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Variation in pathogenicity among single-oospore isolates of *Sclerospora graminicola*, the causal organism of downy mildew in pearl millet*

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Variation in pathogenicity (virulence and aggressiveness) of various single-oospore isolates of *Sclerospora graminicola*, the causal organism of downy mildew in pearl millet, was studied. Oospore isolates were maintained as sporangial progenies on the susceptible pearl millet genotype 7042-S through asexual generations. Twenty single-oospore isolates obtained from samples of oospore inocula from three locations (the ICRISAT Centre, Coimbatore, and Hisar) in India, were tested for pathogenicity on 7042-S. All the isolates were virulent on 7042-S and they varied considerably, between and within locations, in infection efficiency (11–44% infection). The *S. graminicola* populations from the ICRISAT Centre and Hisar were significantly more aggressive than those from Coimbatore. Considerable variation was found among the 10 single-oospore isolates from the ICRISAT Centre in infection efficiency and for latent period on a set of resistant and susceptible genotypes. Seven representative isolates, two each from the ICRISAT Centre (ICOS) and Coimbatore (CBOS), and three from Hisar (HSOS), exhibited variation in infection efficiency, sporangial production capacity, and oospore production rating on the susceptible genotypes 7042-S and NHB 3. A pathogenic fitness index (PFI) was calculated for these isolates as the product of the reciprocal of the latent period × percentage infected seedlings × $\log_{10}$ sporangia per cm^2 leaf area × oospore production rating. Aggressiveness of these isolates, measured as PFI, varied greatly. CBOS-1 was the least aggressive with a PFI of 8·5 on NHB 3 and 69·6 on 7042-S; the PFI of the other isolates ranged from 81·1 to 370·5. Downy mildew resistance in three of the five known resistant lines (700651, P7-4 and 7042-R) was consistently effective against all 10 ICOS isolates, indicating that the resistance of these three lines is likely to be more stable, at least at the ICRISAT location, than that of the other two resistant lines.

INTRODUCTION

Variation in pathogenicity can be measured in terms of both virulence and aggressiveness. Pathogen isolates virulent to the same host genotypes may vary in the aggressiveness with which they infect them. Vanderplank (1968) defined 'aggressive races' as pathogenic variants that do not interact differentially with varieties of the host. Aggressiveness can be measured as pathogenic fitness or in terms of its components: latent period, infection efficiency and sporulation rate. It is greatly influenced by environmental and host factors. Aggressiveness is measured quantitatively as opposed to virulence, which is measured qualitatively.

Sclerospora graminicola, the incitant of downy mildew in pearl millet (*Pennisetum glaucum*), is an obligate biotroph which induces systemic infection in plants. Leaves of infected plants initially become chlorotic and produce asexual spores (sporangia) for several days. Later the leaves turn necrotic and sexual spores (oospores) are formed if the leaves are infected with pathogen thalli of compatible mating types (Michelmore *et al.*, 1988).

Variation in pathogenicity of *S. graminicola* populations from different locations in Africa and India has been demonstrated (Ball, 1983; Ball & Pike, 1983; Ball *et al.*, 1986). Populations from Burkina Faso, Nigeria, and Niger were generally more aggressive than those from Senegal, Zambia, or India, but no differences in aggressiveness were found between Indian and Zambian populations (Ball *et al.*, 1986). In India, variations in pathogenicity of *S. graminicola* populations were

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reported from Mysore and Gulbarga on the pearl millet cultivar HB 3 (Shetty & Ahmed, 1981); from Mysore, Aurangabad, and the ICRISAT Centre on 852B, MBH 110, and NHB 3 (ICRISAT, 1989); and from ICRISAT Centre and Durgapura on NHB 3 (Singh & Singh, 1987). All these studies tested heterogenous populations of the pathogen in the form of mass oosporic and sporangial inocula, and only one pathogenicity parameter, i.e., percentage seedling infection, was used to measure host-pathogen interactions. Therefore, variations in virulence and aggressiveness were not clearly distinguished.

