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Genetic basis of host - specificity in *Sclerospora graminicola,* the pearl millet downy mildew pathogen

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ABSTRACT: The genetic basis of host specificity in *Sclerospora graminicola,* the causal agent of downy mildew in pearl millet, was studied in a host-pathogen cross-inoculation experiment. Two pathotypes, Path-1 and Path-5 selected from hybrid NHB 3 (genetically uniform) and landrace population 700651 (genetically heterogeneous), respectively, was cross inoculated under controlled conditions in a glasshouse. The pathotypes were maintained for 10 asexual generations by serial passage on the seedlings of their respective hosts. Pathogenicity test with Path-1 indicated an increase in virulence over its new host, 700651, compared with the adapted host, NHB 3. However, it was not true for Path-5 with NHB3. Of 120 RAPD primers tested for polymorphism between generations of the isolates, only two primers (I-1 and I-10) could detect variations in Path-1 and only one primer (J-18) in Path-5. The DNA fingerprinting profile of the isolates obtained after 10 generations revealed differences within the microsatellite probe $(GATA)_4$ compared with the initial generation. The change in virulence in Path-1 and its' adaptation to the new host, 700651, was demonstrated by the change in RAPDs and DNA fingerprinting profile in the two extreme generations. Implications of these results in understanding the genetic basis of emergence of new pathotypes were also discussed.

Key words: Pearl millet, *Sclerospora graminicola,* downy mildew, host-specificity

Downy mildews, as obligate biotrophs, have co-evolved with plant hosts over a period of time and have led to divergent forms of the pathogen adapted to different host taxa. *Sclerospora graminicola* (Sacc.) Schroet causes downy mildew in pearl millet [*Pennisetum glaucum* (L.) R. Br.], an important cereal crop of semi-arid tropical regions of the world. The pathogen is heterothallic, completely host-dependent (Michelmore *et al.,* 1982) and by virtue of its systemic growth, can alter plant growth and manipulate reproduction. Downy mildew has caused substantial yield losses, and several epidemics have occurred during the past 30 years resulting in withdrawal of a number of popular hybrids because of their high susceptibility (Safeeula, 1977; Singh, 1995). New hybrids have failed to give durable resistance due to emergence of new downy mildew pathotypes with matching virulence. Biological pathotyping with respect to host specificity, virulence and molecular markers as well as mapping resistance loci (Thakur and Shetty, 1993; Thakur *et al.,* 1992; Sastry *et al.,* 1995; Sivaramakishnan *et al.,* 1995; Jones *et at,* 1995) have indicated the existence of genetic variation in pathogen populations. High level of genetic variation existing in the natural populations would allow rapid selection of clones that would be resistant to fungicide or virulent on pearl millet hybrids having uniform pathotype-specific resistance.

In recent years, DNA marker techniques have been widely used for pathogen pathotyping, discriminating aggressive and non-aggressive isolates, distinguishing genotypes and identifying various fungal strains, pathotypes, and races (Hassan *et al.,* 1991). DNA fingerprinting makes use of microsatellites or simple sequence repeats (SSR) distributed in multiple tandem arrays throughout the genomes of all eukaryotes. Earlier studies on *S. graminicola* included basic genome analysis and genetic variability studies using microsatellites (Sastry *et al.,* 1995; Sastry *et al.,* 1997). The purpose of this work is to examine the changes in the host-specific pathotypes by inoculating them on non-host cultivars of pearl millet and maintaining them for 10 asexual generations on the same cultivars as well as to work out changes in the pathotypes at the molecular level using DNA fingerprinting and RAPDs as the pathotypes adapted the new host cultivars.

MATERIALS AND METHODS

The two isolates of *S. graminicola* used in this study, were originally derived from the pearl millet inbred line, 7042S (highly susceptible) and hybrid NHB3 (genetically uniform), collected from ICRISAT-Patancheru field and maintained on seedlings of 7042 S in a greenhouse. The host-specific pathotypes were derived by serial passage of sporangia obtained from 7042S to NHB3 and 700651 (heterogeneous breeding line from Nigeria) for more than 15 generations. By this time, the disease causing potential of these two isolates, Path-1 and Path-5 was stabilized on the two cultivars (Thakur *et al.*, 1992). The Path-1 caused consistently much higher level of disease on its host NHB3 (> 80% incidence) than Path-5 on 700651 (< 30% incidence). These two pathotypes were maintained as asexual propagules (sporangia) on young seedlings of their respective host genotypes in polyacrylic isolation chambers in a green house.

