Population Structure and Genetic Analysis of Field Resistance to Thiabendazole in *Gibberella pulicaris* from Potato Tubers

Anne E. Desjardins, Elizabeth A. Christ-Harned, Susan P. McCormick, and Gary A. Secor

First, second, and third authors: Mycotoxin Research, National Center for Agricultural Utilization Research, USDA-ARS, 1815 North University Street, Peoria, IL 61604; Fourth author: Department of Plant Pathology, North Dakota State University, Fargo 58105

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ABSTRACT

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Forty-two strains of *Gibberella pulicaris* (anamorph: *Fusarium sambucinum,* synonym *F. sulphureum*) were obtained from dry-rotted potato tubers collected in North America between 1963 and 1991. Twenty-four of 25 strains collected in 1990 and 1991 were resistant to the fungicide thiabendazole (TBZ), which is widely used to control potato dry rot. The 17 strains collected between 1963 and 1986 were all very sensitive to TBZ. In laboratory tests, most TBZ-resistant and TBZ-sensitive strains were virulent on potato tubers and produced trichothecene mycotoxins in liquid culture and in potato tubers. All 42 strains were characterized for sexual compatibility by crosses with tester strains and for vegetative compatibility by complementation of nitrate-nonutilizing mutants. Twenty-one (50%) of the strains belonged to one widespread vegetative compatibility group (VCG 01). Twelve strains (26%) belonged to two additional overlapping groups (VCG 03 and 04). Forty strains were mating type 1. Two strains were mating type 2 and belonged to a unique group (VCG 02). All TBZ-resistant strains were vegetatively compatible with TBZ-sensitive strains collected in previous years. Genetic analysis indicated that TBZ resistance was stable and inherited as a single gene or as closely linked genes, and that resistance mutations of independently isolated field strains were allelic. These results suggest that TBZ-resistant strains are competitive and have the potential to spread and persist in the *G. pulicaris* population that causes potato tuber dry rot in North America.

Gibberella pulicaris (Fries) Sacc. (anamorphs: *Fusarium sambucinum* Fuckel, *F. sulphureum* Schlecht) is a major cause worldwide of dry rot of potato (*Solanum tuberosum* L.) tubers in the field and in storage (7). All potato cultivars grown commercially in North America are susceptible to dry rot (24), and losses are controlled, in part, by chemical treatment of tubers at harvest or before planting. Early in the 1970s, the benzimidazole fungicide thiabendazole (TBZ) was introduced for use on potato tubers (23) and has proven very effective for control of dry rot in seed and stored potatoes. Although benzimidazole fungicides have developed field resistance (32), TBZ resistance was not reported to be a problem in North America for almost 20 yr after its introduction for dry rot control nor during its extensive use in seed potato production (33). In 1989, however, despite application of TBZ, dry rot was a problem at several sites in the north central United States. Since then, numerous strains of *G. pulicaris* have been obtained from dry-rotted tubers collected from potato-growing regions across the United States; more than three-fourths of these field strains have been found to be resistant to TBZ in vitro (33 and G. A. Secor, *unpublished data*). Because of the absence of effective alternative chemical treatments, potato producers resist abandoning TBZ for dry rot control. Continued use of TBZ will require effective strategies to lessen the occurrence of resistance under field conditions and to reduce disease.

The goal of the present study was to obtain information that might be useful in predicting competitiveness of TBZ-resistant strains and to design strategies to control resistance of *G. pulicaris* to TBZ in the field. One major objective was to evaluate the genetic diversity of TBZ-resistant and TBZ-sensitive strains of *G. pulicaris* with mating type tests and with vegetative compatibility analyses using nitrate-nonutilizing (*nit*) mutants. Both of these techniques have been useful in field population analyses of other plant-pathogenic *Fusarium* species (9,17,19,30,31). This article presents data on the level of fungicide resistance of individual strains, their virulence on potato tuber slices, and their ability to produce trichothecene mycotoxins in liquid culture and in potato tubers. Additional objectives of this study were to determine whether TBZ resistance is due to a transient physiological adaptation or to a genetic mutation, to examine whether TBZ resistance is controlled by polygenes or by major genes, and to determine whether TBZ resistance is allelic among field strains from different sources.

MATERIALS AND METHODS

Cultures. Forty-two strains of *Gibberella pulicaris* were isolated from dry-rotted potato tubers (Table 1). Each strain was reisolated from a single conidium. All strains were grown on V8 juice agar slants or plates (34) with a 12-h 25 C light and 12-h 20 C dark alternating schedule. For all experiments, fresh transfers of the strains were obtained from stored stock cultures on V8 slants held at 4 C.

TBZ resistance. Resistance to TBZ was tested by measuring radial growth of each strain on V8 agar plates amended with TBZ in 1% (v/v) dimethylsulfoxide (DMSO). A 100× TBZ stock solution was thoroughly mixed with the medium after sterilization. The growth of each field strain was measured in duplicate using 60- × 10-mm plastic petri plates containing 5 ml of medium or 100- × 15-mm plates containing 15 ml of medium. Plates of test media were inoculated with plugs (3 mm diameter) cut from the growing margins of *G. pulicaris* cultures 5–10 days old and placed, mycelial surface down, on the surface of the assay medium at the edge of the plate. Plates were incubated at 25 ± 1 C in the dark. The radius (from the inoculum to one typical point on the growing margin) was measured daily for 7 days, or until growth had reached the edge of the plate. All field strains were initially tested on 75, 50, 25, and 5 μg of TBZ per milliliter. Sensitive field strains identified in the first screen were then tested on 25,

5, 1, and 0.1 μg of TBZ per milliliter. Control plates contained 1% (v/v) DMSO only. Resistance for each field strain was calculated as an effective dosage to 50% inhibit radial growth (ED_{50}) by linear regression analysis based on one-half the radial growth of the controls. Resistant field strains were also tested on 100, 200, 300, 400, and 500 μg of TBZ per milliliter in V8 agar medium (5 ml of medium in 35-mm petri plates) to determine the minimum concentration of TBZ at which fungal growth was inhibited.

