Resistance

Incidence of Carboxin Resistance in *Ustilago nuda*

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ABSTRACT

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In planta validation and an appropriate in vitro assay confirmed the existence of carboxin resistance within some European field isolates of *Ustilago nuda*. Carboxin resistance was not found within the 1988–1989 Canadian population (260 field isolates tested) of *U. nuda*, nor within a worldwide population collected before the use of carboxin (96 field isolates tested). Indirect in vitro evidence was obtained, which indicated that the in planta effects of seed treatment with carboxin were fungicidal rather than fungistatic.

Control of loose smut of barley, caused by the fungus *Ustilago nuda* (Jensen) Rostr., has been available for approximately 20 yr in the form of seed treatments that contain the systemic fungicide carboxin. Reports from France (3,4) have suggested that some field isolates of *U. nuda* are resistant to carboxin.

Fungicide bioassays are normally based on either spore germination or mycelial growth in culture (2). Frequently, these two manifestations of growth are affected differently by the same fungicide (2). Carboxin is used as a seed treatment to control loose smut of barley, and therefore can only control the fungus by acting against mycelial growth within already infected seeds. However, the reports of carboxin resistance in field isolates of *U. nuda* from France were based on a teliospore germination assay (3,4). Furthermore, an in planta validation of the teliospore germination assay is lacking.

The objectives of this study were to confirm the status of putative, carboxin-resistant, European field isolates of *U. nuda* and to determine the extent of carboxin resistance in *U. nuda*, particularly within Canada.

MATERIALS AND METHODS

Selection of isolates. A total of 386 field isolates (hereafter referred to as isolates) of *U. nuda* were selected for this study. Each isolate was from a single spike and therefore probably from a single dikaryon (1,8). Fifteen European and five Canadian isolates were studied intensively (Tables 1 and 2). Eight of the European isolates were from the cultivar Viva and four from the cultivar Panda; these isolates were insensitive or resistant to carboxin (D. H. Bartlett, *personal communication*). Three of the Canadian isolates were known, from a previous study (5), to be carboxin-sensitive in vitro. Results from in planta and in vitro tests for carboxin resistance in these 20 isolates were compared to determine if in vitro tests alone could be used to screen the remaining isolates.

A total of 260 Canadian isolates were collected in 1988 and 1989 and were screened to determine if carboxin resistance occurs in the current Canadian population of *U. nuda*. A worldwide sample of 96 isolates of *U. nuda*, collected before the first use of carboxin in 1970, was also screened. In addition, eight isolates from South Australia, collected in 1978, and two from Denmark, collected in 1984, were examined.

Maintenance of isolates. Isolates were stored as single-smutted spikes at 4 C and low relative humidity. Most isolates in this study were increased by inoculating teliospores from single spikes to a susceptible host in the greenhouse or in growth chambers. Such increases are useful because they promote genetic homogeneity (the teliospores in one plant usually result from one dikaryon [1,8]); eliminate contaminating microorganisms that commonly occur in field-collected material; and renew viability lost because of age and storage environment.

In planta study. The 20 isolates (Tables 1 and 2) selected for intensive study were increased simultaneously to equalize their viability and physiological status. Plants of the cv. Regal (a universal suscept for *U. nuda*), grown in growth-chambers, were inoculated at anthesis with an aqueous suspension of teliospores (500 mg/l of sterile distilled water). Nine spikes were inoculated with each of the 20 isolates. Seeds from inoculated spikes were harvested and air-dried at room temperature. Each lot of approximately 50 seeds to be treated with dry Vitavax (Uniroyal Chemical Ltd., Ontario, Canada) 75WP Red (75% carboxin), was weighed in a small flask. In the first experiment, the weight of Vitavax that was calculated to give an application rate of 900 μg of carboxin per gram of seed was added to each flask. Flasks were manually agitated for 60 sec after the addition of Vitavax. We assumed that only 750 μg of carboxin would be deposited on 1 g of seed, which corresponds to the commercial application rate of 75 g of carboxin per 100 kg of seed, and that the balance would remain in the flask. However, the actual carboxin-deposition rate was subsequently determined experimentally. The level of carboxin on the treated seeds was determined by difference, because there were insufficient seeds from any given lot for both analysis and planting. For each seed lot, the flask used for treating was analyzed for remaining carboxin by using a methanol extraction and HPLC separation and quantification with a UV detector. The difference between the amount of carboxin initially applied and that recovered from each flask was assumed to be deposited on the treated seed. Additionally, as a cross check, two lots of similarly treated seeds from each of two experiments were analyzed for carboxin by an acetonitrile (first experiment) or methanol (second experiment) extraction and GLC separation and quantification with a flame-ionization detector.

