Aggressiveness, Competitiveness, and Stability of Tolerance of Benzimidazole-Tolerant Strains of *Ceratocystis ulmi*

L. R. SCHREIBER, Supervisory Plant Pathologist, USDA-ARS, Nursery Crops Research Laboratory, Delaware, OH 43015; E. E. CONAWAY, Former Graduate Research Assistant, Department of Plant Pathology, Ohio State University, Columbus 43210; and J. W. PEACOCK, Research Entomologist, USDA-FS Northeastern Forest Experiment Station, Delaware, OH 43015

ABSTRACT

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Benzimidazole-tolerant (T) variants of *Ceratocystis ulmi* were as aggressive or more aggressive than the sensitive (S) wild-type strains from which they were selected when inoculated into nursery-grown American elms. T strains reisolated 34 mo later retained their aggressiveness when tested by reinoculation into both nursery and potted elms. The competitiveness of T and S strains was compared in American elms infested with the smaller European elm bark beetle (*Scolytus multistriatus*), vector of *C. ulmi*. The pathogenic phase of the fungus in the elm xylem and the saprophytic phase in beetle galleries were examined as possible sources of contamination of emerging beetle progeny. T and S strains were both readily transmitted from either the xylem of infected trees or from the bodies of breeding beetles in brood galleries to beetle progeny emerging from elm bolts. Tolerance of T strains to chemicals was stable in vivo and in vitro. Tolerance levels were undiminished when the pathogen was isolated from diseased trees 34 mo after inoculation, after storage on unamended or on benzimidazole-amended PDA for 20 mo, or after 43 serial transfers.

Benzimidazole fungicides are systemic and are used to control many plant diseases. They are the only chemicals recommended for control of *Ceratocystis ulmi* (Buisman) C. Moreau, causal fungus of Dutch elm disease (DED) (6–9, 12,18). Failures in the control of some of these diseases are due to the development of pathogen tolerance (2,3,22).

Investigations of benzimidazole tolerance in *C. ulmi* indicate various levels of both natural and induced tolerance (7,17,20). Schreiber and Gregory (17) demonstrated tolerance in *C. ulmi* sufficiently high to negate chemical control when Lignasan BLP (methyl 2-benzimidazole carbamate phosphate) (MBC-P) was injected into elms at five times the recommended therapeutic rate.

Aggressiveness of *C. ulmi* tolerant to MBC may vary from the wild type. Nishijima and Smalley (13) reported that tolerant strains of *C. ulmi* produced more disease symptoms than sensitive ones. Schreiber and Gregory (17) found equal levels of aggressiveness in a wild-type strain and a tolerant strain and concluded, as did Brasier and Gibbs (6), that there was no relationship between tolerance and aggressiveness.

The competitiveness and survival of tolerant *C. ulmi* strains in nature will influence DED control with benzimidazoles. Shabi and Katan (21) reported that strains of *Venturia pirina* Aderh. tolerant and sensitive to MBC were equally competitive. On the other hand, Wuest et al (24) found that benomyl-tolerant *Verticillium malthousei* Ware showed decreased sporulation and reduced survival compared with sensitive strains.

Findings vary on the loss or reduction of tolerance in the absence of the chemical. Bartels-Schooley and McNeill (1) found benomyl-tolerant colonies of *Fusarium oxysporum* f. sp. *meloni* (Leach & Currence) Snyd. & Hans. reverted to the sensitive types, whereas in other colonies, tolerance was stable. Most studies report retention of benzimidazole tolerance after withdrawal of the chemical (4).

We report on the influence of factors associated with benzimidazole tolerance in *C. ulmi* on chemical control of DED, including comparative aggressiveness and competitiveness of tolerant (T) and sensitive (S) strains, and the stability of benzimidazole tolerance.

