Fungicidal and Fungistatic Effects of Carboxin on *Ustilago nuda*

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ABSTRACT

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The systemic fungicide carboxin is a respiratory inhibitor of *Ustilago nuda*, the fungus that causes loose smut of barley. Concentrations of 0.01 to 1,000 μg/ml of carboxin were applied to 24-hr sporelings of three isolates of *U. nuda* growing in agitated liquid culture. Sporelings were not affected by 24 hr of exposure to carboxin concentrations below 0.03 μg/ml. Carboxin concentrations of 1 μg/ml or higher stopped hyphal growth of sporelings without causing death of apical cells. However, lower concentrations (between 0.03 and 1 μg/ml) not only reduced growth but also killed apical cells. An agar growth test showed that most sporelings were dead after 5 days at 0.125 μg/ml, whereas few sporelings were killed by 10 μg/ml for 5 days. Paradoxically, carboxin has a fungicidal effect on hyphae of *U. nuda* at concentrations lower than those at which it exhibits only a fungistatic effect. In contrast, using the same methodologies, the respiratory inhibitors antimycin A and carbonyl cyanide m-chlorophenyl-hydrazone were increasingly fungicidal with increased concentration. Respiratory inhibitors probably have fungicidal effects only when a certain minimum of energy for autolysis is available.

Respiratory inhibitors normally cause acute cell death followed by necrosis (23). Strong inhibition of fungal respiration eventually will be fungicidal (12). The systemic fungicide carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) is known to strongly inhibit respiration (17,19) through interference with electron transport of the mitochondrial succinate dehydrogenase complex (8,25). Carboxin therefore should exert fungicidal effects (12). However, carboxin was shown to be fungistatic to *Ustilago maydis* (DC.) Cda. and *Rhizoctonia solani* Kühn (16). Therefore, it has been assumed that carboxin controls sensitive basidiomycetous fungi (4) through fungistasis.

Control of loose smut of barley, caused by *U. nuda* (Jens.) Rostr., has been available for approximately 20 yr in the form of seed dressings containing carboxin. Recent reports from France (13,14) have suggested that some field isolates of *U. nuda* now have developed resistance to carboxin.

While investigating the effects of carboxin on sporelings of *U. nuda*, we made an interesting observation: Carboxin appeared to be fungicidal to teliosporelings of *U. nuda* at concentrations below those at which it demonstrated the previously recognized fungistatic effect. This highly unusual response of a fungus to a fungicide is examined critically and documented in this report.

MATERIALS AND METHODS

Fungal culture. The three isolates of *U. nuda* that were used were collected in barley fields in the Canadian prairies. These isolates were shown to be genetically distinct by determining their virulence on differential cultivars. They also were collected at different times: 60-16, 72-66, and 82-96 were collected in 1960 (before carboxin came into commercial use), 1972, and 1982, respectively. The isolates were maintained by inoculating teliospores from single spikes to appropriate differential cultivars, thus promoting genetic homogeneity (the teliospores in one plant usually result from one dikaryon [7,21]).

The fungus was grown aerobically in 125-ml flasks containing 10 ml of culture medium in a controlled-environment incubator shaker (PsycroTherm, New Brunswick Scientific Co., New Brunswick, NJ) at 20 C and 200 rpm. The culture medium consisted of 1.3% (w/v) glucose, 0.4% (w/v) yeast extract, 0.09% (w/v) asparagine, and 20 ml of Vogel's complete salt solution per liter of medium (24). To prevent clumping of sporelings of *U. nuda*, polyacrylic acid (MW 4000, Polysciences, Inc., Warrington, PA), autoclaved separately, was added to each flask at a final concentration of 0.2% (11). The pH then was adjusted to 5.7 with sterile 1 M NaOH.

Each flask of medium was inoculated with 0.1 mg of teliospores of *U. nuda* and ultrasonicated for 40 sec to disperse them.

