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Effects of Genetic Transformation on Fitness of *Cochliobolus heterostrophus*

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ABSTRACT

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The fitness of genetically transformed strains of the southern corn leaf blight fungus *Cochliobolus heterostrophus* was compared to that of the isogenic wild-type progenitor in competitive pathogenicity tests on maize plants. Fitness (an individual's contribution to the gene pool of the next generation) was assessed by monitoring the frequency of the transformed phenotype in mixed populations with the wild type after successive disease cycles. Transformants were less fit than the wild type in 92% of the competition tests ($P = 0.05$). However, when the wild type was mock transformed (the transformation procedure without addition of plasmid DNA), transformants were less fit than the wild type in only 40% of the tests ($P = 0.05$). Thus, the transformation protocol alone accounted for some of the reduced fitness although there was evidence that this condition may be transient; an effect of the presence of plasmid DNA on fitness was not ruled out. In contrast to the effect of protocol, several factors had no apparent effect on fitness: size and copy number of the transforming plasmid, site of plasmid integration into chromosomal DNA, temperature at which plant assays were performed, proportion of initial inoculum composed of transformant conidia, and recipient fungal strain.

Methodology for evaluation of the possible environmental impact caused by genetically engineered microorganisms is under development (2,13,32). One concern is the competitive ability of recombinant strains; will populations decline, increase, or maintain themselves in the environment? These concerns are relevant to the study of plant pathogenic fungi because it is now possible to transform many of them with plasmid DNA. This technology provides the means to genetically engineer fungi for improved performance in the field (for example, as agents for biological control of pests) and to produce isogenic lines with which to evaluate effects of individual genes on parasitic fitness.

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As a first step in the evaluation of the pathological characteristics of recombinant fungi, we have quantified parasitic fitness of transformed strains of the southern corn leaf blight fungus, *Cochliobolus heterostrophus* (Drechs.) Drechs. (anamorph *Bipolaris maydis* (Nisikado & Miyake) Shoemaker = *Helminthosporium maydis* Nisikado & Miyake). *C. heterostrophus* was chosen for this study for several reasons. First, the fungus is readily transformed with plasmids that integrate into chromosomal DNA by homologous recombination (35) or at ectopic sites (34). Second, the disease cycle allows easy quantification of fungal populations. Third, it is possible to build on several previous epidemiological studies of this disease (12), including those that have used near-isogenic strains of *C.*

heterostrophus created by backcrossing (17,23). The limitation of backcrossing is that it allows DNA linked to the gene under study (for example, a virulence gene) to remain heterozygous, thus potentially complicating interpretation of fitness studies. Conversely, in a transformed strain, the exact size, location, and sequence of the variable DNA are known. In principle, transformation can produce isogenic pairs of strains that can be used to definitively address the question, "Will a particular gene influence the fitness of an organism?"

In our studies, competitive abilities of transformants of *C. heterostrophus* were assessed by monitoring the frequency of the transformed phenotype in mixtures with the isogenic wild-type progenitor over successive cycles of pathogenesis on maize plants. These competition tests showed that transformation affected fitness of the fungus. However, we found that the transformation procedure itself introduced variation that must be dealt with before genetic effects on fitness can be assessed accurately.

Competition experiments have been used by others to study the parasitic fitness of different phenotypes of fungi on host plants (5,6,10,17,22,23,33). Such tests also have been used to assess fitness attributes in *Drosophila* (18,24,26,36) and bacteria (3,11,20,21). The validity of this method resides in the fact that the fitness of an individual can be measured by its contribution to the gene pool of the next generation (30).

MATERIALS AND METHODS

Plasmids. The plasmids used in this study (Table 1) were propagated and purified by standard methods (4). The selectable marker on pH1S and pH1B (35), pDH25 (8), and pETE (P. Mullin, *unpublished*) was the *hygB* gene of *Escherichia coli* that encodes hygromycin B phosphotransferase, an enzyme that detoxifies hygromycin B (15). The selectable marker on p3SR2 is the *amdS* gene of *Aspergillus nidulans* (Eidam) Winter that encodes acetamidase, an enzyme that allows growth on medium with acetamide as the sole nitrogen source (14).

Transformation. Laboratory strain C3 of *C. heterostrophus* (MAT2, tox1−, American Type Culture Collection 48330 [19]) and field isolate Hm540 (MAT2, tox1−, provided by K. Leonard) were transformed following a standard protocol (35), which is presented in detail here because it is a variable in some of our experiments. Hygromycin B, the selective agent for transformation, inhibits growth of *C. heterostrophus* at 50 μg/ml but is not lethal at that concentration. Wild-type and transformed strains were stored in glycerol (33%) at −80 C (39).

