Effect of Gene Disruption of Trichodiene Synthase on the Virulence of *Gibberella pulicaris*

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Gibberella pulicaris (anamorph: *Fusarium sambucinum*) accumulates the trichothecene, 4,15-diacetoxyscirpenol (DAS), a potent, host-nonspecific phytotoxin and eukaryotic protein synthesis inhibitor. To investigate the role of DAS in plant pathogenesis, we tested the virulence of five hygromycin-resistant (Hyg^r) transformants that carry a disrupted gene for trichodiene synthase (*Tox5*) and no longer produce DAS *in vitro* or *in planta*. Virulence levels of all five DAS$^-$ transformants were reduced on parsnip root slices, but high levels of virulence were retained on potato tuber slices. Tetrad analysis of one transformant, strain BC51, which carried a single copy of the transforming plasmid, resulted in either cosegregation of the Hyg^r DAS$^-$, parsnip reduced-virulence phenotypes or in the simultaneous loss of all three traits.

In contrast, all progeny from these tetrads, whether DAS$^+$ or DAS$^-$, were highly virulent on potato tubers. Moreover, the Hyg^r DAS$^-$ phenotype was recovered at high frequency from mixed populations after one 3-wk cycle in potato tubers, which suggested that loss of DAS production did not reduce competitiveness of the pathogen. We conclude that DAS accumulation increases the virulence of *G. pulicaris* on parsnip root but does not affect virulence on potato tubers. The apparent effect of the host on the importance of DAS in virulence is still unexplained, but it suggests that when assessing the role of trichothecenes in plant disease, one should be cautious in generalizing results from one plant species to another.

Although the biological activity of the trichothecene toxins is known to be inhibition of protein synthesis (McLaughlin *et al.* 1977), their specific function in the fungi that produce them is not obvious. As is common with many other fungal secondary metabolites (Bennett and Bentley 1989), trichothecenes apparently are not essential for fungal growth *in vitro*, because field strains and mutants that do not produce trichothecene grow as vigorously as trichothecene-producing strains (Beremand 1987; Beremand *et al.* 1991). Trichothecenes are potent host-nonspecific phytotoxins (Brian *et al.* 1961; Marasas *et al.* 1971; Cutler and LeFiles 1978; Cutler and Jarvis 1985; Casale and Hart 1988) and are produced by many *Fusarium* species and by other fungi that are virulent plant pathogens (Marasas *et al.* 1984). Accordingly, the trichothecenes may play a role in plant pathogenesis. There is considerable circumstantial evidence to support this hypothesis, including correlations of virulence with ability of field strains to produce trichothecenes *in vitro* (Manka *et al.* 1985; Beremand *et al.* 1991) and isolation of trichothecenes from diseased plant tissues (Marasas *et al.* 1984; Desjardins and Plattner 1989; Snidjers and Perkowski 1990). Chlorosis, necrosis, and other symptoms are produced in a wide variety of plants treated with very low concentrations of trichothecenes (10^{-5}–10^{-6} M) (Marasas *et al.* 1971; Cutler and Jarvis 1985; Kuti *et al.* 1989). On the other hand, virulence has not always been

correlated with trichothecene production *in vitro*, as was observed in one study of auxotrophic mutant strains of *Gibberella zeae* (Adams and Hart 1989).

To analyze more critically the role of trichothecenes in plant pathogenesis, we previously investigated three prototrophic *Fusarium sporotrichioides* mutant strains that were altered in trichothecene biosynthesis (Desjardins *et al.* 1989b). These three strains had been shown by complementation tests and chemical analysis to each be blocked in a different step in the complex trichothecene biosynthetic pathway and to accumulate different end products (Beremand 1987; McCormick *et al.* 1989; Plattner *et al.* 1989). Trichothecenes are synthesized from the hydrocarbon trichodiene by a series of oxygenations, esterifications, and rearrangements. The parent strain of *F. sporotrichioides* accumulated T-2 toxin, which has six oxygenations. One mutant strain accumulated trichodiene; the second accumulated calonectrin analogues with four oxygenations; and the third accumulated 4,15-diacetoxyscirpenol (DAS; Fig. 1) with five oxygenations. When each of the four strains was inoculated on parsnip (*Pastinaca sativa* L.) root slices, only the T-2 toxin producing parent strain and the DAS-producing mutant strain were highly virulent. The mutant strain that produced calonectrin analogues was weakly virulent, as was the strain that produced only trichodiene. The latter two mutant strains complemented each other to restore T-2 toxin production *in vitro* and to restore virulence, at least in part, on parsnip root. These results strongly suggested that production of certain highly oxygenated trichothecenes, such as DAS and T-2 toxin, was required for high virulence of *F. sporotrichioides* on parsnip root.

To determine if trichothecene production is a general virulence factor of *Fusarium* species, we extended our analysis to *Gibberella pulicaris* (Fr.:Fr.) Sacc. (anamorph:

F. sambucinum Fuckel). *G. pulicaris* is a cosmopolitan soil saprophyte and pathogen of many different plants, including parsnip (Desjardins *et al.* 1989a) and potato (Desjardins and Gardner 1989). Economically, *G. pulicaris* is most important as a cause of dry rot of stored potato tubers in North America and in Europe (Boyd 1972; Seppanen 1989). In previous studies (Desjardins and Beremand 1987; Beremand and Desjardins 1988; Beremand *et al.* 1991), more than 70 field strains of *G. pulicaris* were examined for their ability to produce trichothecenes in liquid culture and, in unrelated work (Desjardins and Gardner 1989; Desjardins and Plattner 1989), 31 of these strains were also evaluated for their ability to cause dry rot in potato tubers. When these two sets of data were compared, an association between high trichothecene production *in vitro* and high virulence on potato tubers was revealed; this association suggests a causal relationship. On the other hand, such quantitative correlations among field strains of different genetic backgrounds might be entirely fortuitous.