In this paper we report the interactions of 15 single-oospore isolates with a set of pearl millet genotypes for various components of aggressiveness.

MATERIALS AND METHODS

Establishment of single-oospore isolates

Isolates were established from oospores of *S. graminicola* contained in leaf powder samples of pearl millet obtained from Coimbatore and Hisar (supplied by S. B. Mathur, Indian Agricultural Research Institute, New Delhi) and from the ICRISAT Centre (supplied by S. D. Singh). Plastic pots (10-cm diameter) filled with a mix of autoclaved soil and sand (1:1) were inoculated in a greenhouse by adding 10 ml suspension of oospore-bearing leaf powder in distilled water to the top 5 cm layer of the mix in each pot. The suspension was adjusted to contain approximately 50 oospores/ml. Seeds of the universally susceptible pearl millet genotype 7042-S were surface-sterilized with 2·5% NaOCl for 5 min followed by thorough washing with tap water, and were sown at 20 seeds per pot. Twenty pots were maintained for each of the three inoculum sources. Pots for each set of inoculum were kept isolated in polyethylene chambers to avoid cross-contamination among isolates, and were watered regularly. Fifteen days after inoculation only 2–3% of the seedlings showed downy mildew symptoms with each of the three inocula. With such a low frequency of infected seedlings, each infection was assumed to have resulted from a single oospore. This method and assumption for obtaining single-spore isolates has been used for various studies in *Bremia lactucae*, the causal organism of lettuce downy mildew (Ilott *et al.*, 1987; Michelmore *et al.*, 1988). We selected 20 oospore progeny lines, as sporangia, from individual infected seedlings, 10 from the ICRISAT Centre (ICOS), six from Hisar (HSOS), and four from Coimbatore (CBOS). These were evaluated for aggressiveness by inoculating seedlings of 7042-S, using sporangial inoculum.

The oospore progeny lines (isolates) were maintained on 7042-S seedlings through asexual generations. Infected plants of each isolate were kept separately within individual polyethylene chambers in the greenhouse. Each isolate was inoculated onto a fresh set of 7042-S seedlings, once a month, with sporangia from the previous generation. Plants were kept free from insect pests and were adequately fertilized and watered. The sporangial inocula for experiments were collected from these plants.

Inoculum and inoculation method

On the evening before the inoculation day, infected leaves from individual isolates were collected and washed free of old sporangial growth using wet cotton swabs under running tap water. The leaves were kept, with their abaxial surfaces up, in plastic-tray humidity chambers lined with wet blotting paper and incubated at 21 °C for 6 h, after which the temperature was lowered to 2 °C until sporangial collection the next morning. The sporangia were collected from each isolate separately, in ice-cold distilled water, using a small paint brush, and the sporangial concentration was adjusted to 1×10^5 sporangia/ml.

Seedlings were inoculated at the first leaf stage (3 days after emergence) by injecting 5 μl of inoculum into the base of the unfurling leaf blade with an injection syringe and needle. Inoculated seedlings were incubated inside a polyethylene-lined chamber sprayed with water to maintain high relative humidity (>90%) at 21 °C for 24 h. Pots were later moved to a greenhouse bench at 25 ± 2 °C. To determine the latent period, seedlings were observed daily from the 4th day after inoculation for the appearance of systemic symptoms. Numbers of infected seedlings and total seedlings per pot were recorded 15 days after inoculation to determine the infection efficiency (infection %).

Estimation of sporangial production capacity

The number of sporangia produced per unit area of infected leaves was determined. Samples were taken from young infected leaves of five seedlings from each treatment 35–40 days after inoculation. Leaf samples of approximately 10–25 cm^2

area were incubated for sporulation. Five uniformly sporulating leaf samples were selected, and each sample was put into a separate glass test tube containing 30 ml distilled water and thoroughly shaken with a vortex mixer to dislodge sporangia. Leaf samples were removed and two drops of lactophenol were added to each test tube to inhibit zoospore release. The tubes were sealed with parafilm and stored at 4 C till sporangial counts were made. Leaf areas of individual leaf samples were measured on a LI-COR leaf area meter (Delta-T Devices, Burwell, Cambridge, England). Sporangial counts were made using a haemocytometer. The total number of sporangia/ml of suspension and the number of sporangia produced per cm^2, based on the leaf area of the given sample, were calculated. Sporangial production capacity was computed as $\log_{10}$ sporangia/cm^2.