For production of conidia, strains were grown on complete medium (CM) (19) for 10 days. Conidia were suspended in water, separated from mycelium by passage through two layers of cheesecloth, and transferred to liquid CM (10^5/100 ml) in a 300-ml flask. The flask was shaken (250 rpm) for 12–18 hr, and mycelium was collected by centrifugation. A mixture of cell wall-dissolving enzymes (Novozyme 234 [Novo Biolabs, Danbury, CT] [10 mg/ml], Driselase [Sigma Chemical Co., St. Louis, MO] [10 mg/ml], and chitinase [25 μg/ml]) in 0.7 M NaCl was added to the mycelium, and the resulting suspension was shaken (50 rpm) at 22 C for 2–4 hr. Protoplasts (10^8 to 10^9) were pelleted by centrifugation (8,000 *g* for 10 min at 4 C), washed three times with STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$), and resuspended in STC (10^7 protoplasts/100 μl). Approximately 20 μg of plasmid DNA was added followed by 1 ml of polyethylene glycol (60%, w/v, in 10 mM Tris, pH 7.5, and 10 mM $CaCl_2$). The mixture was incubated for 20 min at 22 C, added to 20 ml of molten nonselective regeneration medium (0.1% yeast extract, 0.1% casein enzymatic hydrolysate, 1 M sucrose, and 2.0% agar), and poured into petri plates (10 cm diameter). After 4–12 hr, plates were overlaid with 10 ml of 1.0% agar containing hygromycin B at a final plate concentration of 50 μg/ml. The plates were incubated at 30 C in darkness for 5–7 days when colonies appeared.

Eleven transformants of *C. heterostrophus* were chosen for this study (Table 1). Each transformant was purified by generating a colony from a single hygromycin B-resistant conidium. Transformants S1 and B1, 10-2-1, and 2-2 were provided by G. Turgeon, P. Mullin, and S. Karcher, respectively. Transformant 2-2 resulted from cotransformation of strain C3 with plasmids pDH25 and p3SR2 and could grow on medium containing hygromycin B and acetamide as the sole nitrogen source (34).

Transformants chosen for competition tests had wild-type colony morphology, color, and growth rate in culture. Genomic DNA was isolated (39) from each transformant, digested with two restriction endonucleases (one that cut once in the transforming plasmid and one that did not), Southern blotted (25), and probed with the transforming plasmid. The pattern of hybridizing bands identified the configuration of plasmid DNA in the fungal genome as indicated in Table 1. Details of these analyses will be published elsewhere.

Phenotypic stability. Each transformant was propagated for five cycles by serial plating on CM agar in petri dishes (10 cm diameter). After the fifth subculture, 50–100 individual conidia (along with hygromycin B-sensitive and -resistant control strains) were transferred to medium containing hygromycin B (50 μg/ml) and scored for growth. In addition, strains S13, S24, S25, and S28 were assayed for phenotypic stability after parasitic growth on plants (16).

Competition tests. Plants of maize inbred W64AN were grown from seeds in clay pots (12.5 cm diameter, four seeds per pot) containing a mixture of sand, compost, and peat moss (1:2.5:2.5) in a controlled-temperature chamber maintained at 25 C and 16 hr of light per day. When plants were 3 wk old, they were inoculated with a suspension of conidia (10^5/ml) in 0.5% Tween 20. Inoculum was a mixture of conidia from a hygromycin B-resistant transformant of *C. heterostrophus* and its hygromycin B-sensitive wild-type progenitor. Five pots of plants were inoculated with each mixture, placed in a mist chamber for 24 hr, and returned to the growth chamber. Lesion development was identical for the wild-type strain and for all transformants. After 10 days in the growth chamber, plants were placed again in mist chambers for 24 hr to induce sporulation. Conidia from sporulating lesions were collected and used to inoculate a fresh set of maize plants. Each mixture of strains was passed through 5 to 10 disease cycles on successive generations of host plants. After various generations, 100 to 150 individual conidia were plated on hygromycin B medium to determine the percentage of resistant conidia (Fig. 1). Mist chambers were disinfested with 0.5% sodium hypochlorite between conidial generations.

TABLE 1. Characteristics of transformants of *Cochliobolus heterostrophus*

Plasmid[a]	Plasmid size (kb)	Wild-type strain[b]	Transformant[c]	Integration site[d]	Copy number[e]
pH1S	6.7	C3	S1	homologous	tandem
		C3	S13	homologous	single
		C3	S24	ectopic	tandem
		C3	S25	homologous	tandem
		C3	S28	ectopic	single
pH1B	12.45	C3	B1	homologous	single
		C3	B12	ectopic	single
		Hm540	540B1	homologous	single
pDH25	7.0	C3	DH3	ectopic	tandem
pDH25 + p3SR2	7.0 8.8	C3	2-2	ectopic	N.D.
pETE	14.6	C3	10-2-1	ectopic	single

[a] Plasmids carrying fragments of chromosomal DNA of *C. heterostrophus* are pH1S, pH1B, and pETE; the others carry only pBR322 and sequences of *Aspergillus nidulans*.

[b] Two strains of *C. heterostrophus*, C3 or Hm540, were transformed with plasmid DNA.