Seed of the two genotypes, NHB3 and 700651 were surface sterilized with 2.5% chlorax for 5 min and planted in pots (20 seeds /pot) filled with autoclaved soil-sand-farmyard manure mixture (1:1:1 by volume). The pots were kept in polyacrylic boxes to avoid cross contamination among the isolates. Seedlings at the first-leaf stage were inoculated with sporangial suspension of each pathotype as described by Thakur and Shetty (1993). The inoculated seedlings after 16 h of incubation at 20°C and >95% RH were returned to a greenhouse at 25 ± 2°C. The seedlings were then observed for downy mildew symptoms from the 4th day after inoculation to determine the latent period and the percentage downy mildew infection.

The pathotypes, Path-1 and Path-5 were also cross-inoculated on to 700651 and NHB3, respectively, and maintained for 10 asexual generations. The generations on new hosts were designated as AG_{10} (altered host generation 10). As a control, both the pathotypes were maintained on their respective original hosts for the same number of generations designated as G_{10}. Virulence and latent periods of both pathotypes were tested on alternate generations with two replications for each treatment with 40 seedlings/replication. The 10th generation (AG_{10}) fungal spores from the infected leaves of plants were used for determining the RAPD profile.

Sporangia were collected from infected sporulating leaves after overnight incubation at 20°C for DNA isolation (Sastry *et al.*, 1995). Restriction enzyme *Msp*I (NEB, USA) was used to digest 8-10 µg of genomic DNA according to supplier's instructions and electrophoresis was carried out on 1.2% agarose gel in TPE buffer (90 mM Tris-phosphate, 2 mm EDTA, pH 7.5). Gels were stained in ethidium bromide, photographed and dried in a gel dryer.

Oligonucleotides were synthesized on a gene assembler plus (Pharmacia), desalted on a NAP column (Pharmacia) and purified on 20% denaturing polyacrylamide gel. The oligonucleotide probe was 5'-end labeled by T4 polynucleotide kinase. Dried gels were denatured, neutralized and hybridized at their Tm-5°C, i.e. 35°C for $(GATA)_4$, and subjected to stringent washing as described by Schafer *et al.* (1988). The hybridized gels were exposed to x-ray films for specific periods, depending upon the intensity of the hybridization signal.

RAPD reactions were performed in a 25 µl volume containing 10 × Taq buffer (Promega), 100 µM dNTPs (NEB), 15 ng primer (10-mer Operon), 50 ng of genomic DNA and one unit Taq DNA polymerase (Promega). Amplification was carried out in a MJ Research DNA engine (PTC 200) thermal cycler programmed for 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. Amplified fragments were resolved by electrophoresis on 2% agarose gel containing 0.5 µg/ml ethidium bromide and the bands were visualized on UV transilluminator.

Data on latent period and disease incidence were subjected to analysis of variance using GENSTAT (Rothamsted Experiment Station, Harpenden, Herts AL5 2JQ, UK) to determine the significance levels of different treatments. Regression analysis was also done to determine the relationship between generations and disease incidence.

RESULTS

A significant increase in disease incidence occurred in case of Path-1 on its new host, 700651 between the first generation (21%) and the 10th generation (45%). The relationship between the asexual generations on the altered host cultivar and the disease incidence was found to be linear and significant (Fig.1a). However, a similar increase in the incidence was not recorded for Path-5 on its' new host NHB3 (Table 1). The incidence on NHB3 was significantly reduced with Path-1 obtained from its' new host, 700651 compared with that from its selected host, NHB3. This decline in the incidence had a significant negative relationship with increasing asexual generations (57% at G2 to 26% at G_{10}) (Fig 1b). There was no such change in disease incidence for Path-5 from NHB3 to its original host, 700651 at different generations. The two controls, Path-

Table 1. Downy mildew incidence and latent period of host specific pathotypes of *Sclerospora graminicola* after 10 asexual generations in a cross-inoculation study

