Breeding for Highly Fertile Isolates of *Nectria haematococca* MPVI that are Highly Virulent on Pea and In Planta Selection for Virulent Recombinants

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ABSTRACT

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The heterothallic ascomycete *Nectria haematococca* mating population VI (anamorph *Fusarium solani*) is a broad host range pathogen. Field isolates of this fungus that are pathogenic on pea tend to be female sterile, of low fertility, and the same mating type (*MAT-1*), whereas female fertile isolates of either mating type that are highly fertile tend to be nonpathogenic on this plant. To facilitate genetic analysis of traits that may be important in the ability of *N. haematococca* to parasitize peas, a breeding project was undertaken to produce hermaphroditic isolates of each mating type that are highly fertile and highly virulent on peas. Al-

though the association of high virulence on peas with female sterility and the *MAT-1* mating type was not completely broken, isolates with high fertility and high virulence on peas were bred within two generations. Highly virulent progeny were also isolated by an alternative method in which pea plants were inoculated with a mixture of ascospores from a cross between two moderately virulent parents. Whereas all ascospores isolated without selection in planta had lower virulence than the parents, many isolates recovered from diseased tissue were more virulent than the parental isolates. Some of the recovered isolates were shown by restriction fragment length polymorphism analysis to be genetic recombinants of the parents, demonstrating that the pea tissue selected virulent recombinants. All highly virulent isolates tested had the ability to detoxify the pea phytoalexin pisatin, again showing a link between this trait and pathogenicity on the pea.

Nectria haematococca (anamorph *Fusarium solani*) is a heterogeneous group of ascomycetes composed of self-fertile (homothallic) and self-sterile (heterothallic) groups (2). The heterothallic forms require opposite alleles at a mating type (*MAT*) locus for a fertile cross and are subdivided into seven incompatible groups called mating populations (MP) (23). Lack of genetic compatibility suggests that these morphologically similar MPs are actually different species, which is supported by analysis of rDNA sequences from representatives of the different MPs (29).

This study deals with mating population VI (MPVI) of *N. haematococca*. Although the natural occurrence of perithecia from MPVI isolates has been reported only in Japan on mulberry branches (22), the sexual cycle can be completed readily in culture (45). Therefore, the organism is amenable to conventional genetic analysis, and laboratory crosses provide a means to identify *F. solani* isolates that are members of MPVI. Although members of MPVI are best known for their pathogenicity on the pea (*Pisum sativum* L.), where they are commonly referred to as *F. solani* f. sp. *pisi*, individual members may cause disease on nine plant species and one animal species, can exist as soil-inhabiting saprophytes, and can be isolated from diseased tissue of 14 plant species of which they are not primary pathogens (45).

Genetic investigations into the mechanisms of pathogenicity of MPVI have focused primarily on diseases of garden pea. Field isolates obtained from different biological habitats vary in pathogenicity on pea and in traits thought to be related to virulence on pea. Initial investigations found a relationship between virulence

on peas and the ability to detoxify the pea phytoalexin pisatin (38) via demethylation (48), a phenotype called Pda. Genetic studies identified eight *PDA* genes (*PDA1*, 2, 3, 4, 5, 6-1, 6-2, and 7) that confer the Pda phenotype (8,14,18,27). When a cloned *PDA* gene (*PDA9*) was used in Southern analysis, it was possible to distinguish genetically characterized *PDA* genes as well as additional active and inactive members of the *PDA* gene family (8,11,19,26). It has also been shown that one of the *PDA* genes, *PDA6-1*, is on a meiotically unstable 1.6-megabase chromosome (25).

Molecular genetic transformation methods have greatly facilitated the ability to manipulate the genotypes of pathogenic fungi. The ability to do conventional genetic analysis in combination with molecular techniques, however, provides significant advantages for identifying genes involved in pathogenicity. Field isolates of *N. haematococca* highly pathogenic on peas are relatively infertile in laboratory crosses. Most can act only as males and many are of the same mating type (*MAT-1*) (43; H. D. VanEtten, unpublished data), making conventional genetic studies difficult. The goal of this study was to develop hermaphroditic laboratory strains of each mating type that were highly fertile and highly virulent on pea. This paper reports the results of a breeding program aimed at developing such laboratory strains and describes an alternate method in which the pea plant is used to select highly virulent isolates from a mixed inoculum of ascospores. As a consequence of this study, information on the genetic control of virulence, fertility, mating type, and sex was also obtained.

MATERIALS AND METHODS

Growth and maintenance of fungal isolates. Parents used in this study (Table 1) and other isolates were maintained as previously described (43). For short-term storage, cultures were grown

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on slants of V8-juice agar (M-29) (34), sealed with polyethylene tape, and stored at 4°C in the dark. For long-term storage, suspensions of spores in 50% glycerol were made and stored at -130°C (45).

Field isolates were designated with a T followed by a number (T7). Tetrad progeny were designated with three hyphenated numbers representing the cross number, ascus number, and ascospore, respectively (86-4-4). Randomly isolated ascospores were designated with two hyphenated numbers: cross number and ascospore (34-18). Single conidia isolated from plant tissue infected with an inoculum composed of ascospores from a cross were designated with cross number, a letter denoting the lesion, and spore number (352-B-10).

Crosses. Reciprocal crosses were performed as described previously (37,43) except for cross 352, for which sterile filter paper containing approximately 1 mg of linoleic acid was added to the crossed cultures to increase fertility (7). Random ascospores were collected as described previously (37).