Test solutions. Carboxin was added after 24 hr, at which point the sporelings had completed meiosis and were in the mycelial phase of development that is characteristic of colonization of the host. Effects were scored 18 hr to 6 days later. The carboxin used was a 97% technical grade material obtained from Uniroyal Chemical Ltd. (Elmira, Ont.). Fresh stock solutions of carboxin in methanol were made every 2 wk and stored at 4 C. The most

TABLE 1. Effects of carboxin (added after 24 hr of incubation) on growth and viability of teliosporelings of *Ustilago nuda*

Concentration (μg/ml) of carboxin in medium	Percentage of dead apical cells[a]	Growth of sporelings[b] after addition of carboxin			Growth between 18 and 42 hr as percent of control
		18 hr	42 hr	144 hr	
0	7	12.8	51.2	...[c]	100
0.01	9	19.9	54.6	...	90
0.03	23*[d]	13.4	26.9*	...	35
0.125	29*	4.3*	6.5*	6.5	6
1.0	4	4.3*	4.3*	4.3	0
10.0	4	4.4*	4.3*	4.3	0
1,000.0	4	4.1*	4.1*	4.1	0

[a] Mean of eight samples, 100 apical cells/sample, 24 hr after adding carboxin.
[b] Mean number of apical cells/sporeling, nine samples of at least 100 apical cells/sample.
[c] Not determined.
[d] Means followed by an asterisk are significantly different at $P < 0.01$ (Kruskal-Wallis test) from the control (0 μg/ml).

TABLE 2. Survival of sporelings exposed to carboxin (added after 24 hr of incubation) in liquid shake culture

Concentration of carboxin (μg/ml)	Percentage of surviving sporelings after different exposure times[a]		
	36 hr	5 days	6 days
0	99	89	87
0.125	99	19*	10*
10	99	88	88

[a] Mean of 200 sporelings filtered, washed, and plated on carboxin-free water agar. Means followed by an asterisk are significantly different from the 36-hr mean at $P < 0.05$.

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TABLE 3. Effect of three experimental conditions on the interaction between teliosporelings of *Ustilago nuda* and carboxin

Concentration (μg/ml) of carboxin[a]	Polyacrylic acid		Ultrasonication		Yeast contamination	
	In medium	Not in medium	Used	Not used	Present	Absent
Percentage of dead apical cells[b]						
0	5	6	7	6	5	5
0.1	26*[c]	29*	26*	26*	28*	27*
10.0	6	5	7	5	6	7
Growth of sporelings[d]						
0	31.6	29.4	30.0	31.7	28.8	30.6
0.1	5.6*	5.9*	5.6*	5.6*	5.8*	5.6*
10.0	4.3*	4.4*	4.2*	4.3*	4.2*	4.3*

[a] Added after 24-hr growth.
[b] Means of eight samples of 100 apical cells/sample, 24 hr after addition of carboxin.
[c] Means followed by an asterisk are significantly different at $P < 0.05$ from the control (0 μg/ml) (Kruskal-Wallis test).
[d] Mean number of apical cells/sporeling, eight samples of at least 100 apical cells/sample, 24 hr after addition of carboxin.

TABLE 4. Effects of the respiratory inhibitors antimycin A and carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) on growth and viability of teliosporelings of *Ustilago nuda*[a]

Compound	Concentration (μg/ml)	Percentage of dead apical cells[b]	Growth of sporelings[c]
Antimycin A	0	7	28.2
	0.1	8	10.9*
	1.0	17*[d]	6.8*
	100.0	46*	6.2*
	200.0	45*	7.1*
	250.0	48*	5.3*
	500.0	52*	5.5*
CCCP	0	7	28.6
	0.1	6	5.3*
	0.5	14*	5.0*
	1.0	43*	4.7*
	10.0	82*	4.7*
	100.0	89*	4.9*

[a] Inhibitors added 24 hr after inoculation of flasks.
[b] Mean of four samples of 100 apical cells/sample, 24 hr after addition of inhibitor.
[c] Mean number of apical cells/sporeling, four samples of at least 100 apical cells/samples, 24 hr after addition of inhibitor.
[d] Means followed by an asterisk are significantly different at $P < 0.05$ from the control (0 μg/ml).

concentrated stock solution was serially diluted by volume to provide other necessary stock solutions. Each flask received 0.05 ml of the appropriate methanol stock solution, depending upon the final carboxin concentration desired (that is, from 0.01 to 1,000 μg/ml). Control flasks received 0.05 ml of methanol. All experiments were conducted on at least three separate occasions.

Effects of carboxin on mycelial growth. Mycelial growth may be measured in many different ways (9). We counted hyphal tips, or apices per sporeling (22), because this was done conveniently in conjunction with apical cell death determinations. Dry-weight determinations lack precision at low organism concentration (2). Also, hyphal tip counts would minimize the problems due to microbial contaminants in collections of teliospores of *U. nuda* from farm fields.

Determination of apical cell death. Evans blue is a vital stain which has not been used previously with fungi but has been shown to distinguish living from dead plant cells (6,20). It is a large molecule and thus does not penetrate the intact biological membranes of living cells (6).