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MATERIALS AND METHODS

Aggressiveness studies. The *C. ulmi* strains included three wild-type S strains (DS, WS, and IS) and three T strains (DT, WT, and IT). T variants were selected by serial exposure of S strains to methyl 2-benzimidazole hydrochloride incorporated in potato-dextrose agar (PDA) at 25 μg/ml. The aggressiveness of the six strains was compared on the basis of foliar symptoms induced in inoculated American elms (*Ulmus americana* L.). Ninety field-grown elms (15–25 cm in diameter at breast height [dbh]) were arranged in completely randomized blocks for the six inoculation treatments. Inoculum was a conidial suspension grown in potato-dextrose broth (PDB) on a rotary shaker. A final concentration of 10^6 conidia per milliliter was prepared with distilled water. Trees were inoculated on 15–16 June 1978 by introducing 1 ml of suspension into a secondary branch on each tree by the method of Schreiber and Stipes (19). Aggressiveness was determined by visually estimating the percentage of foliage symptomatic on 31 July and 29 August 1978 and on 31 August 1979.

To determine the stability of aggressiveness in T and S strains, stock cultures of the strains used in 1978 were compared with cultures reisolated from those trees on acidified PDA in 1981. Samples for reisolation were obtained with an increment hammer from the bases of two trees inoculated with each isolate.

Suspensions of reisolated cultures and stock cultures were grown on PDB and inoculated into nursery elms, as described previously, on 1–2 June 1981. The same suspensions were used to inoculate potted American elms 1–1.3 m tall on 11 June 1981 by placing a drop of conidial suspension in a 4-mm-wide chisel wound in the base of the stem. There were 10 replicate trees per treatment in both the nursery and the potted elms. Foliar symptoms were evaluated 2 and 6 wk after inoculation.

Competitiveness of T and S strains. We determined the relative competitiveness of T and S strains of *C. ulmi* by comparing the frequency of their isolation from bodies of smaller European elm bark beetles (*Scolytus multistriatus* Marsham) that emerged from bolts of American elms. The trees (6.5–10 m high and 15–25 cm dbh) had been inoculated with T or S strains or were uninoculated. Parent beetles, reared in the laboratory, were used to infest bolts from these trees and were contaminated with the T or S strain or both or were uncontaminated.

To recover and identify strains from emerging beetle progeny, elm logs were

cut into bolts 35 cm long. The presence and chemical tolerance of *C. ulmi* was determined by removing 10 increment samples from the circumference of each bolt. These were placed on acidified PDA or acidified PDA amended with 5 or 100 μg/ml of MBC-P. The ends of the bolts were then waxed to prevent excessive drying during the experiments. One to three bolts were placed in cylindrical cardboard ice cream cartons 37.5 cm long and 27.5 cm in diameter. Four to 14 replicated cartons were used per treatment. The closed cartons were placed on shelves under continuous light at 25 C and 50% RH. Beetle progeny, attracted by the light, were collected in translucent plastic bottles in the carton lids (15).

Emerged beetles were killed by freezing at −10 C for 24 hr, then counted and placed on elm-extract agar (EEA) to determine the presence of *C. ulmi*. EEA was prepared by boiling 80 g of elm xylem tissue in 700 ml of distilled water for 1 hr, adding 13.5 g of agar to the decanted liquid, and sterilizing the mixture. To 100 ml of sterile distilled water was added 200 μg of cycloheximide and 100 μg each of novobicin and streptomycin sulfate. The suspension was agitated 1 hr and stirred into the molten agar. The chemical tolerance of strains growing from beetles was determined by subculturing from EEA onto PDA amended and unamended with MBC-P. To ensure that beetles were initially free of *C. ulmi*, a 100-beetle sample was plated on EEA at the beginning of the experiments.

To determine if cross-contamination by *C. ulmi* occurred between beetles after they emerged from the bolts, 200 fungus-free beetles, reared in the laboratory, were placed on PDA in petri dishes with *C. ulmi* for 15 min at 27 C; 200 similar beetles were placed in dishes with PDA only. Twenty beetles from each treatment were then plated on EEA to determine the percentage of fungus-contaminated beetles. Ninety contaminated and 90 uncontaminated beetles were then placed in each of two rearing cartons with a debarked elm bolt under continuous light. Beetles from carton 1 were collected individually in gelatin capsules as they emerged into the collecting bottle. Beetles from carton 2 were collected en masse at the end of each of 2 days and the intervening night. Beetles that emerged overnight from carton 1 were considered a mass collection as were those from carton 2. All beetles were killed by freezing and plated on EEA to detect *C. ulmi*.