Virulence assay. Virulence was assessed gravimetrically as previously described (13). Slices of Russet Burbank tubers were inoculated with mycelium plugs and incubated in the dark at 25 ± 2 C. Six days after inoculation, virulence was measured as the average percentage of dry-rotted tuber tissue for three replicate slices. To facilitate comparison with data from previous studies (14,15), strain R-6380 (isolated from potato in Germany) was included as a standard virulent strain in all virulence tests. Its virulence was 60 ± 8% in the present study and 79 ± 24% or 71 ± 10% in previous studies.

Trichothecene toxin assays. Liquid cultures of *G. pulicaris* were grown and analyzed for 4,15-diacetoxyscirpenol (DAS) using gas-liquid chromatography (GLC) without derivatization as previously reported (15). A 2.5-ml aliquot from a 7-day-old liquid-shake culture growing in 25 ml of a medium containing 0.1% yeast extract, 0.1% peptone, and 5% glucose (YEPD) was extracted with 5 ml of ethyl acetate on a vortex mixer for 90 sec. The organic layer was removed and taken to dryness under a stream of nitrogen and then resuspended in 1 ml of ethyl acetate and analyzed by GLC with flame ionization detection on a gas chromatograph (HP 5890, Hewlett Packard, Palo Alto, CA) fitted

TABLE 1. Characteristics of field strains of *Gibberella pulicaris* used in this study

Original strain numbers	FRC[a]	Source[b] Geographic region	Source[b] Year collected	Cultivar	Mating type	VCG[c]	Diacetoxyscirpenol in liquid cultures (μg/ml)[d]	Virulence (% of tuber rotted)[e]	Trichothecenes in rotted tissue μg/g	TBZ resistance (ED_{50} μg/ml)[f]
TBZ-sensitive strains										
NRRL 13707	R-9148	ND	1963	Unknown	1	01	104[g]	22 ± 12[h]	trace[h]	1-2
	R-738	NY	1969	Unknown	1	01	44 ± 22	59[i]	134	1-2
	R-2116	PA	1972	Kennebec	1	03	55 ± 22	51[i]	216	1-2
	R-2633	ID	1975	Unknown	1	01	298[g]	10 ± 8[h]	0[h]	1-2
	R-2634	ID	1975	Unknown	1	0X	44 ± 35	59[i]	26	1-2
	R-2744	FL	1975	Unknown	1	0X	17 ± 17	67[i]	33	1-2
	R-2750	NY	1975	Unknown	1	0X	28 ± 12	69[i]	36	1-2
	R-2949	PA	1976	Unknown	1	03	43 ± 23	57[i]	76	1-2
	R-2951	PA	1976	Unknown	1	0X	28 ± 10	68[i]	32	1-2
	R-6039	MD	1981	Unknown	1	01	54 ± 18	9[i]	23	1-2
A-27940	R-9147	NB	1982	Unknown	2	02	32[g]	72	47	1-2
DAOM 192963	R-9285	PEI	1985	Unknown	1	0X	93[g]	34 ± 10[h]	5[h]	1-2
NRRL 13700	R-9146	NB	1985	Fundy	2	02	204[g]	40 ± 15[h]	trace[h]	1-2
DAOM 192966	R-9286	PEI	1985	Unknown	1	0X	53[g]	8 ± 18[h]	1[h]	1-2
NRRL 13500	R-9288	WI	1985	Unknown	1	01	75[g]	32 ± 24[h]	2[h]	1-2
NRRL 13711	R-9149	CO	1986	Centennial Russet	1	0X	5[g]	16 ± 8[h]	1[h]	1-2
DAOM 196035	R-9287	NB	1986	Shepody	1	01	14[g]	62 ± 10[h]	trace[h]	1-2
RN 5	R-9262	ND	1991	Red Norland	1	01	58 ± 10	50 ± 12	13	1-2
TBZ-resistant strains										
FID 75-1	R-9283	ID	1990	Russet Burbank	1	01	95 ± 63	71 ± 6	15	34
FID 79-1	R-9240	ID	1990	Unknown	1	01	47 ± 17	7 ± 5	58	40
FND 5-1	R-9280	ND	1990	Norchip	1	03	69 ± 22	50 ± 14	10	48
FND 23-2	R-9282	ND	1990	FL795	1	01	62 ± 25	52 ± 10	12	38
FND 26-1	R-9281	ND	1990	Norchip	1	04	56 ± 24	43 ± 9	11	40
FND 31-3	R-9239	ND	1990	Unknown	1	01	53 ± 24	59 ± 13	13	36
FMI 135	R-9256	MI	1990	Steuben	1	03	101 ± 28	64 ± 3	42	39
FMI 136	R-9257	MI	1990	Steuben	1	01	45 ± 15	13 ± 7	40	43
FMI 138	R-9258	MI	1990	Steuben	1	03	88 ± 32	56 ± 5	11	43
FMI 140	R-9259	MI	1990	Atlantic	1	01	85 ± 30	45 ± 9	25	39
FMI 141	R-9260	MI	1990	Atlantic	1	03	85 ± 9	50 ± 14	12	32
YG 1	R-9271	ND	1991	Yukon Gold	1	01	87 ± 34	67 ± 11	19	29
YG 2	R-9272	ND	1991	Yukon Gold	1	01	52 ± 28	63 ± 12	17	37
YG 3	R-9273	ND	1991	Yukon Gold	1	01	89 ± 38	72 ± 2	25	29
YG 4	R-9274	ND	1991	Yukon Gold	1	01	83 ± 40	67 ± 12	24	30
YG 5	R-9275	ND	1991	Yukon Gold	1	03	71 ± 6	57 ± 13	13	53
YG 6	R-9276	ND	1991	Yukon Gold	1	01	79 ± 18	70 ± 8	14	29
YG 7	R-9277	ND	1991	Yukon Gold	1	01	89 ± 38	81 ± 3	14	30
YG 8	R-9278	ND	1991	Yukon Gold	1	04	69 ± 41	46 ± 12	21	22
YG 9	R-9279	ND	1991	Yukon Gold	1	01	116 ± 61	67 ± 1	22	30
RN 1	R-9261	ND	1991	Red Norland	1	01	80 ± 15	75 ± 9	34	32
RN 6	R-9263	ND	1991	Red Norland	1	04	76 ± 30	49 ± 5	42	27
RN 8	R-9264	ND	1991	Red Norland	1	04	76 ± 17	47 ± 23	43	28
RN 9	R-9265	ND	1991	Red Norland	1	04	75 ± 13	33 ± 16	23	26