A solution of 0.05 g of Evans blue (C.I. No. 12790, Allied Chemical and Dye Corp., New York, NY) per 100 ml of distilled water was used. Sporelings were stained by adding 1 drop of the Evans blue solution to test tubes containing 200–300 μl of the sporeling culture. Tubes then were vortexed briefly. Sporelings, mounted on glass slides, then were examined with a Leitz Diaplan microscope (Ernst Leitz Wetzlar GmbH).

To verify that Evans blue is not excluded by dead hyphal cells of *U. nuda*, sporelings were killed by exposure to alcohol, formaldehyde, and ultraviolet irradiation. The dead sporelings were stained uniformly and intensely by Evans blue. In contrast, nearly all cells of untreated sporelings excluded Evans blue.

Determination of dead sporelings. Sporelings were retrieved from both carboxin-amended and control media with sterile Whatman GF/D filters, washed with dilute, sterile culture medium, and transferred to 1.5% water agar. After 24 hr, live sporelings had grown extensively and were distinguished easily from dead sporelings.

Cautionary experiments. A number of experiments were done to ensure that the interaction is not artefactual.

Effect of polyacrylic acid on the interaction. Polyacrylic acid is an anionic polymer that prevents mycelial aggregation of many basidiomycetous fungi when added to agitated liquid culture (11). To ensure that polyacrylic acid was not interfering with uptake of carboxin, sporelings of *V. nuda* were grown with and without the acid in the medium. Carboxin (0.1 or 10 μg/ml) was added as usual 24 hr after inoculation of the flasks, and its effects on mycelial growth and apical cell viability were scored 24 hr later.

Effect of ultrasonication on the interaction. Flasks prepared and inoculated as described previously were, or were not, ultrasonicated. Carboxin (0.1 or 10 μg/ml) was added as usual 24 hr after inoculation of the flasks, and its effects on mycelial growth and apical cell viability were scored 24 hr later.

Effect of contaminants on the interaction. Unidentified yeast species and other microbial contaminants sometimes are associated with collections of teliospores of *U. nuda* and could contaminate agitated liquid cultures of teliosporelings of *U. nuda*. A strain of the 72-66 isolate known to be contaminated with yeast species was compared to an uncontaminated strain of 72-66 using the previously described test system. The effects of carboxin on mycelial growth and apical cell viability were scored 24 hr later. The effects of bacteria on the interaction were not tested because bacterial contaminants were not observed in these experiments.

Effect of unidentified factors on the interaction. Two additional respiratory inhibitors were evaluated. Methanol solutions of antimycin A and carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) (Sigma Chemical Co., St. Louis, MO) were tested in exactly the same manner as was carboxin.

Data analysis. Mean percentages were based on samples consisting of 100 apical cells or 100 sporelings. The significance of differences between means was assessed by the nonparametric Kruskal-Wallis test.