Determination of oospore production rating

Necrotic leaf tissue from infected leaves was collected 30–50 days after inoculation, dried in the shade in brown paper bags, and stored at 10 C until observation. Leaf pieces, 2–4 cm long, were surface-sterilized with NaOCl (2·5%), washed and incubated at 40 C in NaOH (5%) for 12–16 h to clear the tissues. At least five leaf pieces per treatment were examined under a microscope for the presence of oospores. Oospore production rating was scored on a 1–4 rating scale (where 1 = no oospores, 2 = 1–10 oospores/cm^2, 3 = 11–20 oospores/cm^2, and 4 = > 20 oospores/cm^2 of leaf).

Experiment 1

In a greenhouse, the 10 ICOS isolates were inoculated on a set of five downy mildew resistant (700651, 7042-R, 700516, P7-4 and SDN 503) and one susceptible (7042-S) pearl millet genotypes (Singh *et al.*, 1990). The experiment was conducted in a completely randomized block design with 10 isolates × six host genotypes × three replicates with 80 seedlings per replicate. Seedlings were inoculated with a sporangial suspension of each isolate and incubated in the dark for 24 h, as described above; the pots were then moved to a greenhouse bench. Mean infection efficiency and latent period were determined for each replicate, as described before. ANOVA was computed to determine significant differences and interactions.

Experiment 2

Seven single-oospore isolates, two each of ICOS and CBOS, and three of HSOS, were inoculated on two resistant (700651, 7042-R) and two susceptible genotypes (7042-S, NHB 3). The experiment consisted of seven isolates × four host genotypes × three replicates in a completely randomized design. Thirty seedlings were inoculated in each replicate with each isolate, as described above. Observations on infection efficiency were taken 15 days after inoculation and appropriate statistical analyses were performed.

On the susceptible genotypes, the seven isolates were also evaluated for their relative pathogenic fitness parameters, such as latent period, infection efficiency, sporulation capacity, and oospore production according to the procedures described above. For each isolate × susceptible genotype combination, a PFI was calculated as:

$$PFI = (b \times c \times d)/a$$

where a = latent period (days), b = percentage infection, c = sporangial production capacity ($\log_{10}$ sporangia/cm^2), and d = oospore production rating.

RESULTS

Considerable variation was found among 20 single-oospore isolates of *S. graminicola* from three Indian locations with infection ranging from 11 to 44% on 7042-S (Table 1). Variation in aggressiveness was clearly evident among isolates from within and between locations. Isolates ICOS-3, ICOS-12 and HSOS-6 were significantly more aggressive than some of the isolates across locations. The population mean of infection efficiency across isolates at each location showed the ICRISAT Centre (29·2%) and Hisar (25·5%) populations to be significantly more aggressive than that of Coimbatore (16·2%).

The infection efficiency of isolates varied greatly with different host genotypes. *F* values were highly significant ($P < 0{\cdot}001$) for isolates, host genotypes, and isolate × host genotype interaction (Table 2). On two resistant genotypes, 700651 and 7042-R, variation was minimal and the isolates could not be distinguished (Table 3). On the other three resistant genotypes there were significant differences among isolates for infection efficiency. ICOS-3 was most aggressive (23% infection) and ICOS-2 least aggressive (5% infection) on 700516; ICOS-13 was most aggressive (8% infection), and ICOS-3 and ICOS-5 least aggressive (< 1% infection) on P7-4; ICOS-6 was

Table 1. Infection efficiency (infection %) of 20 single-oospore isolates of *Sclerospora graminicola* from the ICRISAT Centre (ICOS), Hisar (HSOS), and Coimbatore (CBOS) on a pearl millet genotype 7042-S in a greenhouse experiment