[a] Deposit numbers for strains at the Fusarium Research Center, The Pennsylvania State University.
[b] Information on geographical region, cultivar, and year of isolation is from the supplier of the strain. Stain R-9148 was previously (15) incorrectly identified as from Maine. CO = Colorado, FL = Florida, ID = Idaho, MD = Maryland, MI = Michigan, NB = New Brunswick, Canada, NY = New York, ND = North Dakota, PA = Pennsylvania, PEI = Prince Edward Island, WI = Wisconsin.
[c] Vegetative compatibility group (VCG), strains that appear to have unique VC types are designated VCG OX.
[d] Means and standard deviations for three replicate tests.
[e] Means and standard deviations for three replicate tests, three slices per test.
[f] Strains assayed in duplicate on V8 agar medium amended with TBZ as described in Materials and Methods.
[g] Data from (3).
[h] Data from (15). Trace indicates less than 0.05 μg/g fresh weight.
[i] One test of three slices.

with a HP1 methyl silicone gum instrument test column (5-m × 0.53-mm × 2.65-μm film thickness). The column was held at 120 C at sample injection, then heated to 210 C at 15 C/min and held for 1 min. The column was then heated to 260 C at 5 C/min and held for 8 min. The concentration of DAS in the extracts was determined by a 50–500 μg/ml purified standard curve. The amount of DAS per milliliter of liquid culture was calculated from this value. No trichothecenes other than DAS were detected in the liquid culture extracts.

Dry-rotted tissues from three tuber-slice replicates were pooled, weighed, macerated, and extracted with ethyl acetate. The extract was dried under a stream of nitrogen, redissolved in 1 ml of ethyl acetate, and analyzed by GLC as described above. The concentrations of DAS and 15-monoacetoxyscirpenol (MAS) in the extracts were determined by 50–500 μg/ml purified standard curves. The amount of toxin per gram of dry-rotted tissue was calculated from this value. To facilitate comparison with data from previous studies (3,15), strain R-6380 was included as a standard strain in all trichothecene tests. In liquid cultures, R-6380 produced 35 ± 11 μg/ml DAS in the present study and 74 μg/ml in a previous study (3). In dry-rotted tubers, R-6380 produced 5 μg/g trichothecenes (DAS and MAS) in the present study and 4.0 μg/g in a previous study (15).

Genetic crosses. Crosses were made between strains of opposite mating type, designated MAT1-1 and MAT1-2; crosses between strains of the same mating type are infertile. Techniques for crossing and for ascospore isolations have been described (12). Sterile mulberry twigs on water-agar slants were inoculated with the female parent, grown for 1–1.5 mo until protoperithecia were well developed, and then fertilized by conidia from the male parent. After fertilization, crosses were incubated at 15 C until perithecia were mature. Femaleness was scored as the ability to produce protoperithecia. Mating type was determined by crosses to two highly female-fertile tester strains: R-6380 (MAT1-1) and 64995 (MAT1-2) isolated from *Brassica oleracea* in Germany. Tester strains of European origin were used because highly female-fertile North American strains are not available. Strain 64995 was also used as the female parent in crosses with TBZ-resistant field strains. All random ascospore and tetrad progeny were screened at 10 μg of TBZ per milliliter; selected progeny were tested further to determine ED_{50} values as described above.