Routine scoring of sex, mating type, and fertility of field isolates was done against ascospore isolates 6-36 (*MAT-1*) and 6-94 (*MAT-2*) (37). These isolates, which carry the white perithecial marker (the wild type is red), resulted from a cross between a colonial mutant (M-37) (46) of a field isolate obtained from infected mulberry branches and a second mulberry field isolate, T7 (43). Isolates 6-36 and 6-94 were used as reference isolates for identifying MPVI members because they are hermaphroditic (denoted as MF) and are highly fertile (rating of 4,8) when crossed with one another. These isolates were used as reference isolates because the perithecial marker allows more accurate scoring of sex

in the unknown isolate since perithecial color is a female-specific trait. Progeny were scored for fertility 4 to 6 weeks after crossing using a numerical rating system. The first rating number (1 to 4) represented a visual estimate of the number of perithecia in a 13 × 100-mm slant culture. The second rating number (5 to 9) represented a visual estimate of perithecia with a mass of ascospores oozing from the ostiole: 1 = 1 to 3, 2 = 4 to 9, 3 = 10 to 30, and 4 = >30 perithecia; 5 = 1 to 3, 6 = 4 to 9, 7 = 10 to 30, and 8 = >30 perithecia with spore masses; 9 = no spore masses (i.e., perithecia appeared to be mature but no ascospores were visible). A cross with no perithecia was scored as 0.

Virulence assay. Virulence is a quantitative measurement of disease and was determined by the test tube assay (47). To score virulence of a given isolate, five to eight plants were inoculated at a wound site on the stem and the resulting lesions were scored by measuring their length along the stem after 4 or 6 days. The inoculum consisted of an agar disk (a 3-mm disk or half of a 6-mm disk) containing mycelia either from a mycelial lawn grown for 2 days from a spore suspension or from the edge of a colony grown for 2 days from a point inoculation.

Statistical analysis of virulence on peas. The analysis described by Hawthorne et al. (9) was used to estimate the number of effective factors (*k*) and the contribution of each allele (*d*). An effective factor is defined as an independently segregating locus affecting virulence (i.e., lesion length) and will be referred to as a virulence gene. A "+" allele is assumed to contribute to virulence and a "-" allele is considered a null allele, making no contribution to virulence. The variance components for progeny within crosses were estimated by analysis of variance using the SAS program

TABLE 1. Isolates of *Nectria haematococca* mating population VI used as parents for crosses in this study

Isolate number ^a	Pda ^b	PDA ^c	Sex ^d	Mating type ^d	Perithecia color ^d	Virulence on pea (mm) ^e	Source
M37	nd ^f	nd	M	2	Red		(46)
T7	+	PDA5,PDA7 ^g	MF	1	Red	2.9 ^h	T. Matuo (SUFII-7) Japan
T10	+	PDA1,Phd ^{gi}	M	2	White	10.5 ^h	(43)
T33	+	Two ^j	MF	1	Red	3.7 ^h	Fusarium Research Center
T63	+	PDA1,Phd ^{jk}	M	1	Red	9.5 ^l	G. E. Harmon, Geneva, NY
T221	-	None ^k	M	2	Red	2.4 ^l	Fusarium Research Center
6-94	+	nd	MF	2	White	6.3 ^l	(43)
23-5	nd	nd	M	2	nd	3.5 ± 3.0 (26)	This study
23-18	nd	nd	M	2	nd	13.5 ± 3.9 (19)	This study
23-40	+	PDA5,PDA7 ^m	MF	1	White	11.0 ± 2.6 (41)	This study
23-50	nd	nd	M	2	nd	12.3 ± 4.7 (16)	This study
33-48	nd	nd	M	1	nd	8.2 ± 5.0 (8)	This study
34-26	nd	nd	M	1	nd	6.6 ± 0.5 (5)	This study
34-30	nd	nd	M	1	nd	15.3 ± 0.8 (4)	This study
34-42	nd	nd	M	2	nd	17.4 ± 0.5 (5)	This study
36-14	nd	nd	MF	2	White	11.9 ± 2.5 (13)	This study
77-5-7	+	PDA1 ⁿ	M	1	nd	10.8 ± 5.5 (137) ^o	(14)
86-4-4	+	PDA5,PDA7 ^g	MF	2	Red	9.1 ± 4.7 (187) ^o	(8)
101-17	+	nd	MF	1	Red	8.0 ± 1.5 (13)	(37)
126-91	+	nd	MF	2	Red	10.1 ± 1.7 ^p	(37)

^a M37 is a colonial mutant (45). "T" are field isolates while all others are ascospore isolates from laboratory crosses.

^b Pisatin demethylating ability (+ or -) of an isolate.

^c The PDA genotype was determined by Southern analysis using PDA-specific probes (footnotes g, i, j, k, m) and/or by segregation of the Pda phenotype (footnote n).

^d M indicates isolate can act as male; F indicates isolate can act as female in a cross with a compatible isolate. 1 = *MAT-1* allele; 2 = *MAT-2* allele. Wild type perithecia color is red, mutant phenotype is white. For male-only isolates, perithecia color genotype was deduced from perithecia color of progeny.

^e Lesion length was determined by the test tube assay. The total number of plants assayed, when that information is available, is in parentheses. Normally, up to eight plants were assayed per experiment.

^f Not determined.

^g Genotypes determined by Funnell (8); PDA genotype was determined by Southern analysis using a PDA-specific probe.

^h Mean lesion length originally reported in Sweigard and VanEtten (36).

ⁱ Phd (PDA-hybridizing DNA) indicates the presence of DNA that hybridizes with PDA probes but may not confer Pda (11); PDA genotype was determined by Southern analysis using a PDA-specific probe.

^j Contains two uncharacterized PDA genes (11,26); PDA genotype was determined by Southern analysis using a PDA-specific probe.

^k PDA genotypes determined by (11); PDA genotype was determined by Southern analysis using a PDA-specific probe.

^l Mean lesion length originally reported in VanEtten et al. (47).

^m PDA genotype was determined by Southern analysis using a PDA-specific probe.

ⁿ PDA genotype was determined by segregation analysis of the Pda phenotype.

^o Lengths of lesions on epicotyls were measured 6 days following inoculation. All others measured after 4 days.

^p Mean lesion length originally reported in Tegtmeier and VanEtten (38).

(SAS Institute, Cary, NC). Because the number of assays for each progeny of a cross varied, the average sample size was calculated according to the formula of Sokal and Rohlf (33).