The recent cloning of a trichothecene biosynthesis gene (Hohn and Beremand 1989) and the development of a DNA-mediated transformation system in *G. pulicaris* (Salch and Beremand 1988) have allowed a more critical genetic analysis of the role of trichothecenes in pathogenesis. Mutants that do not produce trichothecene have been obtained by means of gene disruption of trichodiene synthase (*Tox5*), which catalyzes the first unique step in the trichothecene biosynthetic pathway. In this paper, we present the effects of *Tox5* gene disruption on the virulence of *G. pulicaris* on parsnip and potato. Details concerning the isolation and disruption of the *Tox5* gene from *G. pulicaris* are presented elsewhere (Hohn and Desjardins 1992).

MATERIALS AND METHODS

Cultures and genetic crosses. A strain of *G. pulicaris* from potato in Germany, R-6380, was the recipient strain for transformation. This progenitor strain was identified and supplied by P. E. Nelson from the collection of the Fusarium Research Center, Pennsylvania State University, and was reisolated from a single conidium before this study. Strain R-6380 produced DAS in liquid culture, was male- and female-fertile, and was mating type Mat1-1 (Desjardins and Beremand 1987). Cultures were grown on V8 juice agar medium (Stevens 1974) slants or plates, on an alternating 12-h, 25° C, light and 12-h, 20° C, dark schedule. For long-term storage, strains were maintained on V8 agar slants at 4 C, and selected strains were deposited in the Agricultural Research Service Collection, Peoria, IL. For all experiments, fresh transfers of the strains were obtained from stock cultures held at 4° C.

The Mat1-2 parent in genetic crosses with transformants was 1810-1-5, which is a fifth generation progeny produced by a successive series of backcrosses to strain R-6380 with selection for Mat1-2, high male and female fertility, and DAS production (Beremand 1989). Techniques for crossing strains on sterile mulberry twigs and for tetrad isolations have been described (Desjardins and Beremand 1987; Beremand and Desjardins 1988). Ascospore progeny were catalogued by a series of three numbers: the cross number, the tetrad number, and the ascospore number.

Construction of *Tox5* mutants. The plasmid used for gene disruption, pGP73-2, was derived from plasmid pUCH1, which was supplied by B. G. Turgeon and O. C. Yoder, Cornell University. Plasmid pGP73-2 contains, as a selectable marker, the *hygB* gene, which encodes hygromycin B phosphotransferase from *Escherichia coli* (Migula) Castellani and Chalmers, and a doubly truncated fragment of the trichodiene synthase gene (Hohn and Desjardins 1992). Strain R-6380 was transformed with plasmid DNA following standard methods (Turgeon *et al.* 1987) as described (Hohn and Desjardins 1992). Hygromycin-resistant colonies appeared in 3–7 days. Individual transformants were reisolated from spores and retested for hygromycin resistance before further study.

Trichothecene toxin assays. Liquid cultures were analyzed for trichothecene toxins by gas-liquid chromatography (GLC). An aliquot (2.5 ml) from a 7-day-old, 25-ml liquid culture in YEPD (yeast extract, peptone, dextrose) medium (Ueno *et al.* 1975) was extracted with 5 ml of ethyl acetate on a vortex for 90 s. The organic layer was removed and dried under a stream of nitrogen. The residue was then resuspended in 1 ml of ethyl acetate and analyzed by GLC with flame-ionization detection on a SP7100 chromatograph (Spectra Physics, San Jose, CA) fitted with a 30-m fused silica capillary column (DB1, 0.25 μm). The detection limit for DAS was equivalent to approximately 2 μg/ml in the original liquid culture.

Single-spore strains from the competition experiment described below were grown on V8 slants. Liquid YEPD medium (1 ml) in 24-well culture plates was inoculated with V8 plugs from the culture slants and was incubated for 7 days on a rotary shaker. Each culture was extracted with 2 ml of ethyl acetate and analyzed with thin-layer chromatography visualized with 4-(*p*-nitrobenzyl)pyridine and tetraethylenepentamine (Hammock *et al.* 1974).
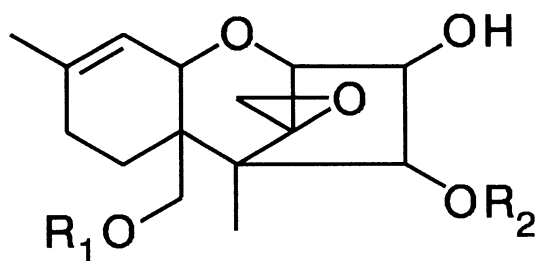
	R$_1$	R$_2$
4,15 - Diacetoxyscirpenol	Ac	Ac
15 - Monoacetoxyscirpenol	Ac	H
4 - Monoacetoxyscirpenol	H	Ac
Scirpenetriol	H	H

Fig. 1. Structures of trichothecenes.

Tissue samples from infected potato and parsnip tissues were macerated and extracted with ethyl acetate. The extract was concentrated under a stream of nitrogen and then analyzed by combined gas chromatography-mass spectroscopy (GC-MS) with a Hewlett-Packard 5979 mass selective detector (Hewlett-Packard Co., Palo Alto, CA). The detection limit for trichothecenes was equivalent to approximately 0.1 μmol/g of plant fresh weight.