Location	Isolate designation	Infection (%)[a]	Mean infection (%)
ICRISAT Centre	ICOS-2	23	29·2
	ICOS-3	38	
	ICOS-4	29	
	ICOS-5	23	
	ICOS-6	34	
	ICOS-7	21	
	ICOS-8	31	
	ICOS-11	20	
	ICOS-12	37	
	ICOS-13	36	
Hisar	HSOS-1	24	25·2
	HSOS-2	17	
	HSOS-3	23	
	HSOS-4	31	
	HSOS-6	44	
	HSOS-7	14	
Coimbatore	CBOS-1	13	16·2
	CBOS-2	19	
	CBOS-4	22	
	CBOS-5	11	
LSD ($P \leqslant 0{\cdot}05$)		16·2	7·1

[a] Based on 65 seedlings in each of the two replications.

Table 2. Analysis of variance for infection efficiency (infection %) of single-oospore isolates from the ICRISAT Centre

Source of variation	d.f.	Mean square	*F* value
Replication	2	1·14	—
Isolate	9	71·91	6·93***
Genotype	5	3835·90	369·79***
Isolate × genotype	45	42·86	4·13***
Error	118	10·37	

*** Significant at $P \leqslant 0{\cdot}001$.

most aggressive (14% infection) and ICOS-11 least aggressive (1% infection) on SDN 503 (Table 3). On 7042-S, infection varied from 22% for ICOS-7 to 42% for ICOS-12.

Latent periods varied considerably for various isolate-genotype combinations (Table 3). Generally, latent periods were longer for isolate-genotype combinations with the lower infection efficiency, but there were some exceptions. For example, ICOS-7 had a latent period of just 7 days on 700651, even though it infected fewer than 1% of the seedlings, and ICOS-8 had a latent period of 15 days on 700516, although it infected 15% of the seedlings. On the susceptible genotype 7042-S, all isolates had a latent period of 6 days, which was less than that of any isolate on a resistant genotype.

In the second experiment, with seven isolates, *F* values (Table 4) for isolates, genotypes, and genotype × isolate interactions were highly significant ($P < 0{\cdot}001$). Only isolates HSOS-2 and HSOS-4 were virulent on 7042-R (Table 5). All seven isolates were virulent on 700651, NHB 3, and 7042-S, but they were less aggressive on 700651 (1–8% infection) than on NHB 3 (7–92% infection). Isolate HSOS-2 was less aggressive than HSOS-4 on 7042-S and it was also significantly less aggressive than all but CBOS-1 on NHB 3. However, there were significantly differ-

Table 3. Infection efficiency (infection % = IP) and latent period (LP) of 10 single-oospore isolates of *Sclerospora graminicola* from the ICRISAT Centre (ICOS) on six host genotypes[a]

	700651		700516		P 7-4		SDN 503		7042-R		7042-S	
Isolate	IP	LP	IP	LP	IP	LP	IP	LP	IP	LP	IP	LP
ICOS-2	1	15	5	9	3	11	8	8	1	15	23	6
ICOS-3	2	9	23	9	< 1	15	4	9	0	—[b]	35	6
ICOS-4	< 1	15	6	8	1	12	3	8	0	—	30	6
ICOS-5	0	—	14	8	< 1	11	6	7	1	10	23	6
ICOS-6	< 1	9	14	7	5	10	14	7	0	—	34	6
ICOS-7	< 1	7	7	9	4	8	5	8	0	—	22	6
ICOS-8	0	—	15	15	2	9	6	9	0	—	31	6
ICOS-11	1	7	6	8	5	10	1	9	0	—	23	6
ICOS-12	< 1	12	13	8	1	10	6	7	0	—	42	6
ICOS-13	0	—	10	8	8	10	7	8	< 1	15	36	6
LSD ($P < 0.05$)	2·1		5·5		3·0		4·2		1·3		10·8	

[a] Mean based on 80 inoculated seedlings in each of three replications, observations taken 15 days after inoculation.
[b] No infection.