In planta selection assay. Lesions were produced on pea epicotyls as described in the test tube assay, except the inoculum originated from a mixture of spores. In the initial test of the assay, inoculum was obtained from conidia of the Pda⁺ isolate 34-18 and the Pda⁻ isolate 55-8 or from mixtures of the conidia of these isolates in ratios from 1:1 (Pda⁺:Pda⁻) to 1:1000 in multiples of 1/10. Approximately 1×10^6 conidia were spread onto the surface of 20 ml of minimal agar medium (medium M-100) (34) contained in 15×100 -mm petri plates and grown for 2 days in the dark at 27°C. When ascospores from cross 352 were used as inoculum, 6 to 10 spore masses ($>10^3$ spores) from the ostioles of perithecia were collected and suspended in 200 µl of sterile water by shaking at 180 rpm for 2 to 3 h before being spread onto the surface of 12 ml of M-100 agar medium contained in 15×60 -mm petri plates. The inoculated plates were inspected under a dissecting scope to ensure that only ascospores were present and incubated for 2 days as above. Pea seedlings were grown and inoculated with plugs of this inoculum as described for the virulence assay, and lesions were measured beginning 6 days after inoculation. Stem pieces containing lesions were placed in a moist chamber at room temperature (43) until conidia appeared on the lesion surface (2 to 5 days). The conidia were collected and plated onto Martin's peptone-glucose agar medium (20), amended with ampicillin (15 mg/100 ml) and streptomycin (6 mg/100 ml), and incubated at 18°C under lights. Single-spore isolations were done by incubating the plates overnight and transferring single-germinated spores to V8-juice agar slants. For isolation of cultures from single colonies, plates

were incubated for 3 days, and then each colony was transferred to V8 juice agar plates or slants.

Assay for pisatin demethylating ability. Pisatin demethylating ability was measured by monitoring the loss of the methyl group from (3-*O*-methyl-¹⁴C)-pisatin (18). Pisatin and (3-*O*-methyl-¹⁴C)-pisatin were obtained by published protocols (35,47).

Preparation of genomic DNA. Genomic DNA was prepared either as described by Miao et al. (26) or by the following modification of the method described by Raeder and Broda (31). The amount of lyophilized, ground mycelium and volumes of solutions were increased fourfold. DNA was precipitated with 0.5 volumes of isopropanol, chilled on ice for ≥ 2 h, collected by centrifugation, and washed with 1 ml of 70% ethanol. The pellet was dissolved in Tris-EDTA (TE) buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0), and 0.1 volume of 3 M sodium acetate was added and the DNA precipitated with 2 volumes of 100% ethanol. The pellet was washed and suspended in TE buffer.

Southern analysis of genomic DNA. DNA digestion was done by standard procedures (32). Restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Restricted DNA was size fractionated by electrophoresis at room temperature in 1% agarose gels (ultraPURE agarose, Gibco BRL, Gaithersburg, MD) with $1\times$ Tris-acetate-EDTA buffer (TAE) (0.04 M Tris-acetate, 0.001 M EDTA). DNA was transferred onto Hybond N⁺ membrane (Amersham Corporation, Arlington Heights, IL), using 0.75 M sodium chloride, and 0.2 N sodium hydroxide as the denaturing and transfer buffer. Membranes were pre-hybridized, hybridized, and washed using stringent conditions (5). The probe used for *PDA* genes was the 1.35-kb *SacI* fragment from *PDA79* (*SacB*) (19). The *Nectria* repeat sequence (*NrsI-2*),

TABLE 2. Sex, mating type, and fertility of field isolates versus virulence on pea

Isolate	Habitat	Geographic location	Lesion (mm) ^a	Sex ^b	Mating type ^b	Fertility ^b
Field isolates highly virulent on peas						
Isolates available for breeding project						
T1	Pea	USA (NY)	11.1	M	1	4,7
T8	Pea	USA (NY)	12.8	M	1	4,5
T9	Pea	USA (NY)	13.3	M	2	4,7
T10	Pea	USA (NY)	10.5	M	2	3,5
T17	Pea	USA (NY)	12.7	M	1	3,5
T18	Pea	USA (NY)	9.4	M	2	4,8
T21	Pea	USA (NY)	13.9	M	1	3,5
T23	Pea	USA	14.6	M	1	4,9 ^c
T29	Chickpea	USA (CA)	13.4	M	1	4,9 ^c
T30	Pea	USA (WA)	15.4	M	1	4,7
T54	Pea	USA (NY)	9.6	M	1	4,7
T57	Pea	USA (NY)	11.8	M	1	4,5
T63	Pea	USA (NY)	9.5	M	1	4,6
Isolates obtained subsequently						
T220	Barley	USA (ID)	11.4	M	1	4,6
T295	Red clover	USA (WI)	9.6	M	2	4,7
T302	Red clover	USA (WI)	9.8	MF	1	3,7
T316	Red clover	USA (WI)	10.5	M	2	3,6
T466	Soil	Europe (UK)	22.9	M	2	4,6
Field isolates with high fertility						
Isolates available for breeding project						
T6	Mulberry	Japan	6.3	MF	2	4,8
T31	Mulberry	Japan	4.4	MF	1	4,8
T36	Potato	Japan	6.1	MF	2	4,8
T76	Alfalfa	USA (CT)	2.0	MF	1	4,8
T95	Tulip tree	USA (SC)	2.7	MF	2	4,8
T201	Corn	USA (NY)	3.6	MF	1	4,8
T204	Corn	USA (PA)	1.9	MF	2	4,8
Isolates obtained subsequently						
T296	Red clover	USA (WI)	4.2	MF	2	4,8
T301	Red clover	USA (WI)	4.0	MF	1	4,8

^a Virulence determined as in Table 1 (footnote e). Lesions were measured 4 days after inoculation except for T466 which was recorded after 8 days (Ciuffetti and D. Funnell, unpublished data). Virulence of isolates numbered T1 to T204 were originally determined by Van Etten (43) and Van Etten et al. (47).

^b Sex and MAT determined as in Table 1 (footnote d). Reference isolates 6-36 and 6-94 were used to determine sex, mating type, and fertility. Data of isolates numbered T1 to T204 originally determined by Van Etten (43) and Van Etten et al. (47).

