Differential Selection on *Rhynchosporium secalis* During Parasitic and Saprophytic Phases in the Barley Scald Disease Cycle

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ABSTRACT

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Competition among eight *Rhynchosporium secalis* isolates was assessed during parasitic and saprophytic phases of the disease cycle in field experiments conducted at two locations and over two growing seasons. The eight isolates were inoculated onto six barley populations exhibiting varying degrees of resistance. Microsatellite analysis of 2,866 isolates recovered from the field experiments showed significant, and sometimes opposite, changes in the frequencies of *R. secalis* genotypes during the growing season (parasitic phase) and between growing seasons (saprophytic phase). Isolates that showed the most complex virulence in greenhouse

seedling assays had the lowest fitness in the field experiment. Significant differences in isolate fitness were found on different host populations and in different environments. Selection coefficients were large, indicating that evolution can occur rapidly in field populations. Although inoculated isolates had the lowest overall fitness on the moderately resistant landrace cv. Arabi Aswad, some isolates were more virulent and consistently increased in frequency on this landrace, suggesting a risk of directional selection and possible erosion of the resistance following its widespread deployment in monoculture. These results provide the first direct evidence that *R. secalis* pathogen genotypes differ in their saprophytic ability and parasitic fitness under field conditions.

Additional keywords: microsatellites, population genetics.

Leaf blotch or scald, caused by the fungus *Rhynchosporium secalis* (Oudem.) J. J. Davis, occurs wherever barley is grown and is one of the most economically important diseases of barley worldwide (2,25). Yield losses of 10 to 40% are common (25), and losses of nearly 100% can occur on susceptible barley cultivars (32). *R. secalis* affects barley on several million hectares in North Africa, West Asia, Central Africa (Eritrea and Ethiopia), Yemen, Central Asia, the Andean countries (Peru, Colombia, Bolivia, and Ecuador), and the Far East (33). Scald is particularly damaging in areas where barley is cultivated continuously such as in West Asia, in central and southern parts of North Africa, the highland regions of Peru and Nepal, and large areas in Eritrea and Ethiopia. Farmers in these countries cannot afford fungicide treatments on a regular basis; therefore, host resistance is the preferred method for controlling scald. Unlike in Western Europe and North America where scald is also a damaging disease, breeding programs in many developing countries are not efficient enough to ensure a rapid replacement of scald-resistant cultivars following the breakdown of resistance. For example, Rihane was released as a scald-resistant cultivar in several North African countries in 1983 but is now widely regarded as susceptible to local *R. secalis* populations (32). However, the cultivar continues to be cultivated over large areas and currently covers over 40% of the acreage grown to barley in these countries.

The evolution of pathogens in response to host resistance genes has received considerable attention, especially in pathosystems characterized by the rapid breakdown of race-specific resistance (4,20,22,30,36–38). The partial scald resistance found in barley landraces and many improved cultivars is thought to be reasonably durable because there is less selection on the pathogen population (7). However, it is possible that strains with higher aggressiveness will increase in frequency due to directional selection on partially resistant cultivars that are grown over a large area for many years, eroding the effectiveness of the resistance (19). In addition to changes in genotype frequencies that occur due to differences in fitness among strains during the parasitic phase of the disease cycle, significant changes may occur during the saprophytic phase as isolates compete with each other and with other fungal species colonizing the barley straw between seasons. If fungal isolates differ in fitness for survival during the saprophytic phase on stubble, this may alter the genetic structure of the population as much as competition during the parasitic phase. Since *R. secalis* survives saprophytically on stubble (25), the fitness of individual fungal isolates in the field will also be a function of their ability to survive, compete, and reproduce on stubble between growing seasons. However, it is not known if the fitness of *R. secalis* isolates on stubble is influenced by the genotype of barley cultivars or their level of resistance.

R. secalis is a haploid deuteromycete fungus. Primary inoculum is thought to consist of conidia dispersed by rain-splash or mycelia in infected seeds and crop debris (25). Despite the absence of a known teleomorph, a wide pathogenic variation exists in most populations of the pathogen (3,10) and *R. secalis* populations have been shown to be highly variable for several genetic markers (14,16,18,21,24). The virulence structure of *R. secalis*

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populations can change over a relatively short period of time, and major resistance genes deployed in barley to control scald have frequently exhibited a finite life span (1,30; and references therein).

Few field experiments have been conducted to test specific inferences (or hypotheses) regarding pathogen evolution under controllable and repeatable environments (4,22,38–40). All of these experiments have focused on selection occurring during the parasitic phase of the disease cycle. Zhan et al. (38) measured how quickly *Mycosphaerella graminicola* populations adapted to different wheat genotypes and showed results consistent with disruptive selection operating in mixed host populations. But they did not consider selection occurring during the saprophytic phase of the disease cycle. In this study, we took knowledge gained from the wheat/*M. graminicola* pathosystem and applied it to the barley/*R. secalis* pathosystem, adopting a similar experimental approach but using microsatellite markers (17) to measure the adaptive responses of experimental pathogen populations to host selection during both parasitic and saprophytic phases of the disease cycle. The principal objectives of our experiments were to (i) determine the relationship between the reproductive fitness and virulence spectrum of pathogen isolates; (ii) determine whether pathogen isolates were better adapted to the hosts from which they originated; and (iii) determine whether pathogen isolates would differ in their ability to compete and survive during parasitic and saprophytic phases of the disease cycle.

MATERIALS AND METHODS

Source of experimental isolates used in the experiments. In March 2002, a collection of *R. secalis* was made from a farmer's field planted to barley cv. Rihane in Al Bab-Aleppo Province, North Syria. This field had uniform infection and was planted to cv. Rihane in the previous year. Collections were made along transects, with at least 1 m between samples. The total area sampled was $\approx 15 \times 50 \text{ m}^2$. In total, 68 isolates were obtained from this field and characterized with microsatellites (17). Of those, eight *R. secalis* isolates with rare alleles for at least one of the eight characterized microsatellite loci were chosen for the glasshouse and field inoculation experiments. The frequencies in the farmer's field of six of the eight chosen isolates were low (0.01 to 0.04), while Sy34 and Sy40 were found at frequencies of 0.12 and 0.15, respectively.

Field experiment design. Field experiments were conducted at the main experiment station of the International Center for