[c] Designation of transformants used in this study. Isolate 2-2 was produced by cotransformation with plasmids pDH25 and p3SR2.

[d] Plasmids integrated into the fungal genome either at the site of sequence homology or at an ectopic (nonhomologous) site.

[e] Each transformant had either a single plasmid or tandemly repeated plasmid copies arranged head to tail. N.D. = not determined.

Evaluation of fungal fitness. *Mock transformation.* To ascertain whether the transformation procedure alone affected fitness, wild-type cells either were subjected to the transformation procedure without exposure to hygromycin B (competition tests 1–5); subjected to the transformation procedure, exposed to hygromycin B, and rescued to nonselective medium after 2 days (competition tests 6–11); or not subjected to the transformation procedure (competition tests 12–35). No plasmid DNA was used in these protocols. Single conidial strains then were tested for fitness by comparing them with hygromycin B-resistant transformants.

Adaptation. For two transformed strains, B1 and S1 (Table 1), the competition test was repeated such that the initial transformant inoculum for each succeeding competition test was grown from a single hygromycin B-resistant conidium chosen from the last cycle of the previous competition test. Wild-type inoculum was grown from glycerol stocks for each of these tests (competition tests 12–16).

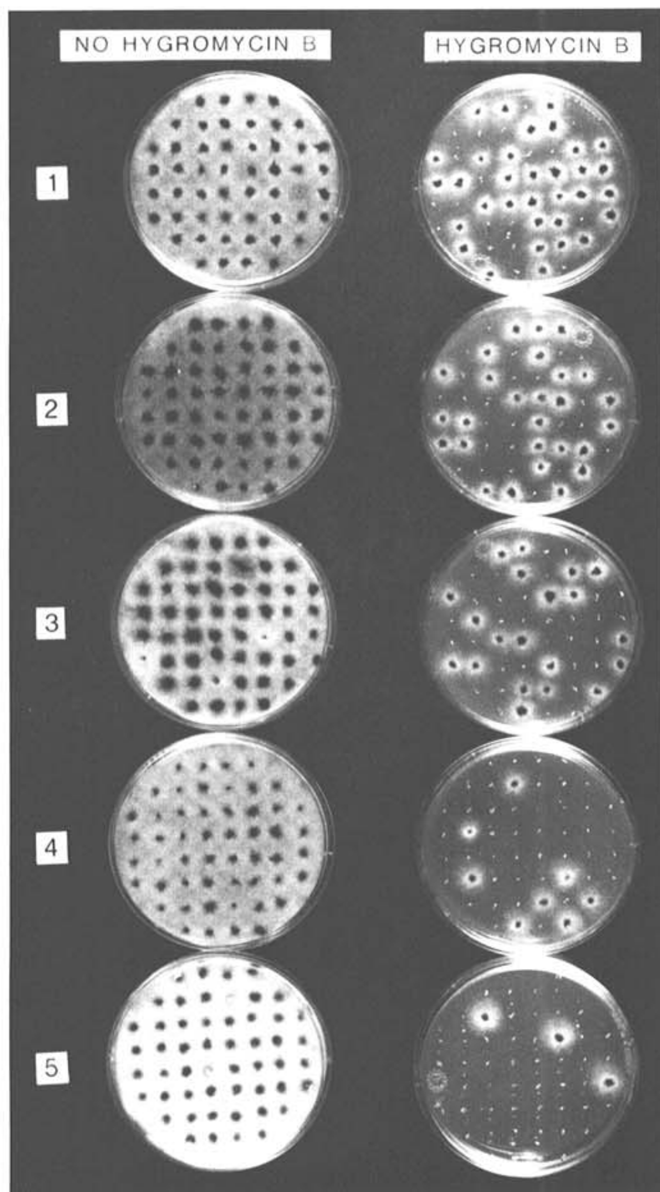

Fig. 1. Frequency of transformed conidia in a mixed population of transformant and wild-type conidia of *Cochliobolus heterostrophus* declines after successive cycles of pathogenesis on maize plants. Conidia from sporulating lesions were plated on both hygromycin B-amended and unamended media. Only transformed conidia grew on medium containing hygromycin B. The frequency of transformed conidia decreased after each cycle of pathogenesis. Numbers on the left indicate disease cycles.

Plasmid configuration in the recombinant chromosome. Transformed strains listed in Table 1 were chosen to determine the effects on fitness of plasmid size, site of integration, and plasmid copy number (competition tests 17–35).

Initial inoculum composition. The proportion of transformed conidia in the initial inoculum was approximately 95% in one test and approximately 50% in another test for each of the transformed strains S13, S24, and S25 (competition tests 17–28).

Temperature. Because temperature is known to influence the fitness of various organisms (10,18,36), the fitness tests were conducted both on a greenhouse bench (ambient temperature) and in a controlled-environment chamber (constant 25 C) for strains S13, S24, S25, and S28 (competition tests 17–30). Two mist chambers maintained at greenhouse or growth-chamber temperature were used for incubation and induction of sporulation.