^c Resulted in infertile crosses with reference isolates but crossed with another member of mating population VI.

provided by H. C. Kistler, is a low copy number repetitive *Hind*III DNA fragment, isolated from the *N. haematococca* field isolate T2 (12). Probes were labeled with ^{32}P -dCTP using the Prime-It II Random Primer Labeling Kit (Stratagene Cloning Systems, LaJolla, CA) to a specific activity of at least 1.5×10^8 dpm per mg.

RESULTS

Sex, mating type, and fertility of field isolates highly virulent on pea. All of the highly virulent field isolates were mated with the reference isolates, and although many induced the formation of perithecia in one of the reference isolates, they either induced the formation of perithecia lacking visible ascospore masses (a rating of 1 to 4,9) or only a few of the induced perithecia contained visible ascospore masses on their ostioles (Table 2). One exception was T18, which resulted in highly fertile crosses with tester isolates and was moderately virulent on peas (Table 2). Isolates that failed to induce the production of fertile perithecia (a 0 or 1 to 4,9 rating) when crossed with isolates 6-36 or 6-94 did not qualify as members of MPVI by our genetic criterion. Thus, isolates that exhibited this behavior were included in Table 2 only if they were able to cross with at least one other member of MPVI. All of these crosses, however, were also of low fertility (D. L. Funnell, P. S. Matthews, and H. D. VanEtten, *unpublished data*).

With one exception, all of the highly virulent isolates were also female sterile (M) (Table 2). The exception, T302, was from red clover and was less virulent than many of the isolates from diseased pea tissue (Table 2). In addition, 12 of the 18 highly virulent isolates were of the same mating type, *MAT-1* (Table 2). The tendency for field isolates that are highly virulent on peas to be male only, of one mating type (*MAT-1*), and to have low fertility in crosses prompted initiation of the breeding project. The goal of this project was to produce laboratory strains more amenable to conventional genetic analysis.

Breeding for high virulence on peas and other desired traits. The majority of the field isolates in our collection are M (approximately 74%) (43; H. D. VanEtten, *unpublished data*), but we were able to readily identify field isolates that are MF, of either mating type, and that produce highly fertile crosses with the reference isolates (Table 2 and data not shown). Although these isolates, except T18 (43; Table 2), are low in virulence on peas, they appeared to be likely sources to recombine genes for high

fertility with genes for high virulence on peas in individual progeny. Another available source of genes for high fertility was found in previously bred laboratory isolates that are female fertile (MF) (36).

Previous studies (38) indicated that the inheritance of virulence on peas is multigenic. This was particularly evident in cross 126, in which a number of progeny were more virulent than either of the low-virulence parents, suggesting a recombination of different virulence genes from both parents in these progeny (38). In addition, the apparent linkage between female sterility and mating type with virulence on peas seen in field isolates was not observed in this cross (36), suggesting this association could be broken, although the most virulent progeny were substantially less virulent than the most virulent field isolates.

To start the breeding project, all highly virulent isolates in our collection were mated with all available MF field isolates and MF laboratory progeny of opposite mating type. The objective was to obtain the most fertile combinations from which progeny having the desired traits of female fertility, high fertility, and high virulence could be identified for further crosses.

Selection for high virulence. Laboratory progeny, rather than field isolates, served as the best source of MF isolates for starting the breeding project because they frequently yielded highly fertile crosses when mated with isolates highly virulent on pea (data not shown). In the first round of crosses (level I), if a cross was fertile, approximately 10 progeny were tested for virulence on pea and more progeny from the cross were evaluated only if some of the progeny were more virulent than the parents. Of 10 crosses, cross 23 (Fig. 1) produced the highest percentage of highly virulent progeny (Fig. 2A). These were scored for sex, mating type, and fertility and served as the primary source of parent isolates for subsequent crosses (Fig. 1). Most of the highly virulent progeny from this cross were M or infertile and of the same mating type (*MAT-1*) (Table 3 and data not shown), so the number of possible crosses with these isolates was limited. One isolate (23-40), however, was MF and *MAT-2* and served as the female parent for the second round of crosses (Fig. 1). The progeny from these crosses were evaluated for virulence as above, and the crosses between sibs of cross 23 tended to produce both a greater percentage of highly virulent isolates and a larger number of progeny with a mean lesion length greater than progeny from cross 23 (Fig. 2A versus 2B and Table 4).

Fig. 1. Genealogy of crosses leading to progeny that were highly fertile and highly virulent on pea. The first isolate listed is the female (perithecial) parent and the second acted as the male. Cross 6 is from VanEtten (43) and crosses 101 and 126 from Tegtmeier and VanEtten (38).

Fig. 2. Virulence of parents and progeny from **A**, cross 23 from level I; **B**, cross 34 from level II; and **C**, cross 43 from level III of the breeding project. Virulence is indicated by lesion lengths on pea epicotyls produced in the test tube assay. Lesion lengths were measured 4 days after inoculation. Arrows indicate the lesion lengths produced by the parents and are the averages of several assays (Table 1). T10 and 23-18 are M only while all other parents are hermaphroditic. n = the number of progeny tested; $\bar{X}$ = the mean lesion length $\pm$ standard deviation of all progeny in the cross.

One further round of crosses (level III) was done using progeny from level II crosses (Fig. 1). Again, possible combinations were limited by the relative paucity of virulent MF isolates and by mating type. The mean virulence of the progeny and the percentage of highly virulent progeny were not substantially increased from those of the level II crosses (Table 4). Level III crosses, however, did produce additional highly virulent isolates (Fig. 2C) that were also highly fertile although there were no MF isolates more virulent than those identified in previous crosses. Because several highly virulent isolates that produced highly fertile crosses had been identified, further breeding was not pursued.