After inoculating nursery elms with T strains of *C. ulmi* in 1978, we surveyed for the presence of these strains in nature by assaying the fungus strains on beetles emerging from trap logs in the nursery from 1980 to 1983. Each year, six healthy American elms were cut during late winter or early spring. The log sections of these trees (2–3 m high and 10–20 cm dbh) were supported upright adjacent to the 1978 infection locus and subjected to natural infestation. Hercon controlled-release dispensers containing multilure (16) were affixed to the logs to increase beetle attraction. In late September and early October, the logs were stored at 5 C until late November or December, when they were cut into bolts. The beetles were reared from the bolts, and number of contaminated beetles and chemical tolerance of the *C. ulmi* strains were determined as described before.

We attempted to determine if the primary source of *C. ulmi* contamination of elm bark beetles emerging from diseased bolts was the fungus from the xylem or that brought in on breeding beetles. Nursery elms were inoculated through chisel wounds in the base of the trunk with conidial suspensions of either the T or S strains. After development of foliar symptoms but before beetle attack, these and several healthy elms were cut into bolts. The presence and chemical tolerance of *C. ulmi* was determined by increment sampling. Bolts were placed in rearing cartons as described previously.

Bolts from the T- and S-inoculated trees were each divided into two groups. One group was infested with uncontaminated beetles; in the other group, the T-inoculated bolts were infested with S-contaminated beetles and the S-inoculated bolts with T-contaminated beetles. The bolts from healthy elms were also divided into two groups. Half were infested with S-contaminated beetles, and half were infested with T-contaminated beetles. These laboratory-reared beetles were contaminated with T or S strains as described before. From each treatment, we recorded the percentage of beetle progeny contaminated with *C. ulmi* and the percentage of beetles contaminated with the T strain.

In a second study, S- and T-inoculated nursery elms and healthy nursery elm trunks were subjected to infestation by elm bark beetles in the field. After infestation and larval development, progeny were reared. The percentage of progeny contaminated with *C. ulmi* and chemical tolerance of *C. ulmi* were determined as described previously.

In a third study, we compared the competitiveness of T and S strains when infesting beetles were simultaneously contaminated with both strains. Healthy elms were cut into bolts, air-dried to make them receptive to beetle infestation, and placed in rearing cartons. Laboratory-reared beetles were contaminated by alternately placing them on PDA in petri dishes containing T or S strains. We infested each carton with 100 beetles.

Stability of chemical tolerance. We determined the stability of tolerance of DT, IT, and WT cultures stored at 2 C on unamended PDA and PDA amended with MBC-P at 2 μg/ml. The tolerance level of each isolate was determined before storage and then periodically for 20 mo. Strains DT and WT were evaluated by measuring colony diameter growth on PDA amended with 200 μg MBC-P per milliliter; IT (a less tolerant strain) was evaluated on PDA amended with 5 μg/ml. Tolerance was measured as described before.

Stability of tolerance of these strains to MBC-P was also determined for stock cultures after successive transfers onto unamended PDA. Initial tolerance of DT, WT, and IT was determined as noted before. An unamended PDA plate of each strain was selected at random to serve as a source of mycelial plugs transferred to the next set of amended and unamended plates. After 7 days of incubation, colony diameter growth was again measured and expressed as a percentage of the controls. This process was repeated for 43 serial transfers.

The tolerance of *C. ulmi* reisolated from trunk increments of nursery elms inoculated with T strains was compared, 34 mo after inoculation, with the stock strains used for inoculum. Increment-hammer samples were taken from three to six trees from each of the three T strain treatments and grown on acidified PDA. Stock cultures were grown on PDA. Agar plugs 8 mm in diameter were cut from the colony margins of both reisolated and stock cultures and placed in the centers of plates of unamended PDA or PDA amended with 5 and 100 μg of MBC-P per milliliter. Growth was measured by averaging two diameter measurements after 5 days. Each treatment was replicated three times. Measurements for each treatment were averaged. Growth on amended agar was expressed as a percentage of that on the unamended controls.