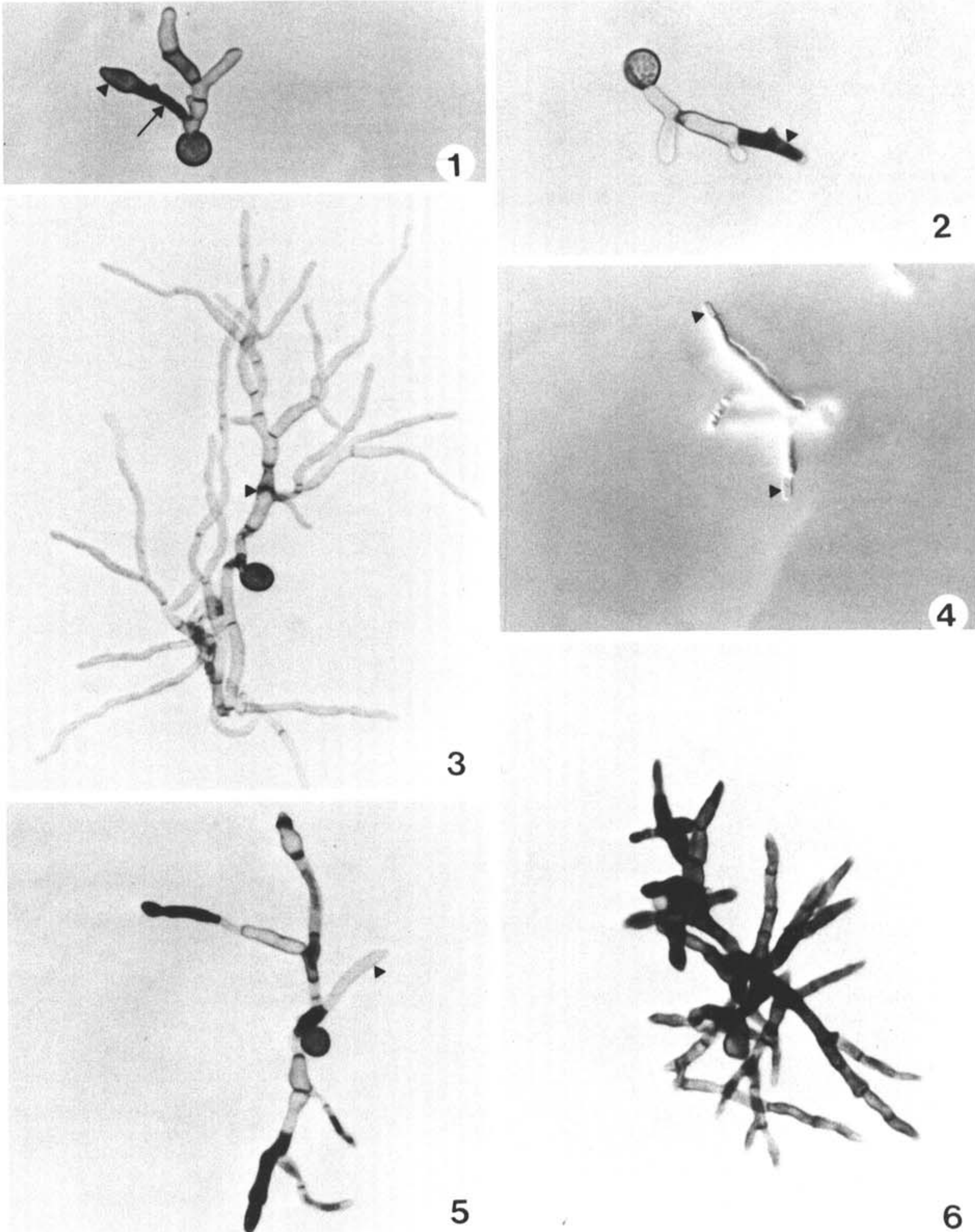
RESULTS

The responses of the three isolates were similar and therefore are averaged in Tables 1–4. The results from one experiment are reported in each table; experiments conducted on at least three separate occasions yielded similar data. The lowest carboxin concentration (0.01 μg/ml) did not reduce the number of apices per sporeling (Table 1). Sporeling growth between 18 and 42

hr after addition of 0.03 μg/ml of carboxin was reduced by 65%. Some growth occurred until 42 hr after exposure to 0.125 μg/ml. No growth was observed after 18 hr in media containing 1–1,000 μg/ml.

Figures 1–3 show sporelings after staining with Evans blue. The densely stained cell in Figure 2 was typical of dead apical cells. The proportion of such dead apical cells observed after 24 hr of exposure to carboxin is given in Table 1. Zero, 0.01, and 1–1,000 μg/ml of carboxin produced the same low level of apical cell death. Mid-range concentrations (0.03 and 0.125 μg/ml of carboxin) resulted in significantly ($P < 0.01$) more apical cell death than lower or higher concentrations.

Cellular hypertrophy (Fig. 1) was correlated with apical cell death; that is, it was not noticeable at low and high concentrations



Figs. 1–6. Light micrographs of sporelings of *Ustilago nuda* (×1,470). **1,** Treated with 0.1 μg/ml of carboxin for 24 hr. Note swollen apical cell (arrowhead) and dead proximal cell (arrow) which has been stained by Evans blue. **2,** Treated with 0.5 μg/ml of carboxin for 24 hr. Note dead apical cell (arrowhead) stained by Evans blue. **3,** Control sporeling grown without carboxin for 48 hr. The older septa (arrowhead) are stained by Evans blue. **4,** Interference contrast micrograph showing empty apical cells (arrowheads) of a sporeling grown in 0.5 μg/ml of carboxin for 24 hr. Empty apical cells are more frequent at 10 μg/ml of carboxin. **5,** Sporelings exposed to 10 μg/ml of carboxin for 7 days. One live apical cell (arrowhead) is not stained by Evans blue. **6,** Sporeling exposed to 0.125 μg/ml of carboxin for 7 days. Intense staining indicates that all hyphal cells are dead.

but was common at 0.03 and 0.125 μg/ml. Empty apical cells (Fig. 4) were more common at a concentration of 10 μg/ml than at the other concentrations tested.