Virulence assays. Virulence on potato and parsnip tissues was assessed gravimetrically as previously described (Desjardins and Gardner 1989; Desjardins et al. 1989a). Tubers of potato cultivar Russet Burbank were obtained from the University of Wisconsin, Lelale Starks Elite Foundation seed potato farm, Rhinelander, WI. Tubers of cultivar Hudson were obtained from E. Lulai, USDA Potato Research Laboratory, East Grand Forks, MN. Tubers were stored at 4° C and were brought to room temperature several hours before use. Slices (0.5–0.7 mm thick and 3 cm in diameter) were prepared aseptically from the medullary tissue of potato tubers. Three tuber slices were placed in a 10-cm plastic petri dish lined with moist filter paper. Slices were inoculated immediately by placing an inoculum plug (5 mm in diameter) at the top edge of each slice. Inoculum plugs were cut from the growing margins of cultures less than 10 days old. The petri dishes were placed in plastic bags and incubated for 6 days at 25 ± 2° C in the dark.

Parsnip roots were obtained from several local suppliers (cultivars unknown) and were peeled, washed with tap water, and surface-sterilized by dipping for 5–10 s in 95% ethanol. For transformant screening tests, roots were cut crosswise into approximately 5-mm-thick slices. To test tetrad progeny and parents, we cut root cortex tissue longitudinally into strips approximately 45 mm long, 15 mm wide, and 5 mm thick. The slices and strips were placed into petri dishes, inoculated, and incubated for 5–6 days as above, except that the inoculum was placed in the center of the parsnip tissue.

On both potato and parsnip tissues, virulent strains produced a dark brown dry rot that had a well-defined edge. Tuber and root pieces were weighed at the end of each experiment; rotted tissue was removed with a spatula, and the remaining tissue was reweighed. Virulence was estimated from the average percentage of tissue rotted for the slices or strips. In all virulence assays, the progenitor strain R-6380 was tested as a control. Virulence of this strain on potato tubers was reproducible over the course of the study, but both the mean and the standard deviation of virulence of this strain on parsnip slices varied between different batches of parsnip roots. Much of this variability in virulence appeared to be due to nonuniformity of the parsnip slices. Statistical analysis of virulence of four transformants and of strain R-6380 on parsnip slices (20 tests each) indicated a significant ($P < 0.01$) negative correlation between grams of dry rot and grams of parsnip slice, which is largely dependent on slice diameter. These data suggest that slices from larger or older parsnip roots are more resistant to G. pulicaris. More reproducible virulence was obtained with parsnip root cortex strips, which were used for analysis of tetrad progeny and parents.

Competition assay. For whole tuber assays, Russet Bur-

bank potato tubers (9–10 cm long and 5 cm in diameter) were scrubbed in running water and air-dried. Each tuber was given a shallow wound (2–3 mm) at two sites by using a disposable pipet tip, then 10 μl of conidia from a culture growing on a V8 agar plate was added to the wound. For the competition experiment, the inoculum was either 10^4 conidia of strain 2357-29-2 (DAS$^+$); 10^4 conidia of strain 2357-29-3 (DAS$^-$); a mixture (20 μl) of 10^4 conidia of each strain; or 10^4 conidia of strain 2357-29-3 followed 24 hr later by 10^4 conidia of strain 2357-29-2. Each wound was sealed with a small piece of Parafilm, and the tubers were incubated in a moist chamber in the dark at 25° C. In all treatments, dry-rot lesions were formed. After 3 wk, tubers were sliced open with a sterile knife, and several small pieces of dry rot from the periphery of each lesion were transferred to a selective medium (Nelson et al. 1983). Cultures were mass-transferred to potato-dextrose agar and carnation leaf agar and identified (Nelson et al. 1983). G. pulicaris was isolated from each lesion. Twenty-five cultures from each lesion were initiated from single conidia on carnation leaf agar: a total of 50 cultures from each single-strain inoculation test and 100 cultures from each co-inoculation test. All cultures were analyzed for hygromycin resistance at 30 μg/ml in YEPD medium. Selected Hyg^r and Hyg^s strains from each lesion were analyzed for DAS production by thin-layer chromatography of extracts of 1-ml liquid cultures as described above.

Trichothecene metabolism. Potato and parsnip slices were prepared aseptically as described for the virulence assays. Potato tuber or parsnip root slices were placed on moist filter paper in 10-cm petri dishes (two slices per dish), and 100 μl of a DAS solution (10 mg/ml in dimethyl sulfoxide) was applied to each slice. In control experiments, the plant tissues were cooked before testing. At intervals (1–72 hr), two slices were removed and placed together in a 50-ml beaker and extracted by soaking in 20 ml of ethyl acetate for 20 min. The extract was dried under a stream of nitrogen, the residue was dissolved in 1 ml of
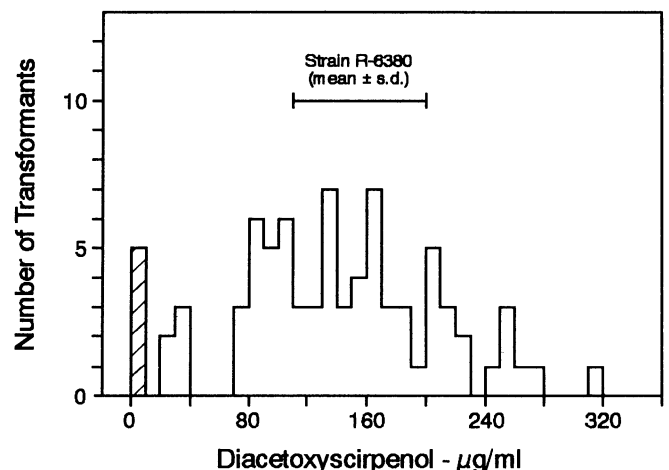

Fig. 2. Distribution of 4,15-diacetoxyscirpenol (DAS) production among 82 hygromycin-resistant transformants of *Gibberella pulicaris*. DAS was analyzed by gas-liquid chromatography as in Table 2. The range for DAS production for the progenitor strain R-6380 is shown. The hatched bar indicates the five transformants that did not produce DAS.