Treatments	DM incidence (%)[1] and latent period (days)[1,2] asexual generations				
	2	4	6	8	10
Path-1 on NHB3	83 (6)	76 (5.5)	84 (5.5)	75 (6.0)	77 (5.5)
Path-1 on 700651	21 (10)	27 (8.0)	33 (8.0)	35 (7.0)	45 (6.5)
Path-1 (ex 700651) on NHB3	57 (8)	56 (8.0)	38 (8.0)	27 (6.5)	26 (6.5)
Path-5 on 700651	29 (8)	39 (7.0)	34 (7.5)	27 (7.5)	32 (7.5)
Path-5 on NHB3	36 (8)	37 (8.0)	37 (7.5)	38 (7.0)	37 96.5)
Path-5 (ex NHB3) on 700651	9 (10)	10 (8.0)	9 (8.0)	8 (7.0)	9 (8.5)
SE (m)	+ 1.9 (±0.0)	1.3 (±0.20)	3.4 (±0.27)	0.7 (±0.32)	1.5 (±0.55)
Mean	39 (8.3)	41(7.4)	39 (7.4)	35 (6.8)	37 (6.8)

[1]Mean of 2 replications; [2]Latent period values in parenthesis

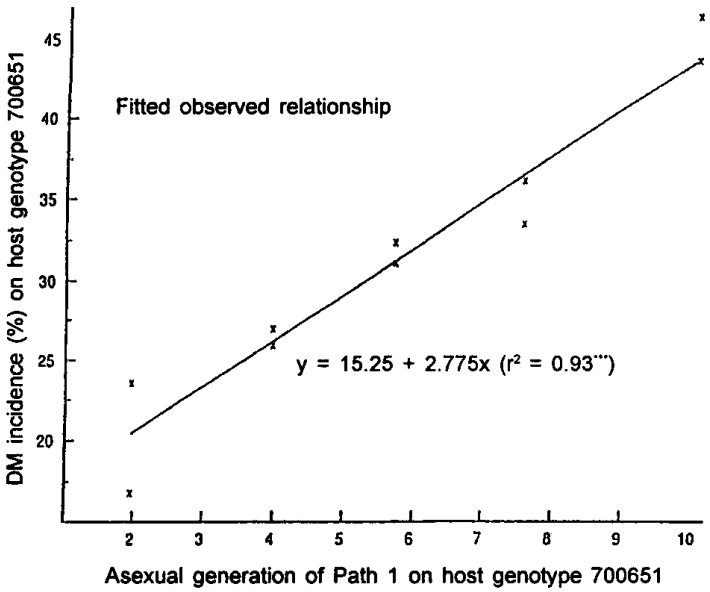

Fig. 1a

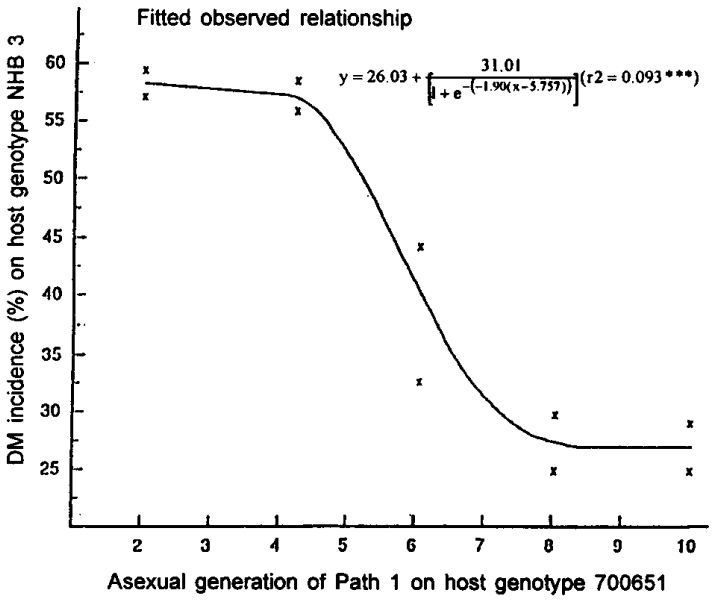

Fig. 1b

Fig.1. Adaptation of a host-specific pathotype (Path-1 on NHB3) to a new host (700651) as indicated by the regression curves of downy mildew incidence as a function of asexual generations: a = Path-1 on new host cultivar 700651; and b=Path-1 (ex 700651) on original host cultivar NHB3. Note that r^2 values are significant in both cases at 1% probability level (***)

1 and Path-5 on their respective hosts showed no significant change for the disease incidence across generations.