Fungal genotype. C. heterostrophus strain C3 and field isolate Hm540 were transformed with the plasmid pH1B and subjected to concurrent competition tests with the respective wild-type progenitor strain (tests 31 and 32).

Mathematical model. Fitness of both the wild-type strain and transformants was assumed to be constant over time, allowing for analysis of the data in the following fashion. Linear regressions of the logits of the proportion of the hygromycin B-resistant population versus generations of selection on maize plants were calculated for each experiment. Selection coefficients were calculated from the following equation:

$$\frac{P_n}{1-P_n} = (1-s)^n \frac{P_0}{1-P_0}$$

where P_n is the proportion of the transformed phenotype after n generations of selection; P_0 is the initial proportion of the transformed phenotype; and s is the selection coefficient. The slope of the line $\ln[P_n/1 - P_n]$ versus n is $m = \ln(1 - s)$. A selection coefficient of 0.35 can be interpreted to mean that the transformant was 35% less fit than the wild type; a negative selection coefficient indicates that the transformant was more fit than the wild type. The theoretical basis of this model has been described previously (22,23). Regression analyses were calculated using the Minitab statistical package (Pennsylvania State University, University Park, PA). Selection coefficients were compared using a paired Student's t test.

RESULTS

Phenotypic stability in culture. Growth rates of transformants in culture were similar to that of their wild-type progenitor. All conidia of each transformant were resistant to hygromycin B after five serial propagations on CM agar, indicating stability of plasmid DNA in the chromosome; an exception was one sensitive conidium (out of 50) of transformant S13 after the fifth cycle on CM agar. Similar stability was observed when transformants carrying pHIS were propagated for five disease cycles on host plants (16). Therefore, we assumed that plasmid DNA was stable in the chromosome when the fungus was growing on plant tissue in competition with the wild-type strain. We thus were able to avoid using another marker (in addition to *hygB*) to track the transformed nucleus. In so doing, complete isogenicity (except for plasmid DNA) was maintained, and there was only one genetic variable (plasmid DNA) in our experiments. It would be interesting, however, to compare results of experiments using marked versus isogenic nuclei; the outcome would depend, of course, on the choice of marker gene.

Effect of mock transformation on fitness. Three general observations were made: when wild-type cells were mock transformed and exposed to hygromycin B, cultures derived from them were less fit than transformants; when wild-type cells were mock transformed but not exposed to hygromycin B, cultures derived from them were as fit as, or more fit than, transformants; and when wild-type cells were neither mock transformed nor exposed to hygromycin B, cultures derived from them were more

fit than the transformants. Data supporting these statements are presented in Tables 2–5.

In competition tests between transformants and mock-transformed strain C3 not exposed to hygromycin B, two transformants showed a significant decline in frequency (Fig. 2A, Table 2, tests 3 and 5), whereas the others did not (Fig. 2A, Table 2, tests 1, 2, and 4). In test 1, the frequency of S24 conidia seemed to increase in the mixture although the selection coefficient (−0.37) did not differ significantly from 0 at $P = 0.05$. When the same transformed strains (S24, S25, and S28) competed against the untreated wild type (Table 4, tests 21–30), they declined in frequency in all but test 24 (strain S24). Results of tests 1–5 (in which transformants were less fit than mock-transformed wild type in 40% of the tests) compared with results of tests 21–30 (in which transformants were less fit than untreated wild type in 90% of the tests) suggested that the transformation procedure itself had a deleterious effect on the fitness of *C. heterostrophus*. The fact that the fitness values of S24 (tests 21–24) and S25 (tests 3 and 4) were not entirely consistent among experiments argues that effects of the transformation procedure on fitness can be variable.

When conidia from transformed strains were mixed in a competition test with those of wild-type strain C3 that had been exposed previously to hygromycin B for 2 days, all of the transformants were substantially more fit (44–73%) than the wild-type strain (Table 2, tests 6–11, Fig. 2B, test 8; graphs of tests 6 and 7 and 9–11 were similar to that of test 8). This was not unexpected because exposure of wild-type cells to hygromycin B caused slow growth and altered morphology in culture (Fig. 3). The detrimental effect of the drug on wild-type *C. heterostrophus* apparently lasts through several conidial generations on plants because the wild-type strain subjected to this treatment was generally less fit after multiple disease cycles (Table 2, tests 6–11) than the wild-type strain mock transformed but not exposed to hygromycin B (Table 2, tests 1–5). Conidia derived from untreated wild-type cells increased in frequency (compared to conidia of transformants) in 21 out of 24 tests (Tables 3 and 4, tests 12–35).