Inheritance of fertility, sex, and mating type and their association with high virulence. Although 6-36 and 6-94 have been routinely used to score MPVI isolates for fertility, sex, and mating type, other MF isolates (113-20 [MAT-1], 113-13 [MAT-2], 23-40 [MAT-1], and 36-14 [MAT-2]) served as tester isolates for some of the progeny in this study. These other isolates were used to potentially initiate the next level of crosses for the breeding project as well as to accommodate the possibility that the fertility exhibited by an isolate was dependent on the tester isolates employed, as had been previously observed (6). Indeed, some isolates that were crossed with more than one set of tester isolates exhibited different levels of fertility with different isolates (Table 3). For example, cross 23 progeny, 23-5, 23-18, and 23-50 (MAT-2), did not cross with 113-20 but were highly fertile (4,8) when crossed with the sibling isolate 23-40.

Because only the highly virulent progeny from each cross were scored for fertility, sex, and mating type and since different tester isolates were crossed with these progeny, conclusions about the genetic control of fertility as well as sex and mating type in these crosses must be tentative. Nevertheless, the data suggest that genetic control of fertility involves several genes. Furthermore, our

TABLE 3. Sex and mating type of laboratory progeny with high fertility and high virulence

Isolate	Lesion ^a (mm)	Sex ^b	Mating type ^b	Fertility
23-5	13.5	M	2	0,0/4,8 ^c
23-18	13.5	M	2	0,0/4,8 ^c
23-40	11.0	M/MF ^d	1	4,8/4,8 ^d
23-50	12.3	M	2	0,0/4,8 ^c
33-3	14.4	MF	1	4,8/3,7 ^e
33-45	16.2	M	2	4,8/2,6 ^f
34-5	16.4	M	1	4,8/0,0 ^e
34-6	13.5	M	1	4,8/0,0 ^e
34-18	16.0	M	1	4,8/4,7 ^e
34-19	16.0	M	1	4,8/4,8 ^e
34-25	17.8	M	1	4,8/0,0 ^e
34-26	16.6	M	1	1,9/4,8 ^e
34-30	15.3	M	1	4,8/3,6 ^e
34-42	17.4	M	1	4,8/4,7 ^e
34-50	15.5	M	1	4,8/4,8 ^e
35-2	19.0	M	1	4,7/1,5 ^e
35-10	13.8	M	1	3,7/4,8 ^e
35-17	16.4	M	1	4,8/0,0 ^e
35-25	14.2	M	1	4,8/4,6 ^e
35-30	12.0	MF	1	4,8/0,0 ^e
35-35	16.6	M	2	4,8/2,6 ^f
35-37	13.3	M	1	4,7/4,8 ^e
35-39	12.0	M	1	4,8/4,8 ^e

^a Lesion length was determined by the test tube assay. All values represent measurements made 6 days after inoculation.

^b M = isolate can act as male and F = isolate can act as female in a cross with a compatible isolate. 1 = MAT-1 allele; 2 = MAT-2 allele.

^c The first fertility score resulted from crosses with 113-20 (37); the second fertility score resulted from crosses with 23-40.

^d The first fertility and sex score resulted from a cross with 113-13 (37); the second fertility and sex score resulted from crosses with compatible siblings from cross 23.

^e Indicates that the first fertility score resulted from a cross with 6-94; the second score resulted from a cross with 36-14.

^f Indicates that the first fertility score resulted from a cross with 6-36; the second fertility score resulted from a cross with 23-40.

results suggest that continued crossing resulted in progeny with increased fertility, possibly because the progeny are genetically more similar. For example, when the 13 highly virulent progeny from cross 23 (level I) were crossed with isolates 113-20 and 113-13, only four isolates (31%) produced fertile crosses and only one was highly fertile. When highly virulent progeny from level II were crossed with the standard tester isolates, 6-36 and 6-94, all but one of the 38 isolates were fertile and 33 (or 86.5%) were scored as highly fertile.

Previous tetrad analyses showed that there are at least two genes involved in determining the female trait in *N. haematococca* (37). Results from this study are also consistent with more than one locus involved in expression of the female trait. For example, in cross 43 (Fig. 2C) both parents were MF but 9 of 23 progeny (39.1%) that produced a fertile cross were M only (data not shown). If only one gene controlled the female trait, all progeny would have the female trait.

Regardless of the number of genes controlling the female trait, the pooled data from the breeding project's most virulent progeny suggest that female sterility is associated with the trait of high virulence on pea (Table 3 and data not shown). In spite of efforts to breed for highly virulent, female-fertile isolates, there was a strong bias toward female sterility in the highly virulent progeny: 47 of the 53 highly virulent progeny (lesion lengths ≥ 10 mm) can act only as male to our tester isolates. This is consistent with what has been observed with highly virulent field isolates: 17 of 18 highly virulent field isolates examined are M only (Table 2).

In contrast with fertility and the female trait, mating type is a single-gene trait and each parent contains an opposite allele (14,18,27,37). Segregation of *MAT-1* and *MAT-2* has been consistent with equal segregation (1:1), even in crosses where there was aberrant segregation of Pda (27,37), presumably due to the loss of supernumerary chromosomes containing *PDA* genes (25). In the breeding project, 45 of 71 highly virulent fertile progeny were *MAT-1*, which was a significant deviation from the 1:1 ratio expected if mating type was segregating independently from high virulence ($\chi^2 = 5.08$; $P = 0.02420$).

Segregation of genes for virulence on pea. The estimates of k varied from 2.38 virulence genes in cross 34 to 11.37 in cross 53 (Table 4). The mean of k for all of the crosses was 5.42 ± 2.75 with a confidence interval of -0.8 to 10.92 . To estimate k , it was assumed that each gene was inherited equally and independently and that a + and a - allele existed, which were also inherited equally (21). The contribution of a + allele toward virulence, is + d and the contribution of a - allele is - d , assuming that each + allele has an additive and equal effect.

In planta selection assay. It was surmised that if a preferential growth advantage was conferred to highly virulent isolates in planta, it might allow for a more rapid means to obtain such isolates. To test this, agar plugs from cultures started with conidial mixtures (1:1 to 1:1,000) of a highly virulent Pda⁺ isolate (34-18) and a low virulence Pda⁻ isolate (55-8) were used to inoculate wounded pea epicotyls. Lesions were allowed to develop for 14 days, and the largest lesions resulting from each spore mixture were excised and the conidia produced from this tissue analyzed for Pda. Pda was used as a marker for the highly virulent isolate, and only Pda⁺ isolates were recovered from these lesions. This included 67 single colonies obtained from three lesions that resulted from the 1:1,000 (Pda⁺/Pda⁻) mixture of conidia.