Nitrate nonutilizing mutants. *nit* mutants were isolated and analyzed by the methods of Correll et al (9) with minor modifications. *nit* mutants were generated on a chlorate medium made by adding 1.6 g of L-asparagine, 2 g of $NaNO_3$, and 45 g of $KClO_3$ to 1 L of basal medium. Plastic petri dishes (100 × 15 mm) containing 20 ml of chlorate medium were inoculated with a mycelial plug at the center of each plate. Approximately five plates per strain were inoculated and placed inside a plastic bag to prevent the plates from drying out. Culture growth was highly restricted until fast-growing sectors appeared, usually in 7–14 days. Fast-growing sectors were transferred from chlorate medium onto minimal medium (basal medium plus 2 g of $NaNO_3$) to check for nitrate utilization and onto V8 agar to check for wild-type growth as compared with the parent strain. Those sectors that were resistant to chlorate and could not utilize nitrate grew as thin, sparse colonies lacking aerial mycelium on minimal medium and were considered *nit* mutants. Fast-growing sectors resistant to chlorate but growing as wild-type on minimal medium when compared to the parent strain were discarded. These mutants appeared to be similar to chlorate-resistant nitrate-utilizing (*crn*) mutants of *Fusarium moniliforme* (19). Phenotype identification of each *nit* mutant was based on the ability to grow, relative to the wild-type parent, on basal medium containing one of five different nitrogen sources: nitrate, nitrite, ammonium, uric acid, and hypoxanthine. Each mutant was reisolated from a single conidium, tested on the five media as described above, and scored for growth type after 5 days. *nit* mutant nomenclature follows that of Correll et al (9) and Leslie (26).

Complementation between *nit* mutants of different strains and between mutants of the same strain was done as follows. Mycelial plugs from V8 agar cultures of mutants were paired 2 cm apart on minimal medium, incubated for 2–3 wks, and scored for appearance of dense aerial mycelia at the point of contact between *nit* mutant colonies. All complementation tests were repeated at least once.

RESULTS

Effect of TBZ. Our collection of *G. pulicaris* contained 17 field strains that were isolated from dry-rotted potato tubers between 1963 and 1986 (Table 1). All of these strains were very sensitive to TBZ; the ED_{50} was 1–2 μg/ml and the lowest concentration at which the fungus did not grow was less than 10 μg/ml. Fourteen additional strains of *G. pulicaris* were isolated in early 1991 from stored, dry-rotted tubers of cultivars Yukon Gold and Red Norland that had been grown in adjacent rows in a potato field near Grand Forks, ND. Although the seed planted for this potato crop had not been treated with TBZ and there was no selection for TBZ resistance during isolation of these strains from dry rot lesions, 13 of the 14 strains were highly resistant to TBZ (Table 1). An additional group of 11 TBZ-resistant strains was collected in 1990 from dry rot problem sites in Idaho, Michigan, and North Dakota. All 24 TBZ-resistant strains were similar in radial growth inhibition by TBZ with ED_{50} of 35 ± 7 μg/ml (Table 1). All TBZ-resistant strains were able to grow on agar medium amended with TBZ at 500 μg/ml, the highest concentration tested. In the absence of TBZ, all 24 TBZ-resistant strains were similar to each other in radial growth on V8 juice agar (6.4 ± 1.4 mm per day), and they were similar to three TBZ-sensitive strains (6.8 ± 0.1 mm) tested at the same time. The average radial growth rate of 15 additional TBZ-sensitive field strains tested in other experiments was 6.4 ± 0.7 mm per day.

Virulence and toxigenicity. To study the potential association of TBZ resistance with other traits of *G. pulicaris*, we compared virulence and toxigenicity of the 24 TBZ-resistant and the 18 TBZ-sensitive field strains (Table 1). Virulence varied from 7 to 81% (mean 54 ± 18%) for TBZ-resistant strains and from 9 to 69% (mean 44 ± 23%) for TBZ-sensitive strains. Among TBZ-resistant strains, mean virulences were 58 ± 22, 55 ± 6, and 44 ± 6% for vegetative compatibility groups (VCGs) 01, 03, and 04, respectively. In all strains tested, DAS was the major trichothecene produced in liquid culture, and it ranged from 45 to 116 μg/ml (mean 76 ± 113) for resistant strains and from 5 to 298 μg/ml (mean 69 ± 72) for sensitive strains (Table 1). MAS was the major trichothecene produced in tuber slices by most strains tested; some strains also produced DAS and trace amounts of other trichothecenes. Trichothecene production in tuber slices ranged from 11 to 58 μg/g fresh weight (mean 23 ± 13) for resistant strains and from 0 to 216 μg/ml (mean 23 ± 35) for sensitive strains.

Mating type. The 42 field strains of *G. pulicaris* in this study were evaluated for their ability to mate with TBZ-sensitive tester strains. Genetic fertility of eight of these strains was reported previously (15). The 24 TBZ-resistant strains were genetically fertile as males and all were MAT1-1. None of these strains produced more than a few protoperithecia, therefore they were all scored as female-sterile. MAT1-1 male-only strains predominated among the 18 genetically fertile, TBZ-sensitive field strains. Only two strains, both from New Brunswick, were MAT1-2, and only four strains (R-9148, R-9286, R-9287, and R-9288) were fertile as females. These results indicate that these TBZ-resistant and TBZ-sensitive strains can mate.

Isolation of *nit* mutants. *nit* mutants were easily obtained from *G. pulicaris*. The fast-growing sectors that were obtained on chlorate selection media were either wedge-shaped or circular. Some sectors were also color mutants ranging from tan to dark orange in comparison with the pale orange of wild-type strains on chlorate medium. Initially, generation of these fast-growing sectors was tested at several concentrations of $KClO_3$. A concentration of 45 g/L was chosen for routine generation of fast-growing sectors because some strains were unable to form sectors at 60 g/L and other strains were not inhibited at 30 g/L. One strain, FID 79-1, was particularly sensitive to chlorate and was tested

at 15 g of $KClO_3$ per liter. Each culture obtained from a fast-growing sector was reisolated from a single conidium before testing *nit* mutant phenotypes except for strain FID 79-1. Single conidia derived from fast-growing sectors of FID 79-1 failed to grow on culture medium and, therefore, mass transfers from sectors were used for further analysis of strain FID 79-1.

