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Selection for host-specific virulence in asexual populations of *Sclerospora graminicola**

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The response to host genotype-directed selection for specific virulence in *Sclerospora graminicola* was studied in the pearl millet–downy mildew system. In greenhouse experiments, seedlings of resistant pearl millet genotypes MBH 110 and 852B were inoculated with sporangia of a field population of *S. graminicola* maintained on susceptible genotypes NHB 3 and 7042. Sporangia from infected seedlings of MBH 110 and 852B were used to inoculate seedlings of MBH 110 and 852B, respectively, in succeeding generations. Within 12 generations of selection on MBH 110 and five generations of selection on 852B, respective host-specific virulences were identified. Selection for 852B-specific virulence was much faster than that for MBH 110-specific virulence. Symptom type, and pathogenic fitness parameters such as latent period, infection index, sporangial and oospore production were measured, and composite fitness index (CFI) was calculated for each selection generation. In general, CFI and individual fitness parameters were positively correlated with selection generation. Virulences of the host-specific pathotypes artificially selected through asexual generations were comparable to the naturally selected host-specific pathotypes AB, an MBH110-specific pathotype from Aurangabad, and MYS, an 852B-specific pathotype from Mysore. The host genotype × pathogen population interaction was highly significant ($P \leqslant 0{\cdot}001$). The results indicate that genetic variation for host genotype-specific virulence exists within field populations of the pathogen, and that selection through asexual pathogen generations can rapidly increase the quantitative virulence of the population to resistant host genotypes. This implies that resistance and virulence in the pearl millet–downy mildew system are controlled by relatively few genes.

INTRODUCTION

Sclerospora graminicola causes downy mildew in pearl millet (*Pennisetum glaucum*), an important cereal crop in the semi-arid tropical regions of the world (Rachie & Majmudar, 1980). *S. graminicola* is an obligate biotroph that reproduces asexually by means of sporangia, which liberate motile zoospores, and sexually by means of oospores. Aerial infection of pearl millet seedlings occurs by zoospores which penetrate the young meristematic tissue and initiate systemic infection. Local lesions are not commonly observed. Asexual sporulation begins 4–7 days after inoculation, and several crops of sporangia are produced from the infected leaf until the tissue turns necrotic (Williams, 1983).

S. graminicola is generally heterothallic (Michelmore *et al.*, 1982; Idris & Ball, 1984), but homothallism may also occur in the fungus (Michelmore *et al.*, 1982). Pathogenic variation in populations of *S. graminicola* has been known to occur (Ball, 1983; Ball & Pike, 1984). Both pearl millet and *S. graminicola* show high levels of outcrossing, and are thus genetically highly variable. It is therefore unlikely that resistance genes in the host and virulence genes in the pathogen will be homozygous and will remain so. Research in India has indicated regular shifts in virulence of the pathogen population with changes in host cultivars (Singh & Singh, 1987). Single-cross pearl millet hybrids (based on cytoplasmic male sterile lines) HB 1, HB 3, BJ 104 and MBH 110 all succumbed to downy mildew within 3–5 years of cultivation in farmers' fields (Anon., 1977–86), indicating the emergence of host-specific pathotypes in the *S. graminicola* population to at least HB 3, BJ 104 and MBH 110.

Clearly, the emergence of a new pathotype in an asexual population is not solely an outcome of genetic recombination, but could be the effect of host genotype-directed selection for specific virulence in the pathogen populations. The hypothe-

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sis under test in the present study was that asexual field populations of *S. graminicola* are heterogeneous and thus highly variable, and that host genotype-directed selection can therefore be an effective mechanism for the evolution of host-specific virulence.

In the context of this research, 'virulence' is defined as the relative capacity of a *S. graminicola* isolate or population to cause disease on a specific pearl millet genotype. Virulence is used instead of 'aggressiveness' to quantify the degree of disease in the host, because the interaction can be shown to be specific and because in heterogeneous pathogen populations, quantitative differences in levels of virulence can be due to differences in frequency of virulence genes with qualitative effects. Pathogenic fitness parameters such as latent period, infection index and sporulation were measured in order to define quantitative virulence of the pathogen populations.