Virulence of in planta selected isolates. Because the test with mixtures of high- and low-virulence conidia had shown that the virulent strain was being selected, the in planta selection approach was employed with ascospores from cross 352, in which both parents were moderately virulent (Table 1 and Fig. 3). Six days after inoculation with agar plugs containing ascospores, lesions were excised and allowed to sporulate. Eleven to 12 single conidial isolates (for a total of 59) from each of the five largest lesions produced, lesion A being the largest and E the smallest

(18.5 and 10.5 mm, respectively, measured 6 days following inoculation), were collected and virulence assays were performed on 56 of these isolates. The mean lesion lengths ranged from 2.0 to 20.6 mm (Fig. 3A), but three single conidial isolates were significantly more virulent (>17 mm) than both parents (Fig. 3A). The most highly virulent single conidial isolates were recovered from the largest pea lesions (Fig. 3A, lesions A and B). Nineteen ascospores were isolated randomly (e.g., without being selected on the plant) from a repeat of cross 352 and tested for virulence on pea. None of these randomly isolated ascospores were more virulent than the parents (Fig. 3B).

Restriction fragment length polymorphism (RFLP) analysis of PDA genes and Nrs1-2 sequences of isolates selected in planta. The difference in virulence among single conidial isolates from a given lesion (Fig. 3A, lesion A) and the recovery of three morphological mutants out of 12 isolates collected from lesion D indicated that more than one genotype and nonparental types were present in a single lesion. In order to confirm this conclusion and to determine whether there was a bias toward specific phenotypes or genotypes, the Pda phenotypes of each isolate were determined and RFLP analyses, using a *PDA* gene probe (fragment *SacB*) and a repetitive sequence (*Nrs1-2*) from *N. haematococca*, were performed on the most highly virulent isolates (lesion length ≥ 12 mm). Both parents (77-5-7 and 86-4-4) from cross 352 are Pda⁺ and contain three different *PDA* genes (Table 1). When the 19 random ascospore progeny from this cross were assayed for Pda, a ratio of 15:4 (Pda⁺/Pda⁻) was observed that is not significantly different from the expected ratio of 3:1 for two independently segregating *PDA* genes ($\chi^2 = 0.12$; $P = 0.73$) or 7:1 for three independently segregating *PDA* genes ($\chi^2 = 1.27$; $P = 0.26$). However, the 59 single conidial isolates obtained after in planta selection were all Pda⁺. To obtain a larger sample of the Pda phenotypes of the isolates selected in planta, 273 isolates obtained from single conidial colonies, including 91 single conidial colonies obtained from three small lesions (3, 4.5, and 5.5 mm, 6 days after inoculation) were assayed; 265 were Pda⁺. The combined single conidial isolates and single conidial colony isolates resulted in a Pda ratio of 324:8 (Pda⁺/Pda⁻), which was significantly different from the expected

TABLE 4. Summary of virulence from breeding project progeny

Cross ^b	n^c	Virulence (mm) ^a		Effective factors	
		Parents ^d	Progeny mean ^e	k^f	d^g
23 ^I	46	8.0 $\pm$ 1.5 11.0 $\pm$ 4.5	8.1 $\pm$ 3.1	8.18	0.83
33 ^{II}	41	11.0 $\pm$ 2.6 3.5 $\pm$ 3.0	9.3 $\pm$ 3.3	3.83	1.54
34 ^{II}	46	11.0 $\pm$ 2.6 13.5 $\pm$ 3.9	11.4 $\pm$ 3.9	2.38	2.65
35 ^{II}	33	11.0 $\pm$ 2.6 12.3 $\pm$ 4.7	10.2 $\pm$ 4.1	4.00	1.85
36 ^{II}	38	11.0 $\pm$ 2.6 10.1 $\pm$ 1.7	7.5 $\pm$ 2.4	3.53	1.12
43 ^{III}	45	11.9 $\pm$ 2.5 11.0 $\pm$ 2.6	8.3 $\pm$ 2.6	5.67	1.01
49 ^{III}	36	11.9 $\pm$ 2.5 8.2 $\pm$ 5.0	8.5 $\pm$ 3.3	8.21	0.96
50 ^{III}	35	11.9 $\pm$ 2.5 6.6 $\pm$ 0.5	10.4 $\pm$ 3.2	3.52	1.55
51 ^{III}	26	11.9 $\pm$ 2.5 15.3 $\pm$ 0.8	10.5 $\pm$ 3.3	3.53	1.74
53 ^{III}	35	11.9 $\pm$ 2.5 17.4 $\pm$ 0.5	9.2 $\pm$ 2.4	11.37	0.53

^a Virulence was based on lesion length measured 4 days following inoculation in test tube assay.

^b Cross levels I to III indicated by superscript (Fig. 1).

^c Number of progeny.

^d Mean of lesion length for parents.

^e Mean of mean lesion lengths of progeny.

^f Estimate of the number of effective factors.

^g Estimate of the contribution of each allele.

ratio of 3:1 ($\chi^2 = 90.36$; $P < 0.000005$) or 7:1 ($\chi^2 = 41.02$; $P < 0.000005$). The eight *Pda*⁻ isolates were obtained from five different lesions, six from small lesions and two from large lesions. These results indicate there is a strong bias for the plant to select for isolates with the *Pda*⁺ phenotype.

RFLP analysis of the *PDA* genes of the 17 most highly virulent progeny (lesion lengths >10 mm) verified that recombinants were present in the lesions. The female parent, 86-4-4, carries two linked *PDA* genes, *PDA5* and *PDA7*, which give *Xho*I-*Bam*HI

restriction fragments of 23 and 6.2 kb, respectively, when probed with the *PDA*-specific probe, *Sac*B (Fig. 4). The male parent, 77-5-7, carries *PDA1* (14) and gives a *Xho*I-*Bam*HI fragment of 3.2 kb (Fig. 4). Although the eight isolates from lesion B contained only *PDA1* like the parent, 77-5-7, all other isolates assayed had nonparental patterns: the five from lesion A and the one from lesion D all carried *PDA1*, *PDA5*, and *PDA7* and the three isolates from lesion C all carried *PDA1* and *PDA5* (Fig. 4; data not shown). All of the highly virulent isolates carried at least *PDA1*, and although *PDA5* and *PDA7* are linked (8), they segregated in the isolates from lesion C.