TABLE 2. Effect of mock transformation on fitness of *Cochliobolus heterostrophus*

	Test no.[a]	Strains in inoculum	Disease cycle[b]	P_0[c]	s[d]	R[e]
Strain C3 not exposed to hygromycin B[f]	1	C3/S24	8	0.82	−0.37, NS	0.75
	2	C3/S24	10	0.72	0.04, NS	0.15
	3	C3/S25	10	0.91	0.52	0.95
	4	C3/S25	8	0.83	−0.06, NS	0.34
	5	C3/S28	10	0.87	0.46	0.95
Strain C3 exposed to hygromycin B[f]	6	C3/S25	9	0.55	−0.68	0.84
	7	C3/S25	9	0.54	−0.44	0.92
	8	C3/S25	9	0.32	−0.73	0.85
	9	C3/S28	9	0.54	−0.47	0.88
	10	C3/S28	9	0.52	−0.49	0.85
	11	C3/S28	9	0.36	−0.72	0.99

[a]Competition tests are numbered consecutively from Table 2 through Table 5.
[b]The number of disease cycles for each pair of fungal strains on maize plants.
[c]P_0 = proportion of transformed conidia in the initial inoculum.
[d]s = the selection coefficient. All selection coefficients were significantly different from 0 at $P = 0.05$ unless labeled NS (not significant). A positive selection coefficient indicates that the transformant was less fit than the wild-type strain; a negative selection coefficient indicates that the transformant was more fit than the wild-type strain.
[e]R = the correlation coefficient of the logits of the proportion of the transformant population versus generations on maize.
[f]Wild-type strain C3 was mock transformed (described in the Materials and Methods section). In tests 1–5, strain C3 was not exposed to hygromycin B; in test 6–11, strain C3 was exposed to hygromycin B for 2 days.

Adaptation of transformants. When transformants B1 and S1 were first tested in a competition test with wild-type strain C3, they were 36 and 56% less fit than the wild-type strain, respectively (Table 3, Fig. 2C, tests 12 and 15). Each strain then was subjected to another set of competition tests with strain C3, but this time the initial inoculum for B1 and S1 was prepared from single hygromycin B-resistant conidia isolated from the last disease cycle of the previous test. In this second set of tests, transformant S1 was 43% less fit than strain C3 (this was significant at $P = 0.1$ but not at $P = 0.05$, test 16). Frequency of transformant B1 conidia did not decline in the second set of tests (test 13), and the transformant was subjected to a third set of tests (test 14). The inoculum for the third set was generated from a hygromycin B-resistant conidium from the last disease cycle of the second set. Again there was no decline in frequency of B1 conidia in the mixed population. The results with B1 suggested that it is possible for a transformant to recover its lost fitness, although evidence for this was not observed in other experiments.

No detectable effects on fitness of integration site and copy number of the plasmid, temperature, or composition of initial inoculum. When wild-type strain C3 was not subjected to the transformation procedure and was placed in competition with each of four transformants (S13, S24, S25, S28) that differed in plasmid copy number and integration site, the frequency of the transformed phenotype decreased in 13 of 14 tests (Table 4, tests 17–30, Fig. 2D, test 26; graphs from tests 17–25 and 27–30 were similar to that of test 26). Selection coefficients for these 13 tests were significantly different from 0 at $P = 0.05$ and indicated that the transformants were 39–64% less fit than the wild-type strain. There was no significant difference in the selection coefficients among the four different strains nor was there an apparent effect on fitness of temperature at which plant assays were done, composition of initial inoculum, or copy number and integration site of the plasmid, with the exception of strain S24 in test 24. When transformant S24 had $P_0 = 0.95$ and was under the ambient temperature regime, the frequency of S24 conidia remained constant over nine disease cycles; the reason for this exceptional result is unknown.

No detectable effects of fungal genotype and different plasmids on fitness. The proportion of hygromycin B-resistant conidia declined in all tests regardless of plasmid construction or fungal genotype (Table 5, tests 31–35; graphs from these tests were similar to that of test 26, Fig. 2D). The proportion of conidia of the transformed Hm540 strain (isolate 540B1) declined from 57 to 2.5% of the population after five disease cycles on maize, and similar frequency changes were seen in the other transformants. The selection coefficients were significantly different than 0 at

TABLE 3. Effects of repeated sets of competition tests on fitness of transformants of *Cochliobolus heterostrophus*

Test no.[a]	Strains in inoculum	Set[b]	Disease cycle[c]	P_0[d]	s[e]	R[f]
12	C3/B1	1	5	0.50	0.36	0.97
13	C3/B1	2	5	0.73	0.03, NS	0.08
14	C3/B1	3	5	0.36	0.02, NS	0.03
15	C3/S1	1	5	0.50	0.56	0.96
16	C3/S1	2	5	0.25	0.43	0.86

[a]Competition tests are numbered consecutively starting with Table 2.
[b]Strain B1 was taken through three sets of competition tests with strain C3; strain S1 was taken through two sets. The S1 and B1 inocula for set 2 were grown from single hygromycin B-resistant conidia from the fifth cycle of set 1; similarly, inoculum for B1 set 3 was grown from a single resistant conidium from the fifth cycle of set 2.
[c]The number of disease cycles for each pair of fungal strains within each set.
[d]P_0 = the proportion of transformed conidia in the initial inoculum.
[e]s = the selection coefficient. All selection coefficients were significantly different from 0 at $P = 0.05$ unless labeled NS (not significant). The s value in test 16 was significant at $P = 0.1$.
[f]R = the correlation coefficient of the logits of the proportion of the transformant population versus generations on maize.