RESULTS

Aggressiveness studies. No significant differences were found in mean percentage of foliage symptomatic on any of the three observation dates between nursery elms inoculated in 1978 with DS and DT or with WS and WT. IT, however, produced significantly higher percentages of foliar symptoms than did IS on all three dates (Table 1).

No significant differences were found in 1981 in aggressiveness between stock cultures and reisolated cultures of T and S strains from 1978 inoculations as measured by percentage of foliage symptomatic in nursery elms. In potted elms, symptoms were less severe than those in the nursery elms. All reisolated cultures, except the IS strain, produced significantly more symptoms than did stock cultures (Table 2).

Competitiveness of tolerant strains. We found no evidence of significant cross-contamination with *C. ulmi* occurring between beetles emerging from rearing cartons into plastic collecting

bottles. *C. ulmi* was isolated from 46% of beetles collected individually in gelatin capsules as they emerged from carton 1; similarly, 39% of beetles mass-collected daily from carton 2 were contaminated with the fungus as were 40% of the beetles mass-collected overnight from cartons 1 and 2.

Of beetle progeny emerging from naturally infested trap logs from 1980–1983, 10, 12, 2.3, and 4.1%, respectively, were contaminated with *C. ulmi*. T strains were not recovered from any beetles.

When uncontaminated beetles were placed on bolts from S- or T-inoculated elms, the percentages of their progeny that were fungus-contaminated were 3.4 and 6.5%, respectively. These progeny carried only the strain used to inoculate the tree. When T-contaminated beetles were placed on S-infected bolts, 13.1% of the progeny were contaminated; 86.4% of these were contaminated with the T strain. When S-contaminated beetles were placed on T-infected bolts, 44.5% of the progeny were contaminated; 89% of these were contaminated with the T strain. When either S- or T-contaminated beetles were placed on healthy elm bolts, the percentages of contaminated progeny were 2.8 and 18.3%, respectively. In both cases, the strain recovered from progeny was the same as that used to contaminate parent beetles.

When healthy elm trunks and T- and S-inoculated elms were subjected to natural infestation by beetles in the nursery (carrying primarily S strains), the percentages of contaminated beetle progeny were 6.5, 11.7, and 3.1%, respectively. These percentages were not significantly different at $P = 0.05$. Only S strains were recovered from progeny from the healthy and S-inoculated elms. The T strain was isolated from 47.5% of contaminated progeny from T-inoculated elms.

When healthy bolts were infested with beetles contaminated simultaneously with both S and T strains, 20.3% of the progeny carried *C. ulmi*. The T strain was recovered from 40.8% of contaminated progeny (Table 3).

When the source of fungus contamination of beetle progeny emerging from elm bolts was diseased wood alone, parent beetles alone, or parent beetle and diseased wood, the average percentages of contaminated progeny were 5, 10.6, and 28.8%, respectively. There were no significant differences in percentages of contaminated beetle progeny when either the parent beetles or elm bolts were the only sources of contamination. The percentage of contaminated progeny was significantly higher ($P = 0.05$) when *C. ulmi* was present both in the elm bolts and on parent beetles.

Stability of chemical tolerance. Before storage, the growth of strains DT and WT on PDA amended with 200 μg of MBC-P per milliliter was 74.7 and 28.1%, respectively, of that of the controls; growth of IT on PDA amended with 5 μg of MBC-P per milliliter was 29.4% of that of the controls. After 20 mo of storage at 2 C on unamended PDA, growth of DT, WT, and IT was 69.1, 35.1, and 32.3%, respectively, of that of the controls; when stored on 2% amended PDA, growth of DT, WT, and IT was 72.9, 32.3, and 31%, respectively, of that of the controls. After 43 successive transfers onto unamended PDA, growth of DT, WT, and IT was 80.6, 33, and 24.9%, respectively, of that of the controls. Thus, no significant changes in tolerance were noted (Table 4).

The tolerance levels of reisolated T strains obtained 34 mo after inoculation did not differ significantly from those of the respective refrigerated stock cultures. Growth of the stock and reisolated culture of DT was 100 and 100%, respectively, of that of the controls at 5 μg/ml and 88.6 and 90.3%, respectively, of that of the controls at 100 μg/ml; WT grew at 98 and 97.3%, respectively, of that of the controls at 5 μg/ml and 35 and 29.7% of that of the controls at 100 μg/ml. Neither the stock culture nor reisolated IT grew on amended agar (Table 5).