Table 4. Analysis of variance for infection efficiency (infection %) of seven single-oospore isolates on four pearl millet genotypes

Source of variation	d.f.	Mean square	*F* value
Replication	2	84·24	
Genotype	3	26510·69	803·553***
Isolate	6	929·56	28·175***
Genotype × isolate	18	733·94	22·246***
Error	54	32·99	

*** Significant at $P < 0.001$.

Table 5. Infection efficiency (infection %) of seven single-oospore isolates of *Sclerospora graminicola* obtained from three Indian locations on four pearl

	Infection (%)[a] on genotype			
Isolate[b]	700651	NHB 3	7042-R	7042-S
ICOS-5	3·2	92·2	0·0	38·1
ICOS-12	1·1	91·1	0·0	50·0
HSOS-2	5·2	74·9	3·3	25·2
HSOS-4	5·4	88·0	2·0	46·9
HSOS-6	7·8	90·9	0·0	42·1
CBOS-1	2·3	7·2	0·0	35·6
CBOS-4	2·0	91·3	0·0	36·5
LSD ($P \leqslant 0.05$)	6·0	8·8	2·8	17·5

[a] Mean based on 30 inoculated seedlings in each of three replications, observations recorded 15 days after inoculation.
[b] Isolates derived from oospores collected from ICRISAT (ICOS), Hisar (HSOS) or Coimbatore (CBOS).

ent levels of infection on NHB 3 between the two CBOS isolates. Isolate CBOS-1 was the least aggressive on NHB 3 with only 7% infection, while the other six isolates caused 75–92% infection.

The PFI and aggressiveness parameters (latent period, infection efficiency, sporangial and oospore production ratings) of the seven isolates on two susceptible genotypes are presented in Table 6. Latent periods for all the isolates were the same (5 days) on genotypes 7042-S and NHB 3. The sporangial production capacity of CBOS-4 on 7042-S was significantly higher than that of CBOS-1 and ICOS-5, but not different from the other isolates. On NHB 3, the sporangial production capacity of HSOS-4 was significantly greater than that of HSOS-2. The oospore production rating varied considerably with the highest rating of 3·6 for HSOS-2 on 7042-S, and 4·0 for ICOS-12 on NHB 3. Generally, there was greater variation of oospore production ratings among isolates on NHB 3 than on 7042-S. Similarly, the PFI values

Table 6. Pathogenic fitness parameters; latent period (LP), infection percent (IP), sporangial production capacity (SPC), oospore production rating (OPR) and pathogen fitness index (PFI) of seven single-oospore isolates of *Sclerospora graminicola* on two pearl millet genotypes

	7042-S					NHB 3				
Isolate	LP	IP	SPC	OPR	PFI	LP	IP	SPC	OPR	PFI
ICOS-5	5	38	5·013	2·13	81·1	5	92	4·923	2·47	223·7
ICOS-12	5	50	5·253	2·33	122·4	5	91	5·090	4·00	370·5
HSOS-2	5	25	5·119	3·60	92·1	5	75	4·880	3·20	234·2
HSOS-4	5	47	5·234	2·60	128·0	5	88	5·132	3·20	289·0
HSOS-6	5	42	5·107	3·40	145·8	5	91	4·889	1·67	148·6
CBOS-1	5	36	5·008	1·93	69·6	5	7	5·045	1·20	8·5
CBOS-4	5	36	5·325	2·87	110·0	5	91	5·099	1·00	92·8
LSD ($P \leq 0·05$)		17·5	0·238	1·58			8·8	0·280	1·04	

LP, time in days between inoculation and sporulation (a).
IP, mean of three replications with 30 seedlings in each replication (b).
SPC, $\log_{10}$ transformed values of sporangial counts/cm^2 of sporulating lesion, mean of five observations (c).
OPR, based on 1–4 rating scale where 1 = no oospores, and 4 = > 20 oospores/cm^2 of leaf tissue.
PFI = (b × c × d)/a.

varied more on NHB 3 than on 7042-S. ICOS-12 had the highest PFI value (370·5) on NHB 3, and HSOS-6 had the highest PFI value (145·8) on 7042-S. CBOS-1, however, had the lowest PFI values on both 7042-S and NHB 3.