MATERIALS AND METHODS

Host genotype

Pearl millet genotypes MBH 110 (an F_1 hybrid from Maharashtra Hybrid Seed Company) and 852B (an inbred line developed at ICRISAT as a maintainer of a male sterile line 852A) were used as test genotypes. MBH 110, once a popular commercial hybrid in Maharashtra State, became susceptible to *S. graminicola* within 5–6 years of its widespread cultivation, and was subsequently withdrawn from cultivation. The hybrid became susceptible to the local population of the pathogen, but it remained resistant to other populations, including that at ICRISAT. The other genotype, 852B, resistant to the ICRISAT Center population of *S. graminicola*, became susceptible to the Mysore population. Two other genotypes, hybrid NHB 3 and inbred 7042, which are both generally highly susceptible in multilocational tests in India, were used as controls. For each genotype the same seed stocks were used throughout the study.

Pathogen population

The asexual population IC of *S. graminicola*, obtained from sporulating leaves of NHB 3 and 7042 from the pearl millet downy mildew nursery at ICRISAT Center, was maintained on 7042 seedlings through asexual generations in a greenhouse. Similarly, the MBH 110-specific population AB (obtained from Aurangabad) and the 852B-specific population MYS (obtained from Mysore) were maintained on MBH 110 and 852B, respectively, through asexual generations. Each population was maintained in separate polyethylene isolation chambers, and extreme care was taken to avoid cross-contamination among isolates.

Inoculum and inoculation method

Infected leaves from pot-grown plants of 7042 in the greenhouse were collected, cut into pieces and washed in running tap water, using a cotton swab to remove old sporangial growth from the leaf surface. These leaf pieces were kept on moistened double-layered blotting paper in covered plastic trays, and incubated in darkness at 21°C for 6 h. The incubation temperature was reduced to 2–3°C after 6 h in order to inhibit release of zoospores from mature sporangia until spores were used as inoculum. Sporangia were washed from the sporulating leaves using a soft brush in ice-cold sterile distilled water, and the spore concentration was adjusted to 1×10^5 sporangia/ml.

Several methods of inoculation have been tested: soaking germinating seeds in the sporangial suspension; spraying young seedlings with sporangial inoculum; and injecting inoculum in seedlings at various growth stages (Singh & Gopinath, 1985). The most effective and reliable method found for this study involved depositing a 5-μl droplet of sporangial suspension, with a microsyringe, inside the rolled portion (base) of the partially unfolding first leaf of a seedling. This method was used throughout the experiments. Pot-grown seedlings were inoculated and incubated in a moist chamber in darkness at 20°C and >95% relative humidity for 24 h and pots were then transferred to greenhouse benches at 25 ± 2°C. Sporangia from MBH 110 and 852B seedlings inoculated with the IC population of *S. graminicola* served as first-generation inoculum. Subsequent generations of inocula were collected from the same host genotype to inoculate new sets of seedlings raised from the same seed lot. Inoculum from MBH 110 to MBH 110 was passed on up to the twelfth generation and that from 852B to 852B up to the fifth generation. At each generation about 100 seedlings were inoculated. Seedlings of 7042 were also inoculated at each generation as controls. The numbers of infected seedlings were counted 15 days after inoculation and the infection percentage was calculated. The infection percentage data were transformed into arcsin values, and an infection

index percentage was calculated as the ratio of infected seedlings in MBH 110 or 852B to that of 7042, multiplied by 100.

Fitness parameters

Observations were made for latent period (time from inoculation to sporulation), symptom type, infection index, sporangial production and oospore production. The symptoms were quite variable on infected seedlings, and these were rated on a scale of 1–4, where 1 = stunted growth with thick leaves and 4 = normal systemic infection (Table 1). Sporangial production was determined by assessment of sporangial growth on infected leaves. For this purpose, young infected leaf pieces from inoculated seedlings were collected and incubated as described above for inoculum increase. Incubated leaf pieces were examined under stereo binoculars, and sporangial production was rated on a scale of 1–4, where 1 = very scanty sporulation and 4 = abundant sporulation. Necrotic lesions from infected plants were used for oospore observations. Necrotic leaf tissues were cleared in 5% NaOH solution and examined under a microscope. Oospore counts were made for at least five leaf pieces in each treatment and were rated on a scale of 1–4, where 1 = no oospore formation and 4 = > 20 oospores/cm^2 leaf tissue (Table 1). The variables infection index, latent period, sporangial production and oospore production were used to monitor changes in pathogenic fitness of the pathogen population over generations. The composite fitness index (CFI), which is the combined effect of the four variables, was calculated for each generation as:

$$\mathrm{CFI} = (b \times c \times d)/a$$

where a = latent period (days), b = infection index, c = sporangial production and d = oospore production. The reciprocal of latent period was used to represent the rate of development of the pathogen. Symptom type was not included in calculations of CFI, because symptoms represent a host response rather than a component of pathogen fitness. Furthermore, the severity of symptoms was highly correlated with sporangial rating. Thus the inclusion of both symptom rating and sporulation rating would give undue weight to the sporulation component of fitness.

Table 1. Effect of selection in asexual generations of *Sclerospora graminicola* on pathogenic fitness parameters in pearl millet hybrid MBH 110

Asexual generation	Symptom type[a]	Latent period (days) (a)	Infection index[b] (b)	Sporangial production[c] (c)	Oospore production[d] (d)	CFI[e]
1	1	10	13	1	1	1·3
2	2	10	18	1	1	1·8
3	2	10	25	2	2	10·0
4	3	7	41	3	3	52·7
5	4	7	31	4	2	35·4
6	4	7	55	4	2	62·9
7	4	6	69	4	2	92·0
8	4	5	110	4	2	176·0
9	4	6	81	4	2	108·0
10	4	5	84	4	2	134·4
11	4	5	51	4	2	81·6
12	4	5	90	4	2	144·0

[a] Symptoms: 1 = stunted growth with thick leaves; 2 = slowly developing chlorotic lesions and narrow leaves; 3 = stunted growth, bunchy leaves leading to necrosis; 4 = normal systemic infection with near normal seedling growth.

[b] Infection index (%) was calculated as the ratio between arcsin-transformed values of percentage infection in MBH 110 and 7042, multiplied by 100.

[c] Sporangial production: 1 = very scanty visible sporulation; 2 = poor sporulation; 3 = moderate sporulation; 4 = abundant sporulation.

[d] Oospore production: 1 = no oospore; 2 = < 10 oospores/cm^2 leaf piece; 3 = 11–20 oospores/cm^2 leaf piece; 4 = > 20 oospores/cm^2 leaf piece.

[e] CFI (composite fitness index) = $(b \times c \times d)/a$.

Comparison of virulence

In another greenhouse experiment, five *S. graminicola* populations, namely, AB (ex. MBH 110), MYS (ex. 852B), IC (ex. 7042, NHB 3), MBH 110/G 12 (the twelfth asexual generation) and 852 B/G 5 (the fifth asexual generation), were compared with regard to their virulence by inoculating four pearl millet genotypes, MBH 110, 852B, 7042 and NHB 3, with each isolate. The experimental design was completely randomized, and consisted of five pathogen populations × four host genotypes × three replicates. Seedlings were grown in autoclaved soil in pots, and at least 50 seedlings were inoculated in each replicate. Inoculated seedlings were maintained in polyethylene isolation chambers, separately for each isolate, in the greenhouse at 25±2°C. Counts of infected and total seedlings were made 15 days after inoculation. Data were subjected to ANOVA to determine the significance of treatments and interaction.

RESULTS

Influence of selection on infection index

The infection index of population IC on MBH 110 was 13% in generation 1. In the repeated asexual generations in MBH 110 it increased to 110% in generation 8. From generations 9 to 12, the infection index fluctuated between 90 and 51% (Fig. 1). On 852B, however, the infection index of population IC in generation 1 was 83%; it declined slightly in generation 2, and subsequently increased to 147% in generation 5 (Fig. 1).