Table 1. Percentage of foliage with symptoms in American elms inoculated with strains of *Ceratocystis ulmi* tolerant (T) or sensitive (S) to benzimidazoles[x]

C. ulmi strain[y]	Foliar symptoms (%) 31 Jul. 1978	29 Aug. 1978	31 Aug. 1979
DS	25.7 a[z]	28.3 a	79.0 a
DT	26.7 a	29.7 a	71.2 a
WS	19.0 a	25.3 a	81.0 a
WT	28.3 a	26.3 a	90.3 a
IS	6.0 a	9.0 a	40.0 a
IT	24.0 b	28.0 b	85.7 b

[x] Nursery elms (15–25 cm dbh) were inoculated with T or S strains of *C. ulmi* (1 ml of a 10^6/ml conidial suspension) in a secondary branch on 15–16 June 1978 (15 trees inoculated per treatment).

[y] In *C. ulmi* strain designations, S = benzimidazole-sensitive and T = benzimidazole-tolerant.

[z] Within a column, for each isolate (D, W, or I) on each observation date, percentages followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Table 2. Percentage of foliage with symptoms in nursery and potted American elms inoculated with reisolated and stock cultures of strains of *Ceratocystis ulmi* tolerant (T) and sensitive (S) to benzimidazoles[y]

C. ulmi strain	Foliar symptoms (%) Nursery elms (inoculum source) Reisolated culture	Nursery elms (inoculum source) Stock culture	Potted elms (inoculum source) Reisolated culture	Potted elms (inoculum source) Stock culture
DT	62.0 a[z]	60.0 a	39.0 a	5.0 b
DS	35.0 a	49.5 a	28.6 a	3.0 b
IT	54.0 a	52.0 a	39.7 a	13.0 b
IS	60.0 a	57.5 a	16.4 a	10.0 a
WT	61.0 a	50.0 a	25.0 a	3.0 b
WS	51.0 a	29.5 a	29.7 a	10.0 b

[y] Nursery elms (15–25 cm dbh) and potted elms (1–1.3 m tall) were wound-inoculated on 1–2 June and 11 June 1981, respectively, with reisolates of T and S strains of *C. ulmi* from elms inoculated on 15–16 June 1978 and with stock cultures of the same strains (10 trees inoculated per treatment).

[z] Within a row, differences between cultures are compared separately for nursery or potted elms. Percentages followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Table 3. Transmission of tolerant (T) and sensitive (S) strains of *Ceratocystis ulmi* to progeny of *Scolytus multistriatus* emerging from American elm bolts

C. ulmi strain[a] In elms	On beetles	No. of beetles recovered	Beetle progeny contaminated (%) with *C. ulmi*	T strain
S	None	8,475	3.4	0.0
T	None	6,560	6.5	100.0
S	T	3,538	13.1	86.4
T	S	8,562	44.5	89.0
None	S	4,381	2.8	0.0
None	T	11,961	18.3	100.0
None	S & T	591	20.3	40.8

[a] American elms (15–25 cm dbh) were inoculated with a conidial suspension of T or S strain of *C. ulmi*. Inoculated and uninoculated trees were cut into bolts that were placed in cardboard rearing cartons. Laboratory-reared beetles were contaminated by placing them on agar cultures of T and/or S strains. Beetles were placed on the bolts in the cartons, and emerging progeny were killed and placed on benzimidazole-amended and unamended PDA to detect the presence of T and S strains.

DISCUSSION

Treatment of high-value elms with benzimidazole fungicides is an effective component of an integrated DED control program. However, both natural and induced chemical tolerance of *C. ulmi* have been demonstrated, in some cases, at levels in excess of chemical concentrations in treated trees (11). We studied three attributes of T strains that might influence the effectiveness of disease control with benzimidazoles.