Analysis showed that the actual deposition rate in the first experiment was only 400 μg of carboxin per gram of seed, rather than the target rate of 750 μg of carboxin per gram of seed. In the second experiment, therefore, sufficient Vitavax was used to give a theoretical application rate of 1,687.5 μg of carboxin per gram of seed. Analysis of seeds and residue in flasks used to treat seeds at this higher application rate showed an average deposition rate of 1,010 μg of carboxin per gram of seed.

TABLE 1. Loose smut infection in barley cv. Regal as affected by treatment with carboxin[a]

Isolate of *Ustilago nuda*	Origin	Infection percentages[b]	
		Treated	Untreated
Carboxin-resistant			
Viva 1	Europe	26	30
Viva 2	Europe	32	32
Viva 3	Europe	32	32
Viva 10	Europe	34	42
Viva 11	Europe	15	20
Viva 12	Europe	25	21
Viva 14	Europe	27	28
Viva 15	Europe	25	20
Carboxin-sensitive			
Fischer's Panda 4	Europe	4	45
Fischer's Panda 5	Europe	0	47
Fischer's Panda 6	Europe	2	63
Harvey's Sonja 7	Europe	0	47
Harvey's Sonja 8	Europe	1	45
Harvey's Sonja 9	Europe	2	30
ICA Panda 13	Europe	2	30
72-66	Canada	1	49
60-16	Canada	0	20
82-96	Canada	0	66
1,355	Canada	0	62
1,359	Canada	0	27

[a] Three separate experiments were conducted. In the first, the treatment was 400 μg of carboxin per gram of seed. In the second and third experiments, the treatment was 1,010 μg of carboxin per gram of seed.

[b] Number of plants with sporulation per total number of plants. Percentages are means of data pooled from the three experiments because a qualitative difference between the putative carboxin-resistant isolates and the others was evident in all three experiments.

TABLE 2. Effects of carboxin on growth and viability of teliosporelings of *Ustilago nuda*

Field isolate of *U. nuda*	Control		0.1 μg/ml carboxin in medium		10 μg/ml carboxin in medium	
	Dead apical cells[a]	Growth[b]	Dead apical cells	Growth	Dead apical cells	Growth
Viva 1	2	34.0	2	26.2	3	4.2*
Viva 2	3	24.8	4	23.6	2	3.8*
Viva 3	5	24.2	5	25.1	2	3.4*
Viva 10	2	33.0	3	25.4	4	4.0*
Viva 11	2	23.6	5	21.3	5	4.0*
Viva 12	6	22.2	5	20.8	5	4.2*
Viva 14	3	19.6	4	19.2	3	3.4*
Viva 15	3	25.3	1	22.2	1	4.1*
Fisher's Panda 4	4	24.5	23*[c]	6.0*	2	4.2*
Fisher's Panda 5	3	22.2	28*	5.2*	6	4.0*
Fisher's Panda 6	2	29.3	25*	6.3*	4	4.4*
Harvey's Sonja 7	6	30.6	18*	5.3*	5	4.5*
Harvey's Sonja 8	5	28.8	24*	7.2*	4	4.5*
Harvey's Sonja 9	1	20.2	22*	4.9*	4	3.9*
ICI Panda 13	3	22.7	19*	5.3*	1	4.2*
72-66	4	29.3	36*	5.5*	5	4.5*
60-16	6	25.2	23*	5.8*	5	4.3*
82-96	6	27.9	23*	6.0*	3	4.2*
1,355	3	28.2	25*	5.4*	3	4.0*
1,359	5	23.6	28*	5.6*	4	3.8*

[a] Mean of eight samples (%), 100 apical cells per sample, 24 hr after adding carboxin.

[b] Mean number of apical cells per sporeling. Nine samples of at least 100 apical cells per sample.

[c] Means followed by an asterisk are significantly different at $P < 0.01$ (Kruskal-Wallis test) from the control (0 μg/ml carboxin in medium).

For each of the 20 isolates, treated and untreated seeds were sown in greenhouse beds on three separate occasions. The beds had been watered before planting so that no further watering was necessary until after seed germination. This was done to prevent the loss of Vitavax through leaching. Plants were scored as infected if they produced one or more smutted spikes.