The relative survival of sporelings exposed to carboxin for longer periods of time (>24 hr) is shown in Table 2. Sporelings were transferred from carboxin-supplemented shake culture to carboxin-free agar to determine their ability to grow. The control (0%) and high carboxin concentration (10 μg/ml) treatments resulted in 87–89% sporeling survival after 5–6 days of treatment, correlating with low apical cell death, whereas the 0.125 μg/ml treatment had only 19 and 10% survival rates after 5 and 6 days, respectively. A live sporeling after 7 days in 10 μg/ml and a dead sporeling after 7 days in 0.125 μg/ml carboxin are shown in Figures 5 and 6, respectively.

Polyacrylic acid, ultrasonication, and a yeast contaminant of teliospores of *U. nuda* did not affect significantly ($P < 0.05$) the fungus-fungicide interaction (Table 3).

Both antimycin A and CCCP produced fungistatic effects at low concentrations but fungicidal effects at high concentrations (Table 4). At 0.1 μg/ml, antimycin A reduced growth of sporelings without killing apical cells. At 1 μg/ml of antimycin A, a further reduction in growth was accompanied by 17% apical cell death. Higher concentrations of antimycin A did not reduce growth further but did increase apical cell death to 52%. Similarly, 0.1 μg/ml of CCCP reduced growth of sporelings without killing apical cells (Table 4). Higher concentrations of CCCP did not reduce growth further but did increase apical cell death to 89%.

DISCUSSION

Carboxin has a fungicidal effect on teliosporelings of *U. nuda* at concentrations below those at which fungistatic effects are noted. This is an anomalous relation. The only similar interaction is the effect of sodium dimethyl dithiocarbamate on mycelial growth of *Aspergillus*; over a restricted concentration range, increasing the external concentration of fungicide elicits a decreasing response from the fungus (10).

Antimycin A and CCCP, as expected (12), were increasingly fungicidal with increasing concentration. These results, because they were obtained with the same experimental protocol, help confirm the anomalous carboxin finding. Moreover, the carboxin finding cannot be explained as an artefact resulting from either polyacrylic acid, ultrasonication, or yeast contamination because none of these factors affected the results (Table 3).

It is not clear why carboxin would be fungicidal at concentrations in the 0.03 to 0.125 μg/ml range and fungistatic at 1 μg/ml or higher concentrations. Normally, when cellular energy production is compromised by carboxin or other respiratory inhibitors, growth slows or stops (15). This initial fungistatic effect may be converted into a fungicidal effect if the balance between synthetic and autolytic cellular activities favors the latter (15). Autolysis in some hyphal compartments could produce substrates for the growth of compartments that are still living (5). Carboxin may not be directly fungicidal at 0.125 μg/ml, but rather may induce fungicidal effects via autolysis. Antimycin A and CCCP also may induce fungicidal effects in this way. The failure of carboxin concentrations of 1 μg/ml or higher to have fungicidal effects appears to be associated with less growth (4.1–4.3 apices/sporeling) than was observed for antimycin A or CCCP (5.4 and 4.6 apices/sporeling, respectively). These results suggest that the initial fungistatic effects of respiratory inhibitors are converted to fungicidal effects only when a certain minimum level of energy for autolysis is available. Further work with additional respiratory inhibitors and a more detailed physiological study of the effects of respiratory inhibitors on *U. nuda* and other fungi are needed to test this hypothesis.

Whether carboxin is fungistatic or fungicidal in planta is not known. The concentration of carboxin in developing barley seedlings infected with *U. nuda* presumably would dictate (as it does in vitro) whether carboxin is fungicidal or fungistatic. The recommended rate of application for complete control of loose smut of barley gives a theoretical carboxin loading of 900 μg/ml/seed (1). However, the in planta carboxin concentration is affected by its rapid oxidation to the sulfoxide (3) which is much less active than carboxin itself (18).