T strains were shown to be as aggressive as the wild-type S strains. Indeed, in the case of strain IT, the T strain produced more severe foliar symptoms than did the S strain. In addition, the T strains surviving in diseased nursery elms as long as 3 yr produced symptoms equal to those produced by stock cultures in nursery elms and exceeded those produced in potted elms. The last result reflects either greater aggressiveness of the reisolated cultures than of the stock cultures or greater resistance of the potted than nursery trees or both.

The spread of T strains will be influenced by their ability to compete in the saprophytic phase under the bark and in beetle galleries in dying elms and by their ability to contaminate and survive on emerging vector progeny. Previous reports indicate that breeding beetles contaminated with *C. ulmi*, and not the fungus in infected xylem, are the main source of contamination of beetle progeny (6,10). Because the strains carried by breeding beetles are primarily or solely S strains, little opportunity would exist for spread of T strains from infected trees. We found that *C. ulmi*, initially inoculated into the xylem, grows saprophytically into breeding galleries and, along with the fungus introduced on breeding beetles, may infest emerging progeny. These results agree with those of Brasier (5) and Webber and Brasier (23).

We also established that T strains, whether present in xylem of diseased trees or on breeding beetles entering brood wood, either in the presence or absence of S strains, are present on emerging progeny. Thus, T strains are competitive with wild-type S strains when both are present in brood wood. This suggests that T strains are readily available for vector transmission. The competitiveness of T strains in the saprophytic fungus stage, combined with their stable aggressive and benzimidazole-tolerant nature, makes them a threat to DED control with benzimidazole fungicides.

Although it has been demonstrated that *C. ulmi* possesses the genetic potential for high levels of tolerance (13,17,20), T strains have not been reported frequently in nature. This may be due to 1) lack of selection pressure because of the limited use of benzimidazoles, 2) sampling only a small portion of a predominantly sensitive population, 3) environmental factors acting against survival of T strains after beetle emergence and flight or in root-graft transmission, and 4) insufficient inoculum concentrations of the T strains on the vector to produce infection and disease. Parker (14) and Webber and Brasier (23) found reductions in fungus survival up to 88% in contaminated beetles after emergence from pupal chambers and subsequent flight. Our study addressed only the qualitative presence of the T strains on individual beetles. Finally, because T strains may represent a small portion of the *C. ulmi* population, and if their adaptibility does not exceed that of the S strains, they may assimilate into or be lost from the wild population through interbreeding and attrition.

Table 4. Stability of benzimidazole tolerance of strains of *Ceratocystis ulmi* stock cultures stored on benzimidazole-amended and unamended PDA[x]

Tolerant strain	Growth of stock cultures on amended PDA as a percentage of controls[y]			
	Initial growth of stock cultures	After 20 mo stored on unamended PDA	After 20 mo stored on amended PDA	After 43 serial transfers onto PDA
DT	74.7 a[z]	69.1 a	72.9 a	80.6 a
WT	28.1 a	35.1 a	32.3 a	33.0 a
IT	19.4 a	32.3 a	31.0 a	24.9 a

[x]Tolerant (T) strains were stored at 2 C on PDA or on PDA amended with 2 μg of methyl 2-benzimidazole carbamate phosphate (MBC-P) per milliliter.
[y]Colony diameter growth was compared initially and after 20 mo of storage on unamended PDA or on PDA amended with 2% MBC-P. DT and WT were tested on PDA amended with 200 μg, and IT, on PDA amended with 5 μg of MBC-P per milliliter. A similar comparison of growth of T strains was made after 43 serial transfers on PDA with a 7-day intervening growth period.
[z]Within a row, percentages followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Table 5. Stability of benzimidazole tolerance of strains of *Ceratocystis ulmi* reisolated from American elms[y]

Tolerant strain	Concentration of MBC-P (μg/ml)	Growth on amended PDA as a percentage of controls[z]	
		Stock cultures	Reisolated cultures
DT	5	100.0 a	100.0 a
	100	88.6 a	90.3 a
WT	5	88.6 a	97.3 a
	100	35.0 a	29.7 a
IT	5	0.0 a	0.0 a
	100	0.0 a	0.0 a

[y]Reisolations were made from American elms 34 mo after inoculation with T strains. Growth was compared with that of stock cultures maintained on PDA at 5 C.
[z]Within a row, percentages followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

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