The latent period showed significant variation at different generations when both Path-1 and Path-5 isolates were inoculated on their new host cultivars. The latent period for Path-1 on 700651 decreased from 10 days at generation 2 (AG_2) to 6.5 days at generation 10 (AG_{10}). Similarly, the latent period for Path-5 on NHB3 decreased from 8 days at AG_2 to 6.5 days at AG_{10}. However, the pathotypes from the alternate hosts did not show any significant change in latent period on their selected hosts even after 10 asexual generations. Analysis of variance showed significant effects of treatments, asexual generations and their interaction on latent period.

Pathotypes were screened with RAPD primers before infecting the new host cultivar (generation G_0) for detecting variations at the molecular level, and after maintaining for 10 asexual generations on a new host cultivar (generation AG_{10}). The control was maintained under the same conditions on its selected host cultivar for 10 generations (G_{10}). Path-1 and Path-5 were screened with 120 oligonucleotide (Operon) primers to detect polymorphism. Two primers (I-1 and I-10) could distinguish AG_{10} and G_0 in Path-1 (Fig 2),

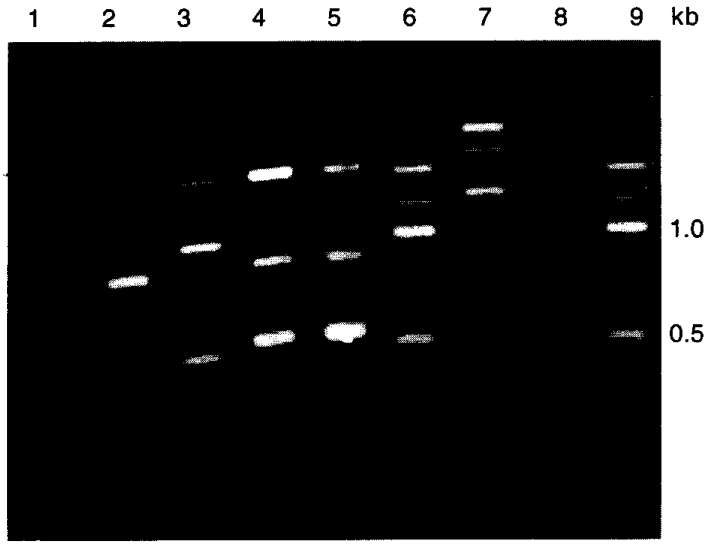

Fig.2. RAPD pattern with different generations of Path-1 and Path-5 using oligonucleotide primers J-18 (lanes 1 & 2), 1-1 (lanes 4,5) 1-10 (lanes 7 & 8)
Lane 1: Path-5 on host 700651 at Go; Lane 2: Path-5 on host NHB3 at AG_{10}; Lane 4 & 7: Path-1 on host NHB3 at Go; Lane 5 & 8: Path-1 on host 700651 at AG_{10}; Lane 3,6 & 9:100 bp ladder molecular weight markers

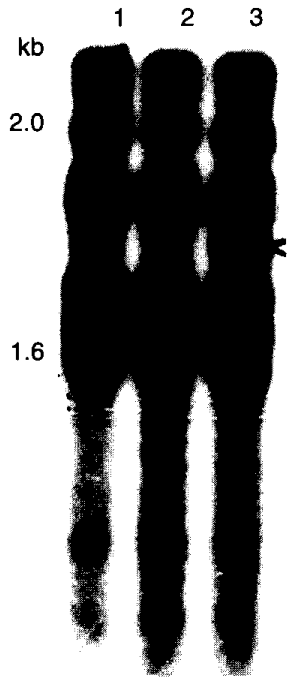

Fig.3. DNA fingerprinting with different generations of Path-1 with (GATA)4. DNA was digested with *Mspl* and DNA hybridization was carried out as described under Materials and methods.
Lane 1: Path-1 on NHB3 at G_{10}; Lane 2: Path-1 on 700651 at Go; Lane 3: Path-1 on 700651 at AG_{10}

whereas only one primer (J-18) could identify the difference between generations of Path-5 (Fig 2). The RAPD data indicated that there were additional bands in the 10th generation which were different from the common ones present in both generations.