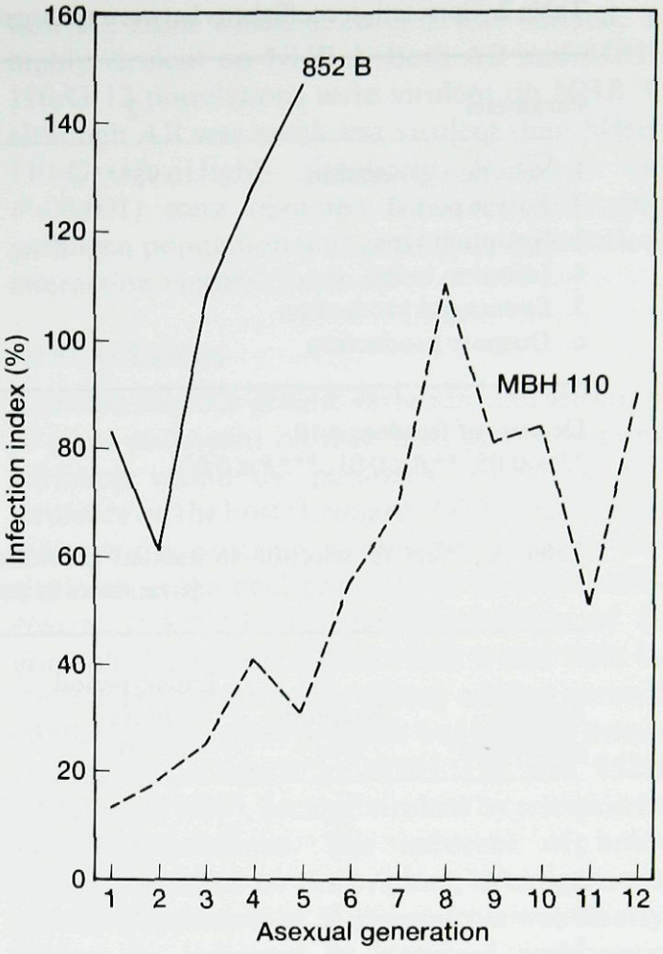

Fig. 1. Change in infection index of an asexual population of ICRISAT field collection of *Sclerospora graminicola* in response to successive generations in resistant pearl millet cultivars MBH 110 and 852B in greenhouse experiments. Infection index (%) was calculated as the ratio between arcsin-transformed values of downy mildew incidence (%) in MBH 110 or 852B and 7042, multipled by 100.

Influence on fitness parameters

Selection on MBH 110 resulted in a reduction in latent period from 10 days at generations 1–3 to 7 days at generations 4–6 and 5 days at generations 10 and beyond. Symptom type ranged from rating 1 in generation 1 to rating 4 in generation 5 and beyond. Oospore production varied from rating 1 in generations 1 and 2 to most commonly 2 in generations 3 and beyond (Table 1). The CFI increased from 1·3 in generation 1 to an average value of 128·8 in generations 8–12. Significant positive correlations were found among all fitness parameters, except for oospore production, which was significantly correlated only with sporangial production (Table 2). Selection generation was significantly correlated with all fitness parameters except oospore production, indicating a general increase in fitness over generations.

Selection on 852B resulted in a reduction in latent period from 7 days in generations 1–2 to 5 days in generations 4–5, and an increase in oospore production from a rating of 2 in generation 1 to a rating of 4 in generations 4–5 (Table 3). Symptom type and sporangial production were of normal susceptible types, with a rating of 4 in each generation. The CFI showed a steady increase from 94·8 in generation 1 to 470·4 in generation 5. Latent period was significantly correlated with infection index, and both were significantly correlated with the generation of selection (Table 4). Oospore production was not correlated with other fitness parameters.

Comparison of artificially selected and natural field populations

Three host-specific original populations, IC, AB and MYS, caused a high level of infection on their

Table 2. Correlation coefficients between pathogenic fitness parameters on pearl millet hybrid MBH 110

Parameter	2	3	4	5	6
1. Selection generation	0·95***	0·83**	0·74**	0·82**	0·37
2. Latent period		0·83***	0·82**	0·83***	0·41
3. Symptom type			0·64*	0·96***	0·55
4. Infection index				0·64*	0·26
5. Sporangial production					0·62*
6. Oospore production					

Degrees of freedom = 10.
$*P \leqslant 0{\cdot}05$, $**P \leqslant 0{\cdot}01$, $***P \leqslant 0{\cdot}001$.