DISCUSSION

Ball *et al.* (1986) demonstrated variation in pathogenicity among populations of *S. graminicola* from India, and several countries in West Africa and southern Africa. Our results further document the high degree of heterogeneity for virulence and aggressiveness that exists within populations and among single-oospore isolates of the pathogen. Twenty single-oospore isolates of *S. graminicola* from three locations in India showed considerable variation for infection efficiency from within and between locations. Clearly, the populations from the ICRISAT Centre and Hisar were more aggressive than those from Coimbatore. Ten single-oospore isolates derived from the ICRISAT Centre field population of *S. graminicola*, maintained on 7042-S, showed considerable variation for infection efficiency and latent periods on a set of resistant and susceptible lines. Among the resistant lines, 700651, P7-4 and 7042-R could not clearly distinguish the various ICOS isolates, but differences in virulence to 700516 and SDN 503 were apparent among the isolates. These results suggest that 700651, P7-4 and 7042-R may have similar genes for resistance to ICOS isolates and that these might prove more stable to the ICRISAT populations of *S. graminicola* than those of 700516 and SDN 503.

The isolates varied greatly for latent period (7–15 days) on resistant genotypes 700651, P7-4 and 7042-R, even though the maximum infection on these genotypes was only 8% on P7–4. All isolates had a latent period of only 6 days on the universal susceptible genotype 7042-S, indicating the similarity in this component of aggressiveness among isolates. However, some isolates showed significantly higher infection levels than others. Host genotype pathogen isolate specificity is evident from the highly significant isolate × genotype interaction factor for infection efficiency (Table 2).

The isolate CBOS-1, with only 7% infection on NHB 3, was clearly distinguishable from the other isolates. Considering 10% infection level as a cut-off point for resistant (< 10%) and susceptible (> 10%) categories, the CBOS-1/NHB 3 combination gave a resistant reaction in contrast to the susceptible reaction of NHB 3 to CBOS-4 and the other isolates. NHB 3 is generally regarded as a highly susceptible genotype, so it was interesting to note that the two isolates from the same location reacted differently to this genotype. At the Durgapura research farm in Rajasthan, NHB 3 was resistant to the local

population of *S. graminicola* when it was grown after being withdrawn from cultivation for several years (Singh & Singh, 1987), suggesting that the pathogen population had lost virulence to NHB 3. At the ICRISAT Centre, a high rate of adaptation of *S. graminicola* to a specific host genotype through selection in asexual generations has been demonstrated (Thakur *et al.*, 1992). These examples clearly indicate the high degree of genetic heterogeneity for virulence in *S. graminicola* populations that enable them to match resistance genes rapidly in the host genotypes. The PFI, which reflects aggressiveness as the combined effect of latency, infection efficiency, and sporangial and oospore production was generally lower on 7042-S than on NHB 3, indicating the lower susceptibility of 7042-S than NHB 3 to these isolates. CBOS-1, the least aggressive isolate, had the lowest PFI values both on 7042-S and NHB 3, suggesting that such isolates are less fit and could eventually be eliminated from the pathogen population. The results suggest that screening of breeding material against the most aggressive isolates, such as ICOS-12 from the ICRISAT Centre, HSOS-6 from Hisar and CBOS-4 from Coimbatore would be useful in identifying resistance that might be highly stable to the pathogen populations, at least at these locations.

We recognize the limitation of our assumption of obtaining single-oospore isolates from low frequency infection of seedlings in this study. Although this assumption may not be valid in some cases, this method has been used in the lettuce downy mildew pathosystem (Michelmore *et al.*, 1988). Now that *in vitro* oospore germination has been demonstrated (Panchbhai *et al.*, 1991), it should be possible to induce infection using single germinating oospores for such studies. Future research will focus on examining pathogenic variability in more samples of *S. graminicola* from diverse host cultivars in major pearl millet growing areas in India.

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