The enzyme-probe combinations used to generate pathotype-specific individual DNA fingerprinting patterns for host-selected pathotypes (Sastry *et al.*, 1995) were tested on Path-1 and Path-5 before and after changing their host cultivars. The DNA fingerprinting pattern after maintaining Path-1 (Fig 3 Lane 3) for 10 generations on the new host 700651 (AG_{10}) showed an additional band of 1.7 KB that was absent in G_{10} (lane 2) and G_{10} (lane 1). No such change in the fingerprint pattern of Path-5 was observed before or after serial passage through the new host cultivar.

DISCUSSION

Serial advancement of generations in the case of Path-1 on its original host cultivar NHB3 did not show much change either in the latent period, or in the percent disease incidence. However, after changing the host cultivar to 700651, the latent period decreased gradually and reached a significantly lower value in the tenth generation similar to that on NHB3. On the other hand, the percent disease incidence on the new host, 700651 was initially low, but increased slowly with advancing asexual generations. When the adapted Path-1 was tested on its original host, NHB3 initial generations showed a higher disease incidence than the later generations. The data on latent period and disease incidence suggested a slow adaptation of Path-1 isolate to the new host cultivar, 700651.

NHB3, a hybrid in India, became susceptible to downy mildew in mid 1970s and was withdrawn from cultivation. This hybrid grown after a gap of 3 years in the same plot at Durgapura, Rajasthan, remained free from disease and with continuous cultivation, downy mildew susceptibility was again seen after 3-4 years (Singh and Singh, 1987). This observation is in agreement with the hypothesis of slow adaptation of Path-1 isolate to its new host, 700651.

In case of Path-5, however, the situation was different from that of Path-1 (Table 1). Path-5 isolate could adapt to the new host NHB3 very easily as revealed by almost similar latent period and disease incidence at each generation. This could be explained by the fact that Path-5 is a selection from the field population maintained on NHB-3. On the other hand, its reverting back to its host, 700651 was not that efficient although the latent period and disease incidence remained unchanged across generations. The initial adaptability of Path-5 to its new host was more efficient than Path-1 although its virulence was lower than Path-1. Thus, it

seems that a pathogen population specific to a particular cultivar adapts slowly to a new cultivar and once adapted to the new cultivar it does not efficiently and rapidly revert back to its original host. The efficiency and rapidity of adaptation, however, would depend on the genetic nature of host cultivar and the pathogen population.

The data based on RAPDs and DNA fingerprinting also suggested the genetic changes that may be associated with the adaptation of the pathogen to a new host. In plants and fungi, reports attributing functions to microsatellites are not known so far. In rice (Gupta *et al.,* 1994) and other plant species (Gortner *et al.,* 1996), it has been shown that microsatellites are transcriptionally active. The increase in the number of trinucleotide repeats, has been shown to be correlated with some genetic diseases, especially in humans. This change in fingerprint pattern may be due to an increase in the number of microsatellite repeats or point mutations in the region of recognition sequence of the restriction enzymes or primer binding sites in the case of RAPD markers. The polymorphism revealed by RAPDs (Fig 2) and appearance of a new band in the fingerprint pattern (Fig 3) need to be further tested to correlate with changes in host specificity and reduced virulence on its original host cultivar.

The emergence of a new pathotype in an asexual population need not be due to genetic recombination alone, but other factors like host cultivar-directed selection for specific virulence in a variable pathogen population could also contribute to this. Specificity could also be the outcome of a number of genetic interactions between the plant and pathogen. When a host-selected pathogen is used to infect a new host cultivar, genes that are involved in the early recognition events and virulence may undergo changes that govern the infection process. Host-race specific recognition may occur by the direct interaction of the "Avr" gene product with the corresponding plant "R' gene product (Keen, 1990). According to Flor's hypothesis, the cultivar specificity within a host species is often determined by gene-for-gene interactions. Newton and Crute (1989) put forth the genetic evidence suggesting that gene-for-gene relationships could form the basis for host species specificity. The fact that downy mildew resistance is a quantitative trait with many loci having variable levels of resistance also lends support to the view that the host adaptation is governed by the different R genes present in the host (Jones et *al.,* 1995). This study would help us in understanding the molecular mechanisms that govern host species/cultivar-race specificity and aid in developing new strategies for management of this biotic stress.

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