Table 3. Effect of selection in asexual generations of *Sclerospora graminicola* on pathogenic fitness parameters in pearl millet inbred 852B

Asexual generation	Symptom type[a]	Latent period (days) (*a*)	Infection index[b] (*b*)	Sporangial production[c] (*c*)	Oospore production[d] (*d*)	CFI[e]
1	4	7	83	4	2	94·8
2	4	7	61	4	4	139·4
3	4	6	108	4	3	216·0
4	4	5	129	4	4	412·8
5	4	5	147	4	4	470·4

[a] Symptoms: 1 = stunted growth with thick leaves; 2 = slowly developing chlorotic lesions and narrow leaves; 3 = stunted growth, bunchy leaves leading to necrosis; 4 = normal systemic infection with near normal seedling growth.
[b] Infection index (%) was calculated as the ratio between arcsin-transformed values of percentage infection in 852 B and 7042, multiplied by 100.
[c] Sporangial production: 1 = very scanty visible sporulation; 2 = poor sporulation; 3 = moderate sporulation; 4 = abundant sporulation.
[d] Oospore production: 1 = no oospore; 2 = < 10 oospores/cm^2 leaf piece; 3 = 11–20 oospores/cm^2 leaf piece; 4 = > 20 oospores/cm^2 leaf piece.
[e] CFI (composite fitness index) = $(b \times c \times d)/a$.

Table 4. Correlation coefficients between pathogenic fitness parameters on pearl millet inbred 852B

Parameter	2	3	4
1. Selection generation	0·95*	0·92*	0·71
2. Latent period		0·97*	0·56
3. Infection index			0·42
4. Oospores production			

Degrees of freedom = 3.
$*P \leqslant 0{\cdot}05$
There was no variation for symptom type and sporangial production over generations in 852B.

specific host genotypes: NHB 3 (97%), MBH 110 (56%) and 852B (59%), respectively (Table 5). The IC population produced < 1% infection on MBH 110 and 5% on 852B, the AB population produced 9% infection on NHB 3 and 16% on 852B, and the MYS population produced 86% infection on NHB 3 and was avirulent on MBH 110. However, these three populations, and MBH 110/G 12 and 852B/G 5 were almsot equally virulent on 7042. Selection for 12 asexual generations on MBH 110 increased infection efficiency from < 1% for population IC to 35% for MBH 110/G 12. Selection for five asexual generations on 852B increased infection efficiency from 5 to 67%. The rate of increase in infection efficiency per generation was lower on MBH 110 than on

Table 5. Comparison of infection efficiency of three original populations of *S. graminicola* and two selected populations on four pearl millet genotypes in the greenhouse

Origin of population[a]	Infection (%)[b]			
	NHB 3	7042	MBH 110	852 B
Original				
IC	97	51	<1	5
AB	9	53	56	16
MYS	86	52	0	59
Selected				
MBH 110/G 12	94	42	35	0
852B/G 5	0	37	0	67
L.S.D. ($P \leqslant 0{\cdot}05$)	12·8	11·6	4·7	18·4

[a] IC = ICRISAT Center field population (ex. NHB 3/7042), AB = Aurangabad field population (ex. MBH 110), MYS = Mysore field population (ex. 852B), MBH 110/G 12 = IC population selected on MBH 110 up to 12 asexual generations, 852B/G 5 = IC population selected on 852B up to five asexual generations.
[b] Mean of three replications with at least 50 seedlings/replicate.

Table 6. Analysis of variance for downy mildew incidence (%) on four pearl millet genotypes inoculated with three field populations and two selections of *Sclerospora graminicola* (Table 5) in the greenhouse

Source of variation	DF	SS	MS	F
Replication	2	50·69	25·34	
Genotype (G)	3	13843·07	4614·35	102·808***
Population (P)	4	3741·67	935·42	20·841***
G × P	12	44762·04	3730·17	83·108***
Error	38	1705·56	44·88	

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

852B. In the latter case, five selection generations produced a population as virulent as, or even more virulent than, the 852B-specific MYS population from the field. Three populations, IC, MBH 110/G 12 and MYS, were also highly virulent on NHB 3. The MYS population differed significantly from the 852B/G 5 population; MYS was highly virulent on NHB 3, while 852B/G5 was avirulent on NHB 3. Avirulence of 852B/G 5 to NHB 3 is particularly notable, because population IC, from which 852B/G 5 was derived, is highly virulent on NHB 3. Both AB and MBH 110/G 12 populations were virulent on NHB 3, although AB was much less virulent than MBH 110/G 12. Highly significant F values (at $P \leqslant 0{\cdot}001$) were obtained for host genotype, pathogen population and genotype × population interaction factors (Table 6).