A total of 229 single-spore *nit* mutants were obtained from the 42 field strains tested (Table 2). *nit3* and *nit1* mutants were the most numerous types identified from the tested sectors at 41 and 34% of the sectors tested, respectively. NitM mutants made up only 7% of the sectors tested and were produced only by strains YG 2, YG 5, YG 9, RN 8, RN 9, FMI 135, FMI 140, A-27940, and NRRL 13707. In initial screens, *crn* (19) mutants were identified as making up 17% of the 229 sectors tested. A major nitrogen regulatory locus, analogous to *nnu* in *F. graminearum* (25), appeared to be mutated in a single sector (from strain YG 3) because it grew only on basal medium containing ammonium.

The numbers and types of *nit* mutants produced from TBZ-resistant and TBZ-sensitive strains were similar (Table 2). Forty percent of mutants from TBZ-resistant strains and 45% from TBZ-sensitive strains were *nit3*. Thirty-five percent of mutants from TBZ-resistant strains and 34% from TBZ-sensitive strains were *nit1*. Seven percent of mutants from TBZ-resistant strains and 5% from TBZ-sensitive strains were NitM. Eighteen percent of mutants from TBZ-resistant strains and 16% from TBZ-sensitive strains were *crn*.

Vegetative compatibility. Complementation between *nit* mutants of different phenotypes was indicated by the appearance of dense growth at the zone of contact between two mutants. As has been observed in other *Fusarium* species (26), heterokaryons that formed between NitM mutants and *nit1* or *nit3* mutants were the most vigorous and gave the most reliable results in complementation tests. Unfortunately, NitM mutants were quite rare and were obtained in only nine field strains. To determine VCGs, a *nit1* or a *nit3* from each of the 42 field strains was paired with a selection of NitM mutants. Forty-eight percent of the field strains complemented the NitM tester from strain NRRL 13707. These 21 strains were designated VCG 01 (Table 1). None of the strains in VCG 01 cross-complemented any of the remaining 21 field strains tested. These data indicate a high degree of incompatibility between the strains in VCG 01 and the strains in other VCGs. Strains NRRL 13700 and A-27940 complemented each other only and were designated VCG 02 (Table 1). Again, there was no cross-complementation between VCG 02 and any other VCG group. Six field strains strongly complemented a NitM mutant of strain YG 5, and these seven strains were designated VCG 03. Three field strains strongly complemented NitM mutants of strains RN 8 and RN 9, and these five strains were designated VCG 04 (Table 1). Weak cross-reactivity was occasionally observed upon pairing certain VCG 03 strains with one of the VCG 04 testers, strain RN 8, and, conversely, upon pairing certain VCG 04 strains with the VCG 03 tester, strain YG 5. In general, these weak reactions consisted of small, scattered clumps or a thin line of mycelia in the region of contact between the two mutants. However, the reaction of strain R-2949 with strains RN 8 or RN 9 was strong, but it occurred 1–2 wk later than with strain YG 5. These data suggest that strains belonging to VCG 03 and 04 are not always incompatible.

TABLE 2. Frequency of nitrate nonutilizing (*nit*) mutants and chlorate resistant nitrate utilizing (*crn*) mutants from 24 thiabendazole (TBZ) resistant and 18 TBZ-sensitive strains of *Gibberella pulicaris*

Phenotype	TBZ-resistant strains	TBZ-sensitive strains
Total sectors tested	173	56
crn mutants	31	9
nit 1 mutants	60	19
nit3 mutants	69	25
NitM mutants	12	3
nnu mutants	1	0

The remaining seven field strains, which had not complemented NitM testers of VCG 01, 02, 03, or 04, were designated VCG 0X (Table 1). *nit1* and *nit3* mutants of these seven strains were tested against each other in all pair-wise combinations, but complementation was not observed. There were no NitM mutants produced from any field strain in VCG 0X. This made analysis more difficult because even self-complementation between *nit1* and *nit3* mutants could be weak and inconsistent. Self-compatibility was evident in four field strains in VCG 0X, suggesting that the incompatibility of these four strains to each other and to the other VC groups was not due to the inability to form hyphal anastomoses, as has been reported for other *Fusariurm* species (26).

Geographical distribution of VCGs in North America is shown in Table 1. VCG 01 (21 strains) was the largest group and the most widespread, being found in potato tubers from Idaho to New Brunswick. VCG 03 (seven strains) was confined to North Dakota, Michigan, and Pennsylvania. VCG 02 (two strains) was unique to New Brunswick, and VCG 04 (five strains) was unique to North Dakota. Multiple VCGs (01, 03, and 04) were recovered from potatoes from one field near Grand Forks, ND.