To further distinguish the genotypes of the single conidial isolates, *Hind*III-digested DNA from these isolates was probed with the repetitive sequence, *Nrs1-2*. The pattern of 77-5-7 consisted of three strongly hybridizing bands at approximately 9.6, 7.4, and 2.6 kb and a less intense band at approximately 4.1 kb, while 86-4-4 lacked these hybridizing bands (not shown). All highly virulent isolates obtained from the lesions had multiple hybridizing bands, and the five isolates from lesions A and D had nonparental patterns, as they had for the *PDA* genes (not shown). The eight isolates from lesion B, however, had the same *Nrs1-2* hybridization pattern as that of 77-5-7, although at least two of these isolates had lesion lengths significantly greater (>17 mm) than those of the parents (10.8 ± 5.5 and 9.1 ± 4.7 mm) (Fig. 3A; data not shown), indicating that they may be genetic recombinants. Although some of the isolates with similar virulence recovered from the same lesion may be clones, nonparental types and different recombinants were obtained from some of the lesions.

DISCUSSION

Some of the traits determining whether *N. haematococca* isolates can be fertile in crosses include compatible mating type alleles, the ability of at least one of the isolates to produce fruiting structures (i.e., to act as an F), and factors that determine the numbers of perithecia and viable ascospores. As observed in previous reports (14,18,27,37), the only mating type was found to be under simple genetic control (one gene with two alleles). Despite this genetic complexity, it was possible by breeding and selection to increase the number of isolates that when crossed produced a large number of perithecia with ascospores. In the first generation, relatively few (4 of 13) of the highly virulent isolates were fertile (three at a low level) when crossed with tester isolates. Since these few isolates were able to cross with one another, they served as the parents for the next generation, yielding crosses 33, 34, and 35. All of the highly virulent progeny resulting from these crosses were fertile and most (33 of 37) scored as highly fertile when crossed with tester isolates. Therefore, highly fertile (as defined by the number of perithecia produced with ascospores) and highly virulent isolates were successfully bred within two generations. A third generation of breeding did not appear to further increase fertility among high-virulence progeny, and in all three generations, the association of high virulence with female sterility and the *MAT-1* allele was not completely broken (Table 3).

Although previous studies on the virulence of *N. haematococca* on chickpeas did not reveal an obvious association between mating type, sex, or fertility (24,28), studies of the rice blast fungus, *Magnaporthe grisea*, found that rice pathogenic field isolates were female-sterile, were unable to cross with one another and, when crossed with fertile field isolates from other grasses, yielded lower numbers of viable ascospores (41). Attempts to increase the fertility of isolates with host-specific virulence were undertaken, and in one study, a backcross series between a rice pathogenic isolate with low fertility, and highly fertile isolates yielded only a few progeny that were female fertile and produced viable ascospores (42). In a second study with *M. grisea*, however, isolates that were pathogenic on rice and produced perithecia with viable ascospores when crossed with siblings of the opposite mating type were bred

Fig. 3. Virulence of in planta selected isolates versus virulence of unselected random ascospores. **A**, Virulence of single conidial isolates obtained from lesions that resulted from a mixed inoculum of ascospores from cross 352. Single conidia were isolated from individual lesions six days following inoculation of plants with germinated ascospores. All isolates were *Pda*⁺. The lesion source from which single conidia were obtained is indicated by a letter (A through E). Lengths of these lesions are A, 18.5 mm; B, 15.0 mm; C, 11.5 mm; D, 11.5 mm; E, 10.5 mm. **B**, Virulence of randomly isolated ascospores from cross 352. Virulence of the single conidial isolates and the random ascospore isolates were assayed as in Figure 2, except lesions were measured 6 days after inoculation. Arrows indicate the lesion lengths produced by the parents (77-5-7 is M only and 86-4-4 is hermaphroditic) and are the averages of several assays (Table 1); n = the number of isolates tested.

in four generations of sib selection (4). Fertility of the southern corn leaf blight fungus, *Cochliobolus heterostrophus*, as defined by perithecia number, was also increased during selective breeding over six generations (16), further demonstrating that enhanced female fertility can be obtained in plant pathogenic fungi by selection for this trait.

A study by Tegtmeier and VanEtten (38) on the inheritance of virulence indicated that one or more genes, not linked to the *PDA* gene, were required for high virulence on pea. Also, the bell-shaped frequency distributions of lesion lengths often observed with progeny from a cross (Fig. 2C, cross 43) suggest that virulence is a quantitative trait involving multiple genes. A continuum in virulence (or aggressiveness) has been described in genetic crosses with *Ustilago hordei* (3), *M. grisea* (42), and *N. haematococca* MPI (9). Such a distribution indicates that multiple genes are involved in expression of the trait and suggests that the genes involved contribute to the trait in an equal and additive manner without dominance (21). With these observed distributions as a basis, the breeding project crosses were analyzed to estimate the number of genes (effective factors [*k*]) segregating in these crosses. In the crosses analyzed, estimates for *k* were from as few as 2 to as many as 11, supporting Tegtmeier and Van Etten's initial observations (38). Several assumptions were made in estimating *k*. One assumption was that the genes involved in determining the virulence on pea segregated equally and independently. Previous results showed a linkage between virulence and *PDA* genes (8,11,15,18,26,38), and recent results (13,49) indicate that several genes required for high virulence on peas, called *PEP* (for pea pathogenicity), are linked to *PDA* (44). Therefore, in this study, an effective factor may represent a cluster of two or more virulence genes acting together in the expression of the trait. A second assumption underlying the estimation of *k* was that each gene had a + and a - allele that were inherited equally (21). With one exception, *Pda*⁻ field isolates lacked a homologous allele to the *PDA* gene (11,26), and 11 of 13 *Pda*⁻ field isolates tested lacked chromosomes in the size range of most of the *PDA*-bearing chromosomes (26). Therefore, it is possible that - alleles for at least some of the genes (i.e., *PEP* as well as *PDA* genes), or even chromosomes homologous to those carrying these genes, were absent in the parents with low virulence initially used in these crosses. It has also been shown that the loss of the *Pda* phenotype in crosses involving *PDA6-1* was, in some cases, due to loss of the entire *PDA*-bearing chromosome (25) and the loss of three *PDA* genes (*PDA1*, *PDA5*, and *PDA7*) associated with virulence on peas was due to loss of a chromosome or part of a chromosome (8). Thus, conventional segregation of + and - alleles of genes may not have occurred in the breeding project, and the loss of one or more chromosomes containing genes for virulence on pea may have resulted in an excess of progeny with low virulence. Even with the limitations in using this method to estimate the number of virulence genes, however, previous work and the results of the breeding project indicate that at least one other gene necessary for pathogenicity segregated independently from *PDA* and the genes tightly linked with *PDA*, because of the occurrence of low virulence *Pda*⁺ isolates.