DISCUSSION

Selection requires genetic variation, and selection of plant pathogens on their host plants requires variation within the pathogen population for virulence on the host (Leonard, 1987). Our results indicate the existence of genetic variation for virulence in the field populations of *S. graminicola* at ICRISAT. The field population of *S. graminicola*, maintained on NHB 3 and 7042 in the ICRISAT pearl millet downy mildew nursery (Williams *et al.*, 1981)), which was initially avirulent or less virulent to MBH 110 and 852B (ICRISAT, 1989), became virulent by selection in asexual generations. The influence of host-directed selection on the original, heterogeneous asexual population of *S. graminicola* was clearly evident, as indicated by increased pathogenic fitness.

In response to selection at various generations, symptom type and all four pathogenic fitness parameters varied in MBH 110, but only three of these varied in 852B. The ratings for symptom type and sporangial production remained the same, 4 in each case, at each generation in 852B, suggesting that the IC population contained less genetic variation for the fitness parameters in 852B than in MBH 110. The CFI followed a similar pattern of increase over generation to the infection index. Lack of oospore production in early generations of the pathogen population on MBH 110 indicates that it could be difficult for this population to become established on MBH 110 in areas where millet is not grown continuously. Sporangia play an important role in the disease epidemiology (Singh & Williams, 1980), and asexual populations of the pathogen can survive in the areas with continuous cropping of susceptible pearl millet cultivars or on volunteer plants. In those areas, a population such as IC could adapt to MBH 110 or other cultivars on which it was initially unable to produce oospores.

Extensive genetic variation in the original asexual population is expected because of the existence of heterothallism in the fungus (Michelmore *et al.*, 1982; Idris & Ball, 1984). It appears that a very low proportion of sporangia were

virulent on MBH 110 in the original IC population, and the proportion generally increased with succeeding generations of selection. This phenomenon is consistent with the concept of qualitative variation for virulence within populations as part of a gene-for-gene relationship between resistance and virulence typical of many host-pathogen systems (Leonard, 1987). The rate of virulence selection was more rapid in 852B than in MBH 110, because a relatively larger proportion of sporangia were virulent on 852B in the original population, and within a few generations a highly virulent population was selected. These results indicate the possibility that two different sets of virulence genes exist in the pathogen populations, corresponding to the resistance genes in the two host genotypes. The results also demonstrate the existence of a mechanism for rapid evolution of a new pathotype in the field population of *S. graminicola*. For example, MBH 110, a popular hybrid grown for several years in the same field, showed increasing levels of susceptibility to downy mildew each year (S.B.K., unpublished data). When both sexual and asexual means are available, the process of change could be more rapid and new recombinants may occur through sexual generations.

The original AB (MBH 110) population showed 9% infection on NHB 3 and 16% on 852B, but the IC (NHB 3/7042) population selected through MBH 110 showed 94% infection on NHB 3 and 0% on 852B, indicating clear differences in virulence between IC and AB populations. A similar trend was observed in the MYS (852B) population and IC population selected on 852B, except that 852B/G 5 showed 9% infection on NHB 3, which is in sharp contrast to MBH 110/G 12, which showed 94% infection on NHB 3. This demonstrates that the selected virulence did not result from contamination by AB or MYS. It also suggests that selection for virulence to MBH 110 also maintained virulence to NHB 3, but that selection for virulence to 852B did not maintain virulence to NHB 3. Thus virulence to MBH 110 may be linked with virulence to NHB 3, but not with virulence to 852B. The loss of virulence to NHB 3 during selection on 852B probably indicates that in the original population IC virulence to 852B was linked with avirulence to NHB 3. These differences may also reflect different resistance loci in hybrids MBH 110 and NHB 3, and in inbred 852B.

Further selection experiments involving both sexual and asexual generations, and pearl millet genotypes with clear resistant and susceptible reaction types, would increase our understanding of the mechanism of evolution of new virulent pathotypes, and would aid the determination of genetic structures of resistance in the host and virulence in the pathogen.

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