Table 1. Characteristics of *Gibberella pulicaris* DAS⁻ transformants

Strain no.	FRC no.[a]	Trichothecene production			Hygromycin resistance	Prototrophic growth	Integrated DNA[d]	
		Liquid culture[b]	Potato tuber[c]	Parsnip root[c]			Sites	Copies
BC51	R-9199	0	0	0	Yes	Yes	1	1
BC81	R-9200	0	0	0	Yes	Yes	1	2 (or more)
BC90	R-9201	0	0	0	Yes	Yes	1	2 (or more)
BC96	R-9202	0	0	0	Yes	Yes	1	1
BC97	R-9203	0	0	0	Yes	Yes	1	1
	R-6380	DAS	DAS[e]	DAS	No	Yes	0	0

[a] Deposit number of strain at Fusarium Research Center, The Pennsylvania State University.
[b] 4,15-Diacetoxyscirpenol (DAS) assayed by gas-liquid chromatography of extracts from 7-day liquid cultures.
[c] DAS assayed by gas-liquid chromatography-mass spectroscopy of extracts from infected tissues.
[d] Data from Hohn and Desjardins (1992).
[e] 15-Monoacetoxyscirpenol and scirpenetriol were detected in infected potato tissues.

ethyl acetate, and the amount of DAS was determined by GLC, as above. To determine the amounts of deacetylated products, the extracts were dried after initial GLC analysis; trimethyl silyl (TMS) ether derivatives were prepared with Tri-Sil/TBT (Pierce Chem. Co., Rockford, IL) and analyzed by GLC.

RESULTS

Effect of genetic transformation on DAS production in liquid culture. Hygromycin-resistant strains were obtained after transformation of *G. pulicaris* strain R-6380 with plasmid pGP73-2. The transformation efficiency (average of two experiments) was 0.2 per microgram of DNA. Transformation did not produce any visible change in phenotype; all transformants were similar to the progenitor strain R-6380 in growth rate, in nutrient requirements, and in sporulation on an agar medium.

Eighty-two randomly selected Hyg^r transformants were tested for their ability to produce DAS after 7 days of growth in liquid culture. Five transformants did not produce detectable levels of DAS or other trichothecenes by GLC or GC-MS analysis of culture extracts (Fig. 2). All five transformants were indistinguishable from the progenitor strain R-6380 in yield of fungal dry weight after 7 days of growth in liquid culture (data not shown). Each of these five potential DAS⁻ transformants was retested three or more times over a period of several months, and all continued to be completely DAS⁻ in liquid culture, suggesting that the plasmid DNA was mitotically stable in the fungal genome (Table 1).

Southern hybridization analysis of the five DAS⁻ transformants demonstrated that plasmid DNA integrated only at the host *Tox5* locus. Transformants BC51, BC96, and BC97 contained one copy of the transforming plasmid; transformants BC81 and BC90 contained two or more tandem copies (Table 1). Details of these analyses are described elsewhere (Hohn and Desjardins 1992).

Effect of *Tox5* gene disruption on virulence on parsnip root. All five DAS transformants and 10 DAS⁺ transformants were tested for their ability to cause dry rot on parsnip root slices. The 10 DAS⁺ transformants were not chosen randomly, but were selected from among transformants that appeared on the same plates of hygromycin selection medium on which DAS⁻
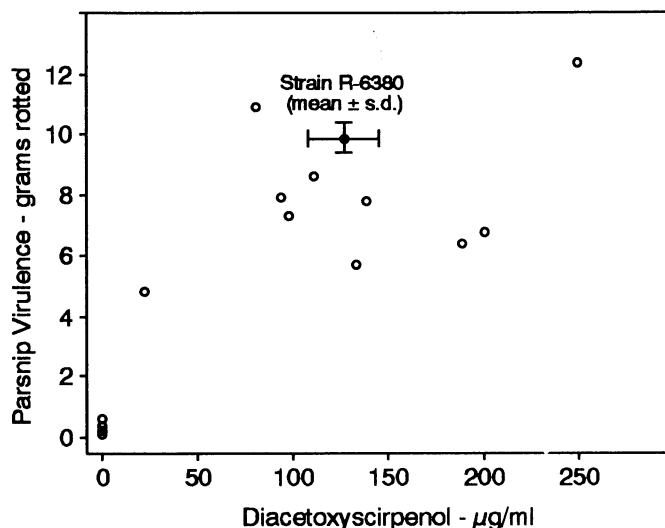

Fig. 3. The relationship between 4,15-diacetoxyscirpenol (DAS) production and virulence on parsnip root slices of 15 hygromycin-resistant transformants of *Gibberella pulicaris*. DAS was analyzed by gas-liquid chromatography as in Table 2. Virulence was assayed as grams of rotted tissue of three parsnip slices after 6 days of incubation. Each point represents one strain. The ranges for DAS production and virulence for the progenitor strain R-6380 are shown.

transformants had appeared. Virulence levels of all five DAS⁻ transformants were reduced compared to the levels of virulence of DAS⁺ progenitor strain R-6380 and the 10 DAS⁺ transformants (Fig. 3). In repeated tests using five different batches of parsnip roots, levels of virulence of all DAS⁻ transformants were always reduced compared to that of the DAS⁺ parent strain (Table 2). From batch to batch, however, there was considerable variation in the virulence of strain R-6380 and in the reduction in virulence of the transformants on parsnip root slices.