When peas were inoculated with mixtures containing varying ratios of asexual spores from an isolate highly virulent on pea and an isolate low in virulence, only spores of the highly virulent isolate were recovered from the plant. This demonstrated that the pea plant could select a specific genotype from a mixed inoculum of *N. haematococca*. That the host plant could be used to select for host-specific virulence had been suggested by studies of downy mildew (*Sclerospora graminicola*) on pearl millet (39) and downy mildew (*Pseudoperonospora cubensis*) on cucurbit (40). However, these results, and ours, are in contrast to those obtained with *Cladosporium fulvum* on tomato, in which an avirulent race was always recovered from plants that were first infected with a virulent race and subsequently inoculated with the avirulent race (10).

When a mixture of ascospores from *N. haematococca* was inoculated on pea, the single conidial isolates recovered after selection in planta had a range of virulence. Nevertheless, compared with the virulence found among unselected random ascospores, there was a very strong bias toward the selection of moderately to highly virulent isolates, illustrating the competitive advantage in planta of the more virulent recombinants. Whether there was a bias toward low fertility in these recombinants was not tested.

Regardless of their virulence, nearly all of the isolates recovered from lesions inoculated with cross 352 ascospores were *Pda*⁺, even though *Pda* analysis of unselected, random ascospores indicated no bias against the *Pda*⁻ phenotype (3:1 or 7:1 *Pda*⁺/*Pda*⁻ ratios). Therefore, there was a strong selective bias for growth of *Pda*⁺ isolates in planta. Additionally, all of the highly virulent isolates recovered from these lesions were *Pda*⁺, as has been consistently observed with highly virulent ascospore progeny (8,15,18,38), suggesting that the *Pda*⁺ phenotype is required for virulence on peas. In addition, all of the most highly virulent isolates contained *PDA1*. Moreover, *PDA1* was found as the only *PDA* gene in some highly virulent isolates. Studies of crosses involving *PDA1* have also shown an absolute correlation between highly virulent progeny and the presence of the *PDA1* gene (15), and transformants that are *Pda*⁻ due to disruption of this gene are reduced in virulence (49). However, these results could account for why most of the isolates obtained from the lesions were *Pda*⁺. Wasmann and VanEtten (49), did an experiment similar to the in planta selection assay with mixtures of a gene-disrupted *Pda*⁻ transformant and its *PDA1* recipient isolate to determine whether *Pda* conferred a competitive advantage on the plant in these presumed near-isogenic isolates. The isolates were co-inoculated onto peas in 1:1 (*Pda*⁻/*Pda*⁺) and 10:1 ratios, and the recovered spores were of approximately the same ratios as the inoculum, suggesting that the *Pda*⁺ isolate could compensate for the *Pda*⁻ isolate in planta. Although all the virulent isolates recovered in the current study were *Pda*⁺, it is unlikely that *PDA* genes are the only factor allowing for the selected enrichment for *Pda*⁺ isolates but rather it is a gene or genes linked to *PDA1* in the selected isolates.

Although it is not unexpected to find that multiple genes control fertility and virulence (9,30) considering the complexity of these processes, it is less clear why genes controlling these processes seem to be affecting both traits. However, the association of high

Fig. 4. The *PDA* genotypes of highly virulent isolates selected by growth in planta. Genomic DNA from representative highly virulent single-conidial isolates obtained from lesions produced by inoculation of peas with a mixture of ascospores from cross 352 was digested with *Xho*I and *Bam*HI and hybridized with *Sac*B, a *PDA*-specific probe.

virulence with low fertility has been observed in *M. grisea* field isolates pathogenic on rice and with low female fertility of laboratory progeny highly virulent on rice (42,41). In *N. haematococca*, the existence of virulence factors for pea, or fertility factors tightly linked with virulence factors, that suppress female fertility might explain the observation that the most highly virulent progeny from the crosses described herein were also female sterile. On the other hand, factors tightly linked with a gene or genes involved in conferring the female fertility trait may modify the expression of the virulence genes resulting in suppression of virulence as had been proposed by Leung et al. (17) for *M. grisea* isolates non-pathogenic on rice. This idea presumes that *M. grisea* isolates, even those nonpathogenic on rice, have the genes necessary to successfully infect rice but that genes associated with female fertility are suppressing full expression of these virulence genes. That one of these suppressive factors may be involved directly in mating was indicated when it was observed that the alpha mating pheromone from *Saccharomyces cerevisiae* was able to suppress virulence of an *M. grisea* isolate pathogenic on rice by inhibiting formation of the appressorium, which is required for successful penetration into the host (1). Whether or not survival pressures have necessitated an interrelationship between the pathways involved in the expression of fertility and virulence can only be speculated, but it is clear that through laboratory breeding and selection, this association can be minimized so that highly virulent and fertile strains useful for further genetic analyses can be obtained.

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