LITERATURE CITED

1. Brooks, D. H. 1972. Results in practice. I. Cereals. Pages 186-205 in: Systemic Fungicides. R. W. Marsh, ed. Longman Group Ltd., London.
2. Calam, C. T. 1969. The culture of micro-organisms in liquid medium. Pages 255-326 in: Methods in Microbiology. Vol. 1. J. R. Norris, ed. Academic Press, London.
3. Chin, W.-T., Stone, G. M., and Smith, A. E. 1970. Metabolism of carboxin (Vitavax) by barley and wheat plants. J. Agric. Food Chem. 18:709-712.
4. Edgington, L. V., Walton, G. S., and Miller, P. M. 1966. Fungicide selective for Basidiomycetes. Science 153:307-308.
5. Fencl, Z. 1978. Cell ageing and autolysis. Pages 389-405 in: The Filamentous Fungi. Vol. 3. Developmental Mycology. J. E. Smith and D. R. Berry, eds. Halsted Press, New York.
6. Fischer, J. M. C., Peterson, C. A., and Bols, N. C. 1985. A new fluorescent test for cell vitality using Calcofluor white M2R. Stain Technol. 60:69-79.
7. Fullerton, R. A., and Nielsen, J. 1974. Inheritance of buff spore color and of virulence on the cultivar Warrior in loose smut of barley, *Ustilago nuda*. Can. J. Bot. 52:2237-2240.
8. Georgopoulos, S. G., Alexandri, E., and Chrysayi, M. 1972. Genetic evidence for the action of oxathiin and thiazole derivatives on the succinic dehydrogenase system of *Ustilago maydis* mitochondria. J. Bacteriol. 110:809-817.
9. Griffin, D. H. 1981. Fungal Physiology. John Wiley & Sons, New York. 383 pp.
10. Hassall, K. A. 1982. The Chemistry of Pesticides. Macmillan Press Ltd., London. 372 pp.
11. Jones, P., Shahab, B. A., Trinci, A. P. J., and Moore, D. 1988. Effect of polymeric additives, especially Junlon and Hostacerin, on growth of some basidiomycetes in submerged culture. Trans. Br. Mycol. Soc. 90:577-583.
12. Kaars Sijpesteijn, A. 1972. Effects on fungal pathogens. Pages 132-155 in: Systemic Fungicides. R. W. Marsh, ed. Longman Group Ltd., London.
13. Leroux, P. 1986. Characteristics of strains of *Ustilago nuda*, the causal agent of barley loose smut, resistant to carboxin. Agronomie 6:225-226.
14. Leroux, P., and Berthier, G. 1988. Resistance to carboxin and fenfuram in *Ustilago nuda* (Jens.) Rostr., the causal agent of barley loose smut. Crop Prot. 7:16-19.
15. Lyr, H. 1977. Effect of fungicides on energy production and intermediary metabolism. Pages 301-332 in: Antifungal Compounds. Vol. 2. M. R. Siegel and H. D. Sisler, eds. Marcel Dekker, Inc., New York.
16. Mathre, D. E. 1968. Uptake and binding of oxathiin systemic fungicides by resistant and sensitive fungi. Phytopathology 58:1464-1469.
17. Mathre, D. E. 1970. Mode of action of oxathiin systemic fungicides. I. Effect of carboxin and oxycarboxin on the general metabolism of several Basidiomycetes. Phytopathology 60:671-676.
18. Mathre, D. E. 1971. Mode of action of oxathiin systemic fungicides, structure-activity relationships. Agric. Food Chem. 19:872-874.
19. Ragsdale, N. N., and Sisler, H. D. 1970. Metabolic effects related to fungitoxicity of carboxin. Phytopathology 60:1422-1427.
20. Taylor, J. A., and West, D. W. 1980. The use of Evans blue stain to test the survival of plant cells after exposure to high salt and high osmotic pressure. J. Exp. Bot. 31:571-576.
21. Thomas, P. L. 1978. Potential for natural hybridization between strains of *Ustilago nuda*. Can. J. Bot. 56:721-726.
22. Trinci, A. P. J. 1984. Regulation of hyphal branching and hyphal orientation. Pages 23-52 in: The Ecology and Physiology of the Fungal Mycelium. D. H. Jennings and A. D. M. Rayner, eds. Cambridge University Press, Cambridge.
23. Trump, B. F., Berezesky, I. K., and Osornio-Vargas, A. R. 1981. Cell death and the disease process. The role of calcium. Pages 209-242 in: Cell Death in Biology and Pathology. I. D. Bowen and R. A. Lockshin, eds. Chapman & Hall, New York.
24. Vogel, H. J. 1956. A convenient growth medium for *Neurospora* (medium N). Microb. Genet. Bull. 13:42-43.
25. White, G. A. 1988. Furan carboxamide fungicides: Structure-activity relationships with the succinate dehydrogenase complex in mitochondria from a wild-type strain and a carboxin-resistant mutant strain of *Ustilago maydis*. Pestic. Biochem. Physiol. 31:129-145.