Effect of *Tox5* gene disruption on virulence on potato tubers. All five DAS⁻ transformants and 66 randomly selected DAS⁺ transformants were tested for their ability to cause dry rot of potato tuber slices. All 71 transformants tested were highly virulent on potato tubers (Fig. 4). In repeated tests, the five DAS⁻ transformants were indistinguishable from the progenitor strain R-6380 in virulence on cultivars Russet Burbank and Hudson (Table 2; Fig. 4).

Table 2. Virulence of *Gibberella pulicaris* DAS⁻ transformants on potato tuber and parsnip root

Strain no.	Normalized potato virulence[a]				Normalized parsnip virulence[b]					
	1	2	3	Mean ± SD	4	5	6	7	8	Mean ± SD
BC51	90	95	117	101 ± 14	4	22	21	21	13	16 ± 8
BC81	103	105	66	91 ± 22	4	16	16	NT[c]	NT	12 ± 7
BC90	94	112	82	96 ± 15	1	40	15	34	5	19 ± 17
BC96	97	126	76	100 ± 25	2	15	9	54	9	18 ± 21
BC97	110	105	83	99 ± 14	6	38	12	32	3	18 ± 17

[a] Mean of three tuber slices for each test, cultivar Russet Burbank for tests 1 and 2 and cultivar Hudson for test 3. Virulence of each strain was assessed after 6 days incubation and was normalized to that of strain R-6380, which was tested simultaneously. Virulence of R-6380 was 95, 73, and 68% of tuber rotted for tests 1, 2, and 3, respectively.
[b] Mean of three (tests 4–6), twenty (test 7), or five (test 8) root slices for each test. Virulence of each strain was assessed at 6 days and was normalized to that of strain R-6380, which was tested simultaneously. Virulence of R-6380 was 66, 54, 56, 28, and 32% of root rotted for tests 4, 5, 6, 7, and 8, respectively.
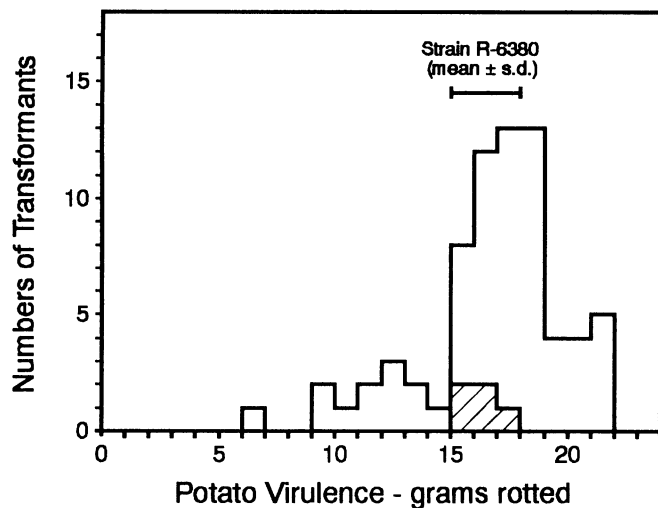[c] Not tested.

Fig. 4. Distribution of virulence on potato tuber slices of 71 hygromycin-resistant transformants of *Gibberella pulicaris*. Virulence was assayed on cv. Russet Burbank as grams of rotted tissue of three tuber slices after 6 days of incubation. The range for virulence of the progenitor strain R-6380 is shown. The hatched area indicates the five transformants that did not produce 4,15-diacetoxyscirpenol.

Table 3. Metabolism of 4,15-diacetoxyscirpenol (DAS) by potato tuber and parsnip root slices

Plant material	Time (hr)	DAS (μg)[a]	15-MAS (μg)[b]	4-MAS (μg)[b]	Scirpenetriol (μg)[b]
Potato	1	211.6	0	0	0
	19	0	116.9	0	53.5
	24	0	91.6	0	77.1
	48	0	45.4	0	75.2
	72	0	0	0	0
Parsnip	1	372.3	0	0	0
	19	281.6	0	105.8	0
	24	175.8	0	107.0	0
	48	115.0	0	110.0	55.8
	72	40.0	0	108.0	76.3

[a] Recovery determined by gas-liquid chromatography (GLC), micrograms per slice, average of two experiments.
[b] Recovery determined by GLC of trimethyl silyl ether, micrograms per slice, average of two experiments. 15-MAS = 15-monoacetoxyscirpenol.

Effect of *Tox5* gene disruption on DAS production in parsnip and potato tissues.