The chronology of occurrence of VCGs among 42 strains of *G. pulicaris* is shown in Table 1 and Figure 1. Although definitive conclusions cannot be drawn from our small sample size, some trends are suggested. The two oldest strains available, which were collected from dry-rotted tubers in 1963 and 1969, were members of VCG 01, whereas VCG 03 was first collected in 1972. VCG 02 and 04, which appeared to be more restricted geographically, were also only found in more recent collections: VCG 02 in 1982 and 1985, VCG 04 only in 1990. A significant proportion (7 of 17) of the strains isolated before 1986 and obtained from culture collections were incapable of forming heterokaryons with other strains. In contrast, all strains more recently isolated from the field were vegetatively compatible with several other strains in the collection.

Heritability of TBZ resistance. To determine the heritability of TBZ resistance, field strains that differed in TBZ sensitivity were crossed. Five TBZ-resistant parents were selected on the basis of their high sexual fertility. Because all of the 24 available TBZ-resistant field strains were female-sterile, the TBZ-resistant strain served as the male parent in these crosses. When tested for radial growth inhibition in agar cultures (Table 3), all five crosses produced progeny that fell into only two parental

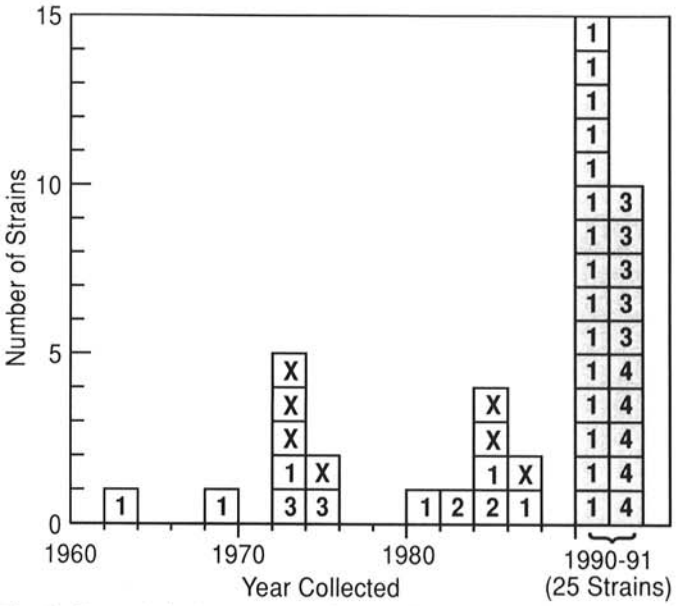

Fig. 1. Chronological occurrence of vegetative compatibility groups of *Gibberella pulicaris* in North America. Each square represents one strain, numbers inside squares indicate the vegetative compatibility group of the strain. Open squares = thiabendazole-sensitive strains. Filled squares = thiabendazole-resistant strains.

TABLE 3. Segregation of thiabendazole (TBZ) resistance[a] among random ascospore progeny from five crosses of *Gibberella pulicaris*

Cross	Parents: Female TBZ sensitive (MAT 1-2)	Parents: Male TBZ resistant (MAT 1-1)	No. of Progeny: TBZ-sensitive	No. of Progeny: TBZ-resistant	Ascospore germination %	χ^2 (1:1 ratio)
2555	64995	FND 31-5	13	5	73	3.56
2556	64995	FID 79-1	17	20	77	0.24
2558	64995	FND 5-1	17	26	90	1.88
2560	64995	FND 23-2	36	3	81	27.92[b]
2612	64995	FMI 135	15	14	91	0.03

[a] Concentration of TBZ in agar medium was 10 μg/ml.
[b] Chi-square value rejected at $P = 0.05$.

phenotype classes: resistant (ED_{50} = 30–60 μg/ml TBZ) or sensitive (ED_{50} 1–2 μg/ml TBZ). The discontinuous variation among progeny argues against polygenic control of TBZ resistance and suggests that each of the TBZ-resistant parental strains differs from strain 64995 by one gene or by closely linked genes.

Among random ascospore progeny of crosses 2555, 2556, 2558, and 2612, the 1:1 ratio of TBZ-resistant to TBZ-sensitive progeny fits the expectation for one major gene segregating for this trait (Table 3). For confirmation, tetrad analysis was performed on cross 2612. Progeny of the seven complete tetrads that were tested segregated in a 4:4 ratio (TBZ-resistant to TBZ-sensitive) (Figure 2). This result also is consistent with single-gene segregation for TBZ resistance. In cross 2560, the majority of progeny recovered were sensitive to TBZ. These data might suggest that multiple genes are required for TBZ resistance or that TBZ resistance is linked to genes that cause ascospore abortion in this cross. Tetrad analysis of crosses 2555 and 2560 was attempted, but none of the several dozen asci that were observed microscopically or dissected contained a complete set of eight mature ascospores. Genetic analyses of other traits in *G. pulicaris* have often been hampered by low sexual fertility and high frequency of ascospore abortion in many crosses (3,14,15).

TBZ-resistant field strains could not be crossed to each other to test directly for allelism of TBZ resistance because all of these field strains were female-sterile and of the same mating type. Therefore, for allelism tests, 13 TBZ-resistant MAT1-2 strains were selected from the progeny of crosses 2555, 2556, 2558, and 2560 and assessed for female fertility. Only one MAT1-2, highly female-fertile progeny was obtained—strain 2560-R-1, which was a progeny of parental strains 64995 and FND 23-2 (VCG 01 from North Dakota). Strain 2560-R-1 was used as the female parent in crosses to eight independently isolated TBZ-resistant field strains: strains YG 1 and FND 5-1 (VCG 01); strain YG 5 (VCG 03), and strain RN 6 (VCG 04) from North Dakota; strains FID 75-1 and FID 79-1 (VCG 01) from Idaho; and strains FMI 136 (VCG 01) and FMI 141 (VCG 03) from Michigan. These parental strains were selected to represent the widest range of geographical locations and VCGs. No TBZ-sensitive recombinants appeared among a combined total of 2,236 random ascospore progeny from the eight crosses. This suggests that the TBZ-resistance genes of the eight independently isolated field strains are allelic or closely linked. These data also argue indirectly that the 36:3 (TBZ-sensitive to TBZ-resistant) segregation ratio of cross 2560 does not indicate that TBZ resistance requires two or more unlinked genes. If that were so, TBZ sensitivity would have been likely among some of the progeny of strain 2560-R-1.