In vitro study. The in vitro reactions of the 20 isolates (Tables 1 and 2) to carboxin were determined by using the methods developed in an earlier study (5). Flasks that contained 10 ml of liquid culture medium (1.3% glucose, 0.4% yeast extract, 0.09% asparagine, 0.2% MW 4×10^6 polyacrylic acid, and 20 ml of Vogel's complete salt solution per liter of medium) were inoculated with teliospores of *U. nuda*. After 24 hr of growth at 20 C and 200 rpm, carboxin was added. At this time the sporelings had completed meiosis and were in the mycelial phase of development, which is characteristic of colonization of the host. Each of the 20 isolates was tested at both fungicidal (0.1 μg/ml) and fungistatic (10 μg/ml) concentrations of carboxin. In the earlier study (5), carboxin was shown to have a fungicidal effect on hyphae of *U. nuda* at concentrations lower than those at which it exhibits only a fungistatic effect. The cultured teliosporelings were microscopically examined 24 hr after the addition of carboxin. As described previously (5), measurements of both the viability (percentage of dead apical cells and hyphal compartments) and growth (number of apices) of sporelings exposed to carboxin were made.

The remaining 366 isolates in the collection were screened at the fungicidal concentration of 0.1 μg/ml of carboxin. Two controls were run with each set of 12 isolates: flask cultures of the 12 isolates to which no carboxin was added, and a single flask culture of a carboxin-resistant isolate to which carboxin, at 0.1 μg/ml, was added.

RESULTS

In planta study of the 20 selected field isolates. Similar results from the three separate experiments were obtained; they have been pooled in Tables 1 and 2. A qualitative difference between the eight isolates from cv. Viva and the other 12 isolates (seven European and five Canadian) was evident in all three experiments. The level of infection of the putative carboxin-resistant isolates, obtained originally from Viva, was unaffected by seed treatments with Vitavax (Table 1). Combined data from the putative carboxin-resistant isolates showed that 214 of 802 (27%) plants treated with Vitavax were infected, while 258 of 998 (26%) untreated plants were infected (Table 1).

In the first experiment, at the Vitavax-deposition rate of 400 μg of carboxin per gram of seed, seven of 297 plants from seed inoculated with four of the carboxin-sensitive isolates, were infected. In the subsequent two experiments at the deposition rate of 1,010 μg of carboxin per gram of seed, only one of 616 plants was infected. The overall infection percentage of treated plants for all carboxin-sensitive isolates was only 0.9%. In contrast, 42% (518 of 1,224) of untreated plants were infected by the carboxin-sensitive isolates.

The mean infection percentage for untreated plants of the carboxin-resistant field isolates from Viva varied from 20 to 42%, while that attributable to the untreated carboxin-sensitive isolates varied from 20 to 66%. Such variability in infection levels after inoculations with *U. nuda* is common. Similar variability occurs with inoculations of *U. tritici* (6).

In vitro study of the 20 selected isolates. As in the in planta work, a qualitative difference between the isolates from Viva and the other 12 was evident (Table 2). At the fungicidal carboxin concentration of 0.1 μg/ml, the putative carboxin-resistant isolates from Viva were unaffected when compared to the untreated controls, while viability and growth of the other 12 were significantly reduced. At the fungistatic carboxin concentration of 10 μg/ml, there was no difference between the response of the isolates from Viva (carboxin-resistant in planta) and the others (carboxin-sensitive in planta); all of the isolates stopped growing after the addition of carboxin, but they were not killed. Teliospores from the eight infected plants in the treated, carboxin-sensitive material were tested in vitro, and all were carboxin-sensitive.

Survey of *U. nuda* for carboxin resistance. After the in planta and in vitro studies of the 20 selected isolates, it was clear that carboxin-resistant isolates could effectively and efficiently be identified by an in vitro assay at the fungicidal concentration of 0.1 μg/ml. As Table 3 indicates, all isolates surveyed in this manner were sensitive to carboxin. Control cultures (no carboxin) of the surveyed isolates grew normally while treated (0.1 μg/ml of carboxin) cultures displayed the reactions of the carboxin-sensitive isolates of Table 2. In all cases, the isolates from Viva in the control cultures were resistant to the fungicidal concentration of carboxin.

DISCUSSION

The carboxin-resistant status of certain European isolates of *U. nuda* was confirmed both in planta and in vitro. It is noteworthy that the in planta validation was done in cv. Regal. Carboxin resistance has been reported in isolates of *U. nuda* from cvs. Panda and Viva in which the results of Vitavax seed treatment were occasionally unsatisfactory (M. Puttock, *personal communication*). Thus, there was some doubt as to whether reported "carboxin resistance" was not, in fact, a problem of adequately applying Vitavax to the seeds of some cultivars. The unequivocal observation of carboxin resistance in isolates obtained originally from Viva when tested in Regal, as well as in vitro, has clarified this issue.

The eight infected plants in the treated, carboxin-sensitive material were clearly "escapes." Seven of the eight occurred at the lower carboxin-deposition rate of 400 μg/g of seed. Even at the higher deposition rate of 1,010 μg/g of seed, and with measures to avoid leaching of carboxin in the greenhouse beds, an escape was still seen. The fact that such escapes could be easily identified in vitro as carboxin-sensitive emphasized the utility of the in vitro assay used in this study.