All five DAS⁻ transformants and the DAS⁺ progenitor strain R-6380 were tested for their ability to produce trichothecenes in parsnip root slices and in potato tuber slices. None of the five transformants tested produced DAS or any other detectable trichothecenes in parsnip or potato (Table 1). DAS was detected only in parsnip tissues infected with strain R-6380. 15-Monoacetoxyscirpenol (15-MAS) and scirpenetriol, with a lower level of DAS, were detected in infected potato tissues (Table 1). 15-MAS and scirpenetriol have not been found in pure cultures of *G. pulicaris* grown on liquid media or on autoclaved solid substrates. Consequently, their production in infected potato tubers may result from metabolism by potato enzymes. To test this hypothesis, we followed the activity of DAS applied to potato tuber and parsnip root disks over a period of 3 days (Table 3). In potato tissue, DAS was first deacetylated at C-4 to 15-MAS and then at C-15 to scirpenetriol. No DAS was detected after 19 hr, and after 72 hr no trichothecenes were recovered from the potato tissue. In parsnip tissue, the
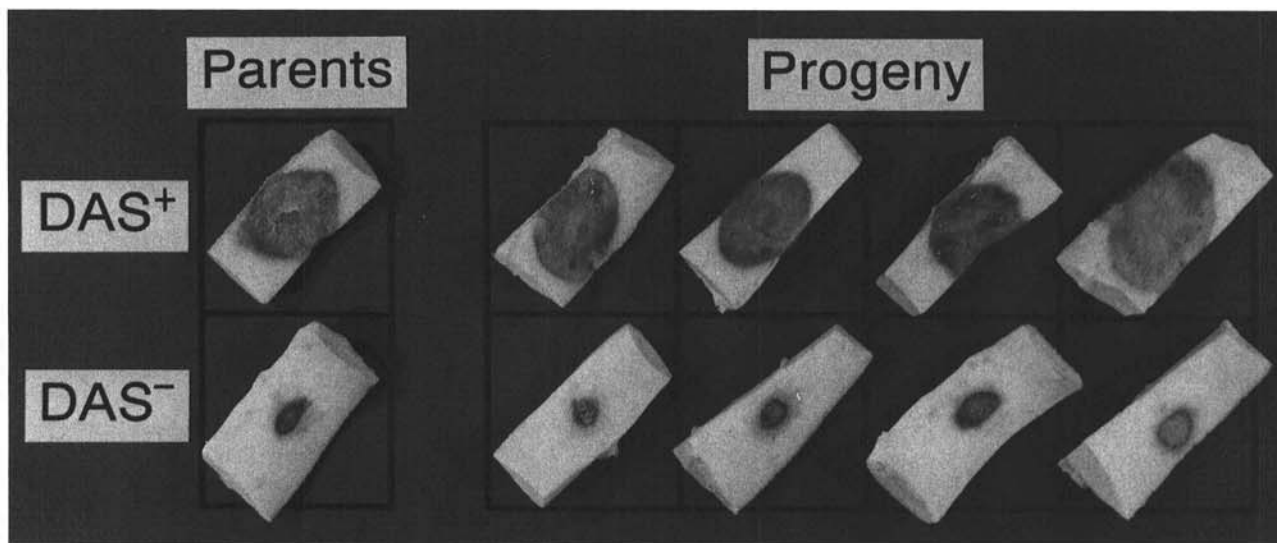
Table 4. Segregation of 4,15-diacetoxyscirpenol (DAS) production and other traits among tetrad progeny of cross 2357 (BC51 × 1810-1-5)

Tetrad no.[a]	Virulence on potato[b] High/low	Virulence on parsnip[c] High/low	Segregation ratios[d]	DAS (μg/ml) Mean ± SD (n)[e]
2	8:0	4:4	4:4	73 ± 7 (4)
3	8:0	4:4	4:4	52 ± 7 (4)
15	8:0	4:4	4:4	35 ± 17 (4)
17	8:0	NT[f]	4:4	72 ± 3 (4)
25	8:0	8:0	8:0	32 ± 16 (8)
26	8:0	4:4	4:4	42 ± 5 (4)
28	8:0	NT	4:4	66 ± 21 (4)
29	8:0	NT	4:4	54 ± 8 (4)
30	8:0	8:0	8:0	47 ± 15 (8)

[a] Strain BC51 was the male parent for tetrads 2–26, and the female parent for tetrads 28–30.
[b] Virulence on potato was assayed as in Table 2. Virulence greater than 75% of the R-6380 control was scored as high. Virulence of strain R-6380 was 95 ± 8% of potato tissue rotted (n = 3).
[c] Virulence on parsnip was assayed on root cortex strips, four per test for progeny and four to six for parents. Virulence was assessed after 5 days incubation. Virulence greater than 50% of the R-6380 control was scored as high; less than 5% of the control was scored as low. Virulence of strain R-6380 was 48 ± 11% of parsnip tissue rotted (n = 2).
[d] Hygromycin-sensitive and DAS⁺/hygromycin-resistant and DAS⁻.
[e] DAS was assayed as in Table 1; value given is the mean of all DAS-producing progeny (four or eight) in each tetrad. DAS production of parent strains was 0 μg/ml (n = 9) for BC51; 54 ± 14 (9) for 1810-1-5; and 57 ± 17 (8) for R-6380.
[f] Not tested.

Fig. 5. Segregation of 4,15-diacetoxyscirpenol (DAS) production and virulence among progeny of cross 2357 tetrad 3 (strain 1810-1-5 × strain BC51). For each strain, four parsnip root strips were inoculated and incubated for 5 days at 25° C in the dark. A representative strip is shown for each strain. Inoculum plugs were removed before photography.

rate of metabolism was much slower. The level of DAS recovered after 72 hr was less than 20% of the DAS recovered after 1 hr. Recovery of DAS from cooked potato and parsnip tissues after 72 hr was greater than 70%.