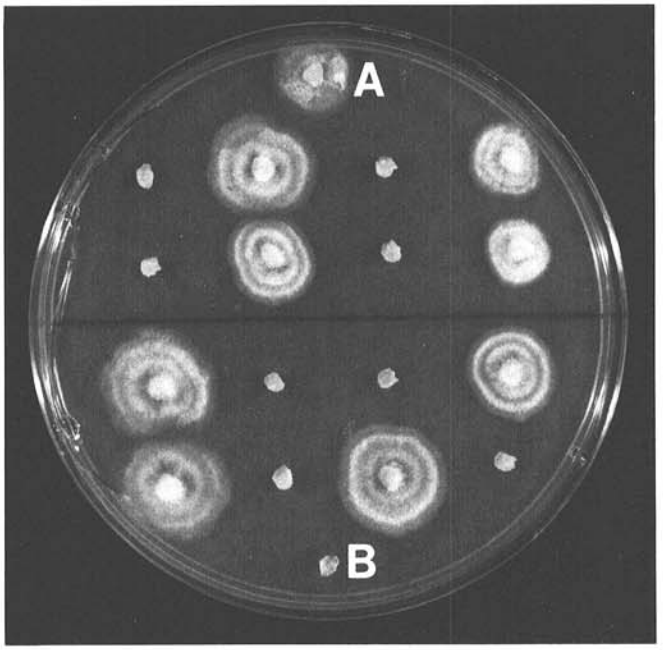

Fig. 2. Segregation of thiabendazole-resistance in two complete tetrads of cross 2612. **A,** thiabendazole-resistant parent, FMI 135. **B,** thiabendazole-sensitive parent, 64995. 10 μg/ml thiabendazole concentration in agar medium. Photo taken 3 days after inoculation.

DISCUSSION

Fusarium dry rot has a long history as a potato storage disease in North America. In 1919, Link and Gardner wrote "Taking the potato crop as a whole, *Fusarium* tuber rots caused greater losses in storage and transit than all other potato diseases combined" (28). Carpenter (8) and Bisby (4) particularly noted the isolation of a *Fusarium* species they called *F. discolor sulphureum*, which is a synonym for *G. pulicaris* (5,29), from dry-rotted potato tubers in Minnesota and the Dakotas. Throughout this century, *G. pulicaris* has continued to be of significant economic importance as a dry rot agent in North America (7). In the absence of control measures, or with adverse weather conditions, disease can be severe, as witnessed by epidemics on Prince Edward Island in 1946, 1947, and 1960 (1). The widespread cultivation of the potato and its long association with dry rot would suggest the potential for substantial genetic diversity in the population of *G. pulicaris* in North America. To the contrary, our introductory study of mating type and vegetative compatibility indicates that genetic diversity may be quite limited in this population.

Vegetative compatibility has been used extensively to analyze genetic diversity of *Gibberella* and *Fusarium* species. For example, vegetative compatibility analysis indicated a unique type for each of 25 sexually fertile strains of *G. fujikuroi* from corn and corn-based feeds from eight southeast and midwest states (27). *Gibberella zeae* from wheat head scab in Kansas was likewise very diverse: each of 24 sexually fertile strains belonged to a distinct VCG (6). Bowden and Leslie (6,26) concluded from the latter study that such high genetic diversity might indicate the occurrence of sexual recombination in the field because an outcross between strains that differ at VCG loci should produce offspring of many VCGs. It should be noted, however, that sexual recombination is apparently not necessary for the generation of a complex VCG pattern, as illustrated by the extreme diversity of the apparently asexual fungus *Aspergillus flavus* in a single cotton field in Arizona (2).

Vegetative compatibility analyses have revealed a contrasting pattern, one of limited genetic diversity, in many *Fusarium* species that have no known sexual stage. Such analyses have been most extensive for *F. oxysporum*, which is divided into forma speciales on the basis of host range. In general, vegetative compatibility of *F. oxysporum* does not occur between strains from different forma speciales (31). However, within a forma speciales, such as strains causing banana wilt (30) or melon wilt (17), individual VCGs can be geographically widespread. Local populations of *F. oxysporum* on each host plant species are often dominated by a small number of VCGs (17,30). The pattern of VCG diversity observed in the present study was similar to that reported for several asexual forma speciales of *F. oxysporum*. Specifically, in *G. pulicaris*, one VCG accounted for 50% of all strains tested. Two additional overlapping VCGs included a further 26% of all strains tested.