The isolates from Viva were resistant in vitro to carboxin at the fungicidal concentration of 0.1 μg/ml and sensitive at the fungistatic concentration of 10 μg/ml. The carboxin concentration affecting mycelium of *U. nuda* within the embryo of a developing barley seedling is not known. Our results suggest that in planta effects of carboxin are fungicidal because it is only at the 0.1 μg/ml fungicidal concentration that carboxin-resistant and carboxin-sensitive isolates can be distinguished in vitro.

We surveyed *U. nuda* in western Canada more intensively than in eastern Canada because more than 90% of Canada's barley is grown in the west. The observation that all of the Canadian isolates were sensitive to carboxin strongly indicates that carboxin-resistant strains are not yet present in Canada.

Similarly, we did not find evidence that carboxin resistance predates the introduction and use of carboxin-containing seed treatments. Our sampling of the pre-1970 worldwide population (96 isolates) was limited, particularly with respect to Europe. It may be assumed that preexisting carboxin resistance would have

TABLE 3. Origin of field isolates of *Ustilago nuda* surveyed for carboxin resistance and found to be carboxin-sensitive

Number of isolates of *U. nuda*	Origin	Year of isolation
156	Western Canada (10)	1988
90	Western Canada (11)	1989
12	Southern Ontario	1989
1	La Pocatiere, Quebec	1989
1	Harrington, Prince Edward Island	1989
96	Worldwide[a]	pre-1970
8	South Australia	1978
2	Denmark	1984

[a] Collected before use of carboxin (i.e., ca. 1970) in Western Canada, Eastern Canada, South Africa, Israel, Kenya, Czechoslovakia, South Korea, Argentina, and elsewhere in South America.

spread rapidly after the introduction of carboxin-containing seed treatments and that it would have been reported before 1986 (3). However, Skylakakis (7) showed that, in the presence of carboxin, the frequency of the resistant population would take 6 yr to reach 10%, starting from an initial incidence of 10^{-7} and assuming conditions favoring maximal disease spread.

The theoretical slow spread of carboxin resistance in *U. nuda* contrasts with observations of rapid spread of a specific type of virulence in western Canada (9). Virulence on cultivars with resistance derived from cv. Jet was first detected in the western Canadian population of *U. nuda* in 1972, but made up 87% of collections by 1978 (9). The spread of carboxin resistance within the European population of *U. nuda* has undoubtedly been inhibited by the use of other fungicides that control carboxin-resistant isolates (4; M. Puttock, *personal communication*).

We did not assess the relative parasitic fitness of the carboxin-resistant isolates from Viva. Mean infection percentages (Table 1) of the carboxin-resistant isolates were generally lower than those of carboxin-sensitive isolates in the absence of Vitavax treatment. However, given variability in infection after inoculation (6), use of more replications and barley cultivars would be necessary to answer this question. We also did not address the possibility that carboxin resistance is a trait that lowers parasitic fitness in any genetic background.

It was also not clear whether the eight carboxin-resistant isolates from Viva, collected in 1986 and 1987, were genetically distinct from one another. In the case of the 12 carboxin-sensitive isolates, the five Canadian ones are known to be genetically distinct in terms of differential virulence.

LITERATURE CITED

1. 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.
2. Griffin, D. H. 1981. Fungal Physiology. John Wiley & Sons, New York. 383 pp.
3. Leroux, P. 1986. Characteristics of strains of *Ustilago nuda*, the causal agent of barley loose smut, resistant to carboxin. Agronomie (Paris) 6:225-226.
4. 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.
5. Newcombe, G., and Thomas, P. L. 1990. Fungicidal and fungistatic effects of carboxin on *Ustilago nuda*. Phytopathology 80:509-512.
6. Nielsen, J. 1987. Races of *Ustilago tritici* and techniques for their study. Can. J. Plant Pathol. 9:91-105.
7. Skylakakis, G. 1982. Epidemiological factors affecting the rate of selection of biocide-resistant genotypes of plant pathogenic fungi. Phytopathology 72:271-273.
8. Thomas, P. L. 1978. Potential for natural hybridization between strains of *Ustilago nuda*. Can. J. Bot. 56:721-726.
9. Thomas, P. L. 1984. Barley smuts in the Prairie Provinces of Canada, 1978-82. Can. J. Plant Pathol. 6:78-80.
10. Thomas, P. L. 1989. Barley smuts in the Prairie Provinces of Canada, 1983-88. Can. J. Plant Pathol. 11:133-136.
11. Thomas, P. L. 1990. Barley smut survey, 1989. Can. Plant Dis. Surv. 70:38.