 22,500, 24,500, and 24,800, respectively). **4-5)** Haustoria fixed 12 hours after application of oxycarboxin to the soil around roots of host plants (× 24,300, and 24,600, respectively). **6)** Haustorium fixed 24 hours after application of oxycarboxin to the soil around roots of host plants (× 23,900).

ultrastructural appearance of haustoria from the same material. One possible explanation is that by the time of fungicide treatment, that is, when flecking is clearly evident, a considerable amount of sporogenous fungal tissue has already been produced. This sporogenous tissue is isolated, at least relative to haustoria, from the host and thus perhaps also from the fungicide. Therefore, it is able to continue to develop to some extent, and to produce the small uredia that were observed.

One of the principal objectives of this study was to compare the effects of the two systemic fungicides on the ultrastructural morphology of the fungus, and certain important differences were noted. Since physiological studies have shown that the two fungicides differ in their modes of action, it is of interest to consider the ultrastructural differences in light of differences that have been demonstrated physiologically. Oxathiin fungicides, including oxycarboxin, act primarily on the respiratory system of the fungus. Specifically, these fungicides block the oxidation of succinate in the mitochondria, the site of action probably being on succinic dehydrogenase (7, 12).

Benomyl rapidly decomposes to methyl 2-benzimidazolecarbamate (MBC) within plants, and the effectiveness of benomyl as a systemic fungicide is mainly due to the action of MBC (8). In contrast to the oxathiin fungicides, MBC and closely related compounds probably do not directly interfere with primary energy metabolism, but act on DNA synthesis or some closely related process such as nuclear or cell division (1).

My observations on the differences of the effects of oxycarboxin and benomyl on the ultrastructure of the fungus can be correlated with the physiological studies just summarized. Thus, the very rapid and complete breakdown of mitochondria exposed to oxycarboxin is a logical consequence of the direct action of this fungicide on basic energy metabolism in the mitochondria. The severe deterioration of nuclear membranes exposed to benomyl, on the other hand, may reflect the action of this fungicide on nuclear processes. The relatively slow and incomplete breakdown of mitochondria in benomyl-treated haustoria could then be interpreted as a secondary effect.

LITERATURE CITED

1. CLEMONS, G. P., and H. D. SISLER. 1971. Localization of the site of action of a fungitoxic benomyl derivative. Pestic. Biochem. Physiol. 1:32-43.
2. ERWIN, D. C. 1973. Systemic fungicides: Disease control, translocation, and mode of action. Annu. Rev. Phytopathol. 11:389-422.
3. FRANKE, W. W., S. KRIEN, and R. M. BROWN, JR. 1969. Simultaneous glutaraldehyde-osmium tetroxide fixation with postosmication. An improved fixation procedure for electron microscopy of plant and animal cells. Histochemie 19:162-164.
4. GONDRAN, J. 1970. Action de l'oxycarboxine contre la rouille couronnée du ray-grass. Phytiatrie-Phytopharm. Rev. Fr. Med. Pharm. Veg. 19:133-140.
5. HARDISON, J. R. 1968. Systemic activity of Fungicide 1991, a derivative of benzimidazole, against diverse grass diseases. Plant Dis. Rep. 52:205.
6. LYR, H., G. RITTER, and G. CASPERSON. 1972. Wirkungsmechanismus des systemischen Fungicids Carboxin. Z. Allg. Mikrobiol. 12:271-280.
7. MATHRE, D. E. 1971. Mode of action of oxathiin systemic fungicides. III. Effect on mitochondrial activities. Pestic. Biochem. Physiol. 1:216-224.
8. PETERSON, C. A., and L. V. EDGINGTON. 1970. Transport of the systemic fungicide, benomyl, in bean plants. Phytopathology 60:475-478.
9. RICHMOND, D. V., and R. J. PRING. 1971. The effect of benomyl on the structure of Botrytis fabae. J. Gen. Microbiol. 66:79-94.
10. STEMPAK, J. G., and R. T. WARD. 1964. An improved staining method for electron microscopy. J. Cell Biol. 22:697-701.
11. VON SCHMELING, B., and M. KULKA. 1966. Systemic fungicidal activity of 1,4-oxathiin derivatives. Science 152:659-660.
12. WHITE, G. A. 1971. A potent effect of 1,4-oxathiin systemic fungicides on succinate oxidation by a particulate preparation from Ustilago maydis. Biochem. Biophys. Res. Commun. 44:1212-1219.