TABLE 4. Fitness of transformants of *Cochliobolus heterostrophus* was not affected by plasmid (pH1S) integration site, plasmid copy number, temperature at which plant assays were done, and proportion of transformant conidia in initial inoculum

Test no.[a]	Strains in inoculum	Integration site	Plasmid copy number	Temperature[b]	Disease cycle[c]	P_0[d]	s[e]	R[f]
17	C3/S13	homologous	single	constant	5	0.50	0.56	0.87
18	C3/S13	homologous	single	constant	9	0.95	0.45	0.92
19	C3/S13	homologous	single	ambient	5	0.50	0.55	0.88
20	C3/S13	homologous	single	ambient	9	0.95	0.43	0.93
21	C3/S24	ectopic	tandem	constant	5	0.50	0.64	0.92
22	C3/S24	ectopic	tandem	constant	9	0.95	0.39	0.86
23	C3/S24	ectopic	tandem	ambient	5	0.50	0.54	0.88
24	C3/S24	ectopic	tandem	ambient	9	0.95	0.03, NS	0.56
25	C3/S25	homologous	tandem	constant	5	0.50	0.39	0.87
26	C3/S25	homologous	tandem	constant	9	0.95	0.52	0.95
27	C3/S25	homologous	tandem	ambient	5	0.50	0.52	0.95
28	C3/S25	homologous	tandem	ambient	9	0.95	0.52	0.88
29	C3/S28	ectopic	single	constant	9	0.95	0.44	0.88
30	C3/S28	ectopic	single	ambient	9	0.95	0.51	0.97

[a] Competition tests are numbered consecutively starting with Table 2.
[b] Competition tests were conducted either in a controlled-environment chamber (constant 25 C) or in the greenhouse at ambient temperature.
[c] The number of disease cycles for each pair of fungal strains on maize plants.
[d] P_0 = the proportion of transformed conidia in the initial inoculum.
[e] s = the selection coefficient. All selection coefficients were significantly different from 0 at $P = 0.05$ unless labeled NS (not significant).
[f] R = the correlation coefficient of the logits of the proportion of the transformant population versus generations on maize.

TABLE 5. No distinguishable effects of different plasmids of fungal genotype on fitness of transformed strains of *Cochliobolus heterostrophus*

Test no.[a]	Strains in inoculum	Disease cycle[b]	P_0[c]	s[d]	R[e]
31	C33/B12	5	0.73	0.27	0.98
32	Hm540/540B1	5	0.57	0.69	0.95
33	C3/10-2-1	5	0.72	0.76	0.94
34	C3/2-2	5	0.66	0.47	0.97
35	C3/DH3	5	0.55	0.38	0.83

[a] Competition tests are numbered consecutively starting with Table 2.
[b] The number of disease cycles for each pair of fungal strains on maize plants.
[c] P_0 = the proportion of the transformed strain in the initial inoculum.
[d] s = the selection coefficient. All selection coefficients were significantly different from 0 at $P = 0.05$ except for that of test 35 which was significant at $P = 0.1$.
[e] R = the correlation coefficient of the logits of the proportion of the transformant population versus generations on maize.

$P = 0.05$ except for test 35 (isolate DH3) where the selection coefficient was significantly different than 0 at $P = 0.1$.

DISCUSSION

Our results clearly show that transformed strains of *C. heterostrophus* carrying a small plasmid in their genomes are less fit than their wild-type progenitor. Several mechanisms could account for this: injurious effects sustained by the fungus during the transformation procedure; the added energy burden associated with the synthesis of new nucleic acids and proteins; disruption of a metabolically important region of DNA at the site of plasmid integration; and/or harmful physiological effects of novel gene products. In every case the transformed strains chosen for this study grew at the same rate in culture, were morphologically identical to, and caused lesions on plants that were indistinguishable from those caused by their progenitor. We found no detectable effect on fitness due to site of plasmid integration, proportion of initial inoculum composed of transformed conidia, temperature at which plant assays were done, or fungal strain.

At least part of the reduced fitness observed in transformants could be explained by an effect of the transformation procedure itself. Untreated wild type was more fit than transformants in 92% of the competition tests, whereas the mock-transformed wild type not exposed to hygromycin B was more fit than transformants in only 40% of the competition tests, although it grew normally in culture. This apparent effect of the transformation protocol could be due to subtle physiological changes in the fungus caused by stresses imposed during the transformation procedure (see the Materials and Methods section). Perhaps effects of procedure could be circumvented by use of an alternative transformation strategy such as electroporation (29,38), incubation of cells with lithium acetate (1,9,28), agitation with glass beads (7), or particle bombardment (31).