Current information is not sufficient to identify all the factors that affect the population structure of *G. pulicaris*, but some possibilities have been considered. For example, Gordon (16) sampled dry-rotted tubers across Canada to investigate the opportunity for sexual crosses between strains of *G. pulicaris* on potato in the field. In 1954, he reported that the two mating types never occurred together on potato and that perithecia did not form on this substrate. His findings are consistent with our observations that all sexually fertile strains collected from potatoes in the United States are of the same mating type. MAT1-2 strains were found only in New Brunswick, Canada. Most strains are female-sterile and, consequently, cannot cross sexually with each other. It is interesting to note that perithecia of *G. pulicaris* are reported to occur in nature on a wide range of trees and other woody hosts (5) and, in our laboratory, perithecia are formed in abundance only on woody substrates. Even the most highly female-fertile strains have failed to produce any protoperithecia on potato tuber tissues in the laboratory (*unpublished data*). Although our results may be affected by limited sample sizes, they suggest that the absence of sexual crossing and meiotic recombination may contribute to limiting genetic diversity of *G. pulicaris* from potato.

The population structure of *G. pulicaris* and of other potato pathogens may also be affected by potato-farming practices and the movement of seed potatoes. The importance of infected banana rhizomes in the spread of banana wilt has been well documented (30), but the movement of *G. pulicaris* via seed potatoes has received little study. In one field study, Leach (22) demonstrated that infected seed potatoes can transmit *G. pulicaris* to the surrounding soil and to daughter tubers. Undoubtedly, TBZ-resistant strains have spread by way of tubers and soil because of the long-distance movement of seed potatoes throughout North America.

Finally, VCGs with a higher level of virulence may be strongly selected for in a plant-pathogen population. For example, greenhouse pathogenicity tests have indicated a strong correlation between virulence and VCG for *Verticillium dahliae* strains isolated from potato tubers (10,18). Our laboratory virulence tests of 24 recently isolated, TBZ-resistant strains of *G. pulicaris* on potato tuber slices revealed no consistent differences in the average virulence of strains from VCG 01, 03, and 04. Further study under greenhouse and field conditions is necessary to determine whether significant and selectable differences can occur under more natural conditions.

The simple and stable VCG profile of *G. pulicaris* provides a useful baseline from which to address the biology of TBZ resistance in North America. In every case, TBZ-resistant field strains were vegetatively compatible with TBZ-sensitive field strains collected in previous years. In fact, the dominant VCG 01 of TBZ-resistant strains was present in North Dakota as early as 1963, preceding the introduction of TBZ for agricultural use. These data do not support the hypothesis that TBZ resistance originated from the introduction of a new strain of the pathogen. On the contrary, the data are consistent with the hypothesis that TBZ-resistant strains were derived by selection from indigenous TBZ-sensitive populations. Additional studies will be necessary to determine the number of VCG loci and alleles in *G. pulicaris* and to establish whether TBZ-resistant and TBZ-sensitive strains in each VCG are clones of a single parental strain or only share VCG alleles. Genetic crosses between *nit* mutants from different VCGs have been attempted but, to date, have been unsuccessful because of the poor female fertility of *nit* mutants.

Our results indicate that TBZ resistance of *G. pulicaris* is the result of a genetic mutation rather than a transient, physiological adaptation. Resistance was genetically transmitted from either a female or a male parent and was inherited as a single gene or as closely linked genes. Resistant field strains and ascospore progeny were all quite similar in their level of resistance to TBZ, and the resistance mutations of several field strains appeared to be allelic. These data indicate that TBZ resistance is likely to be the result of a mutation in a single gene. Experimentally induced resistance to benzimidazole fungicides in a number of Ascomycetes has been shown to be associated with mutations in a β-tubulin gene (11). Furthermore, field resistance of *Venturia inaequalis* to benzimidazoles was recently shown to be associated with mutations in this gene (20). Sequence comparison of tubulin genes from TBZ-resistant and TBZ-sensitive strains of *G. pulicaris* should identify the putative mutation to resistance in this species.

The high frequency and broad geographical distribution of the predominant VCGs of *G. pulicaris* also indicate that there are likely to be few incompatibility barriers to heterokaryon formation and genetic exchange between strains in the field. Thus, the potential exists for rapid spread of TBZ resistance throughout TBZ-sensitive populations already present in potatoes or in potato-field soil in North America. Such spread appears to have been the case since TBZ resistance was first discovered in 1989 (33). On the other hand, it is possible that TBZ-resistant strains were present before 1989 and were not detected because earlier surveys were too limited in sample size. Resistance to TBZ was reported in *G. pulicaris* from potato tubers in Europe by 1983 (21,35).

TBZ resistance had no obvious effect on biological fitness in preliminary laboratory tests. Resistant strains were, in general, comparable to sensitive strains in all traits analyzed, including growth rates on culture media, virulence on potato tuber slices, and production of trichothecene mycotoxins in liquid culture and in potato tubers. Whether resistant strains are competitive in the field in the absence of TBZ selection pressure remains to be tested. All our results, however, are consistent with the prediction that TBZ resistance has the potential to spread and persist in the population of *G. pulicaris* causing potato tuber dry rot in North America.

In conclusion, the widespread natural occurrence of TBZ resistance is likely to alter potato growers' practices for dry rot control. Growers should not rely solely on postharvest application or seed treatment with TBZ; they should use an integrated management program for disease control. Because most dry rot lesions develop from wounds, the most important aspect of a disease management program should be preventing tuber damage. Many grower practices have been developed to reduce wounds and other tuber injuries associated with potato harvesting and handling operations (33). The integration of fungicide use with sound crop management practices should prolong the effectiveness of TBZ for dry rot control, despite the occurrence of fungicide resistance in field strains of *G. pulicaris*.

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