Genetic analysis of *Tox5* mutant BC51. A classical genetic analysis was conducted to test further the association of reduced virulence on parsnip with the DAS⁻ phenotype of the transformants. Strain BC51 was chosen for genetic analysis, because it contained a single-copy, single-site insertion of plasmid DNA and because it was consistently low in virulence on parsnip root. Strain BC51 could not be crossed to the progenitor strain R-6380, because both were of the same mating type (Mat1-1). Instead, strain BC51 was crossed (cross 2357) to strain 1810-1-5, a Mat1-2, DAS-producing, near-isogenic line for strain R-6380 (Table 4). Strain BC51 crossed successfully both as a male (cross 2357A) and as a female (cross 2357B) to strain 1810-1-5. From cross 2357A, 31 tetrads were isolated, 18 of which were complete (i.e., they contained seven or eight viable ascospores). Six complete tetrads were randomly selected and analyzed for hygromycin resistance, for DAS production in liquid culture, and for virulence on potato tubers. All progeny were virulent on potato tubers. In five of these tetrads (2, 3, 15, 17, and 26), four progeny were Hyg^s DAS⁺ and four progeny were Hyg^r DAS⁻ (Table 4). In one tetrad (25), all eight progeny were Hyg^s and produced DAS in amounts approximately equal to that produced by strains R-6380 and 1810-1-5 (Table 4). In cross 2357B, four tetrads were isolated, three of which contained eight ascospores. All of these tetrads (28, 29, and 30) were analyzed for hygromycin resistance, DAS production, and virulence (as above). All progeny were virulent on potato tubers. Tetrads 28 and 29 gave a 4:4 segregation of Hyg^s DAS⁺ to Hyg^r DAS⁻ phenotypes (Table 4). In tetrad 30, all eight progeny were Hyg^s and produced DAS in amounts approximate to that of the progenitor strain (Table 4). In cross 2357, hygromycin resistance was meitotically unstable in two of nine tetrads

Table 5. Competition of hygromycin-resistant and -sensitive siblings of tetrad 2357-29 in whole potato tubers

Test no.	Strains in inoculum	Number of strains recovered[a] Hyg^r : Hyg^s				
		Lesion 1	Lesion 2	Lesion 3	Lesion 4	All lesions
1	2357-29-2 (Hyg^s DAS⁺)	0:25	0:25	NT[b]	NT	0:50
2	2357-29-3 (Hyg^r DAS⁻)	25:0	25:0	NT	NT	50:0
3	2357-29-2 and 2357-29-3 (mixed)	25:0	20:5	25:0	25:0	95:5
4	2357-29-2 and 2357-29-3 (delay)	0:25	25:0	25:0	25:0	75:25

[a] Twenty-five single-spore strains were isolated from each potato lesion 3 wk after inoculation.
[b] Not tested.

analyzed. Southern hybridization analysis confirmed that, in the progeny of tetrad 2357-2, plasmid DNA cosegregated with hygromycin resistance and the DAS⁻ phenotype (Hohn and Desjardins 1992).

Inheritance of virulence on parsnip root cortex strips was investigated among progeny of six tetrads of cross 2357. Root strips were used for these experiments, because they gave more reproducible results than root slices. Among the 48 progeny from these six asci, 32 produced DAS and were high in virulence (mean 41 ± 14% of parsnip root strip rotted), whereas 16 produced no DAS and were low in virulence (mean 1 ± 1%). Levels of virulence of the parental strains 1810-1-5 and BC51 were 50 ± 27% ($n = 2$) and 1 ± 0% ($n = 2$), respectively, when tested simultaneously with progeny strains (Table 4; Fig. 5).

Competition between DAS⁺ and DAS⁻ strains in potato tubers. One Hyg^s DAS⁺ progeny (ascospore 2) and one Hyg^r DAS⁻ progeny (ascospore 3) were selected from tetrad 2357-29 to compare their ability to compete in plant tissue. Tetrad siblings were used for this study in an attempt to randomize possible negative effects of the transformation

protocol itself. We used conidial suspensions of each progeny either alone or mixed in equal numbers to inoculate wounded, whole potato tubers. Dry-rot lesions formed at all 12 wound sites. As expected, all 50 single-spore strains isolated from the two lesions on the potato inoculated with strain 2357-29-2 were Hyg^s (Table 5), and all 50 strains isolated from the two lesions on the potato inoculated with strain 2357-29-3 were Hyg^r (Table 5). From six of eight co-inoculated lesions, only Hyg^r strains were recovered. From one co-inoculated lesion, only Hyg^s strains were recovered. A mixture of sensitive and resistant strains was isolated from the eighth co-inoculated lesion (Table 5). Four to eight strains of each phenotype (Hyg^r or Hyg^s) were selected from each of the four treatment groups and analyzed by thin-layer chromatography for DAS production in liquid culture. All of the Hyg^s strains tested produced DAS, whereas all of the Hyg^r strains did not. These results, although from a small number of lesions, suggest that plasmid DNA was stable during growth in plant tissue under these conditions and that the DAS$^-$ strain was able to compete successfully with the DAS$^+$ strain in potato tuber tissue.

DISCUSSION

By inserting a plasmid containing a truncated coding region of the *Tox5* gene, we disrupted the gene encoding trichodiene synthase in the chromosome of *G. pulicaris*. Five of the 82 hygromycin-resistant transformants that were tested produced no detectable trichothecene toxins in liquid culture or in plant tissues. The virulence of the five DAS$^-$ transformants on parsnip root slices and strips was significantly reduced when compared to the virulence of progenitor strain R-6380. Furthermore, reduced virulence on parsnip root strips cosegregated with the DAS$^-$ phenotype among tetrad progeny of a cross between a DAS$^-$ transformant and a DAS$^+$ parent. These observations are consistent with our previous finding that the virulence of a UV-induced strain of *F. sporotrichioides* that did not produce trichothecenes was highly reduced on parsnip root (Desjardins *et al.* 1989b). Thus, all of the results from parsnip virulence tests are consistent with the hypothesis that trichothecene toxins can be virulence factors in some *Fusarium* diseases (i.e., they can affect "the amount or extent of disease caused") (Yoder 1980). This hypothesis could be tested further by construction of a double mutant via transformation of a DAS$^-$ hygromycin-resistant strain with a functional trichodiene synthase gene. However, this experiment must await the development of an additional selection system for *G. pulicaris*.

Trichodiene synthase, to our knowledge, is the second putative pathogenicity or virulence gene isolated from the genus *Fusarium*. The first *Fusarium* pathogenicity gene isolated was pisatin demethylase, which detoxifies pisatin, a fungitoxic compound produced by pea in response to infection. The pisatin demethylase gene was initially isolated from *Nectria haematococca* by expression in *Aspergillus nidulans* (Weltring *et al.* 1988), and the gene was subsequently used successfully to transform a pisatin-sensitive, avirulent strain of *N. haematococca* to pisatin tolerace and virulence on pea (Ciufetti *et al.* 1988).

Unexpectedly, although the virulences of all five DAS$^-$ transformants of *G. pulicaris* were reduced on parsnip root slices, they were indistinguishable from the virulence of the DAS$^+$ progenitor strain on potato tuber slices. Furthermore, transformant BC51 was as able as the progenitor strain R-6380 to cause dry rot in potato tubers (data not shown). The high frequencies of recovery of the DAS$^-$ phenotype from mixed populations in dry-rot lesions suggest that DAS$^-$ strains might be more competitive than DAS$^+$ strains in potato tissue. A similar phenomenon was well-documented in *Cochliobolus heterostrophus* (Klittich and Bronson 1986) in which presence of the *Tox1* gene reduced competitiveness or pathogenic fitness on maize in the greenhouse and in the field. A more thorough analysis of the effect of gene disruption on virulence of *G. pulicaris* is necessary and should take into account possible negative effects of the transformation protocol itself, effects of the presence of plasmid DNA, and possible rearrangements of plasmid DNA *in planta* (Dickman and Partridge 1989; Keller *et al.* 1990).

The apparent effect of the host on the importance of DAS as a virulence factor of *G. pulicaris* is an intriguing observation. Trichothecenes have been well-documented to be host-nonspecific in their toxicity and to inhibit protein synthesis in a wide range of eukaryotic organisms, including animals, fungi, and higher plants. Plant species tested to date, whether monocotyledons or dicotyledons, have proven to be very sensitive to trichothecenes *in vitro* and *in vivo* (Brian *et al.* 1961; Marasas *et al.* 1971; Cutler and LeFiles 1978; Cutler and Jarvis 1985; Adams and Hart 1989). Nevertheless, there is one rather unusual exception to this general rule, the Brazilian shrub, *Baccharis coridifolia*, which is resistant to the effects of trichothecenes and actually accumulates trichothecenes in its seed coats (Jarvis *et al.* 1988). The mechanism of *Baccharis* resistance to trichothecenes is unknown but could possibly be a trichothecene-resistant ribosomal 60s subunit, as is the case in the trichothecene-producing fungus *Myrothecium verrucaria* (Hobden and Cundliffe 1980). It is possible that protein synthesis or some other trichothecene target site in parsnip and potato cells differs in sensitivity to trichothecenes. To address this question directly, it will be necessary to compare the effect of trichothecenes on protein synthesis in cell-free preparations from parsnip root and potato tuber tissues.

Another possible explanation for the apparent host specificity of DAS as a virulence factor could be differential accumulation in parsnip and potato tissues. We have, on the contrary, observed that trichothecenes accumulate to potentially phytotoxic levels in *Fusarium*-infected tissues of both parsnip and potato. After inoculation with *F. sporotrichioides*, levels of T-2 toxin in parsnip root were 10^{-5}–10^{-6} M (Desjardins *et al.* 1989b), which is within the range known to be toxic to other plants. Comparable levels of trichothecenes accumulated in parsnips and potatoes infected with *G. pulicaris* strain R-6380 in the present study (data not shown) and in potatoes dry-rotted by eleven other field strains of *G. pulicaris* in a prior study (Desjardins and Plattner 1989).

Analysis by GC-MS has revealed one consistent difference between parsnip and potato tissues infected with *G.*

pulicaris. In parsnips infected with strain R-6380 or with DAS⁺ transformants, the only trichothecene detected was DAS. In potato dry-rot tissues, the predominant trichothecenes were 15-MAS and scirpenetriol (extracts were not analyzed for scirpenetriol in the earlier study; Desjardins and Plattner 1989). Because 15-MAS and scirpenetriol were never detected in pure cultures of *G. pulicaris* in liquid media or on solid substrates, their production in infected potato tubers is likely to have resulted from metabolism of DAS by potato enzymes. This conclusion was supported by the observation that uninfected potato tissues were able to rapidly metabolize pure DAS to 15-MAS and then to scirpenetriol. Metabolism of 15-acetyldeoxynivalenol to deoxynivalenol by corn leaf tissue was previously demonstrated by Miller *et al.* (1983).

The preceding argument suggests that deacetylation and further metabolism of DAS by potato tuber tissue may affect the importance of DAS in potato dry rot. These studies should be interpreted with caution, however, because additional studies indicate that DAS also disappears, although more slowly, after it is added to parsnip root tissues. It remains to be shown if different rates or pathways of DAS metabolism affect virulence on these different plants.

In conclusion, our results support the hypothesis that production of trichothecene toxins is important for virulence of *G. pulicaris* and *F. sporotrichioides* on parsnip roots but has no role in virulence of *G. pulicaris* on potato tubers. Additional work is needed to identify the basis of the apparent effect of the host on the importance of trichothecenes in the expression of virulence. Our findings show that, in the assessment of the role of trichothecenes in plant disease, generalizing results from one plant species to another should be done cautiously.

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