Exposure of wild-type cells to a nonlethal dose of hygromycin B for 2 days had a drastic inhibitory effect on subsequent growth under nonselective conditions in culture or on maize plants. However, it is not clear that exposure to hygromycin B actually caused reduced fitness in transformants because they, in contrast to the wild-type strain, were able to detoxify the drug and showed no adverse effects when subsequently grown on nonselective culture medium.

The apparent impact of procedure on fitness may have obscured a possible effect of the presence of foreign DNA in the fungal genome. Such an effect, if any, must have been small because transformants carrying several copies (≤5) of the plasmid were not measurably less fit than those with a single copy. It may be necessary to introduce a large amount of foreign DNA before an effect is detectable. For example, cells of *E. coli* bearing plasmids can be less fit than plasmid-free cells, and the reduced fitness is correlated with large plasmid size (80–150 kb) and high copy number (>20 copies/cell) (40).

One of our experiments suggested that a transformant with reduced fitness eventually recovered and in later tests competed equally with the wild type on maize plants. Transformant B1 was 36% less fit than the wild type in the first set of five disease cycles. However, when purified transformant inoculum obtained from the fifth disease cycle was analyzed in a second and third set of five cycles, it was as fit as the wild type. Evidence for adaptation was not observed in other experiments. For example, in some cases involving a single set of nine disease cycles, the ratio of transformed to wild-type conidia declined little, if any, in early disease cycles but dropped off rapidly in later ones (Fig. 2D). This suggests that the selection coefficient(s) is not constant in all mixed populations and that a quadratic model may provide a better description of the data from certain experiments. Because adaptation would have caused the data points to drop off more slowly in later disase cycles than in early ones, it appears that a single set of nine disease cycles is not sufficient to detect the phenomenon if it exists.

The experimental strategy used here is relevant to analysis of the concept of stabilizing selection (the cost of carrying an

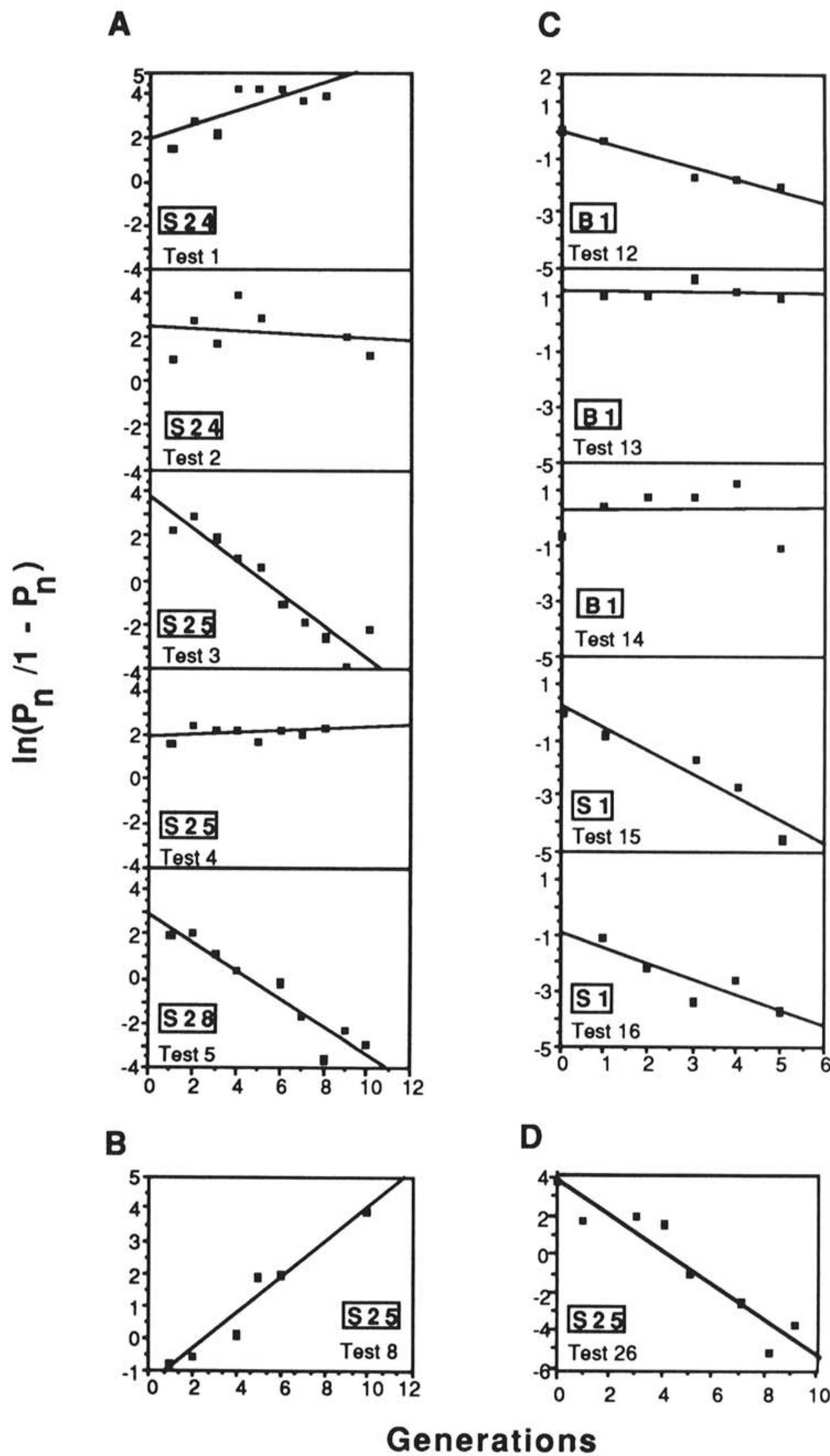

Fig. 2. Linear regressions of the logits of the proportion of transformed conidia of *Cochliobolus heterostrophus* in a mixed population with the wild-type strain [ln ($Pn/1 - Pn$)] versus generations on maize. The test number identifies the transformant population used in each competition test, the selection coefficient(s), and the correlation coefficient (R), as described in Tables 2–4. **A,** Competition tests 1–5 (Table 2) where wild-type strain C3 was subjected to mock transformation but was not exposed to hygromycin B. **B,** Competition test 8 (Table 2) where the wild-type strain was subjected to mock transformation and exposed to hygromycin B for 2 days. **C,** Competition tests 12–16 (Table 3) where transformants were subjected to repeated sets of competition tests with the untreated wild-type strain. **D,** Competition test 26 (Table 4) where the wild-type strain was untreated. Designations S24, S25, S28, B1, and S1 refer to particular transformants (Table 1).

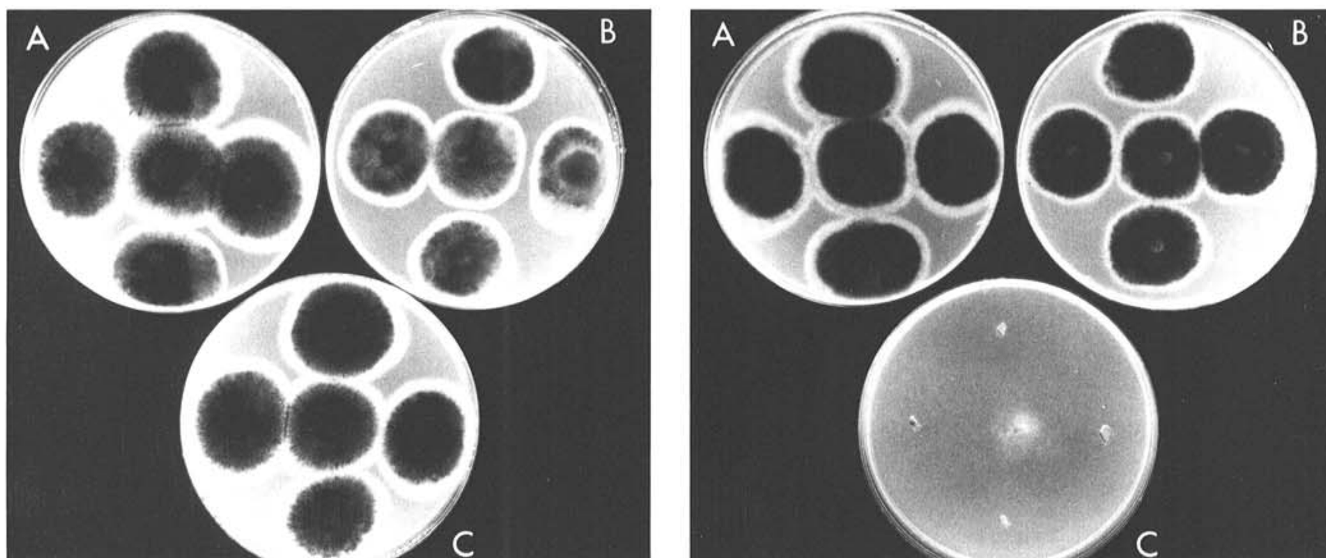

Fig. 3. Growth of transformant S25 of *Cochliobolus heterostrophus* (left photograph) and wild-type strain C3 (right photograph) on complete medium (CM) after three different treatments: A, continuous propagation on CM; B, mock transformation but no exposure to hygromycin B; and C, mock transformation followed by exposure to hygromycin B for 2 days. Photographed after 5 days of growth.

unnecessary virulence gene) as described by others (27,37). To rigorously determine whether or not a virulence gene carries a genetic load, it is necessary that the entire genomes of the two strains being compared are isogenic except for different alleles at the virulence locus. This is impossible to achieve by sexual backcrossing, but it can be done readily with the tools of molecular biology. However, our results show that molecular procedures themselves can introduce variation into the system. Therefore, procedural effects on fitness must be eliminated before genetically engineered isogenic genomes can be compared for the effect of a single virulence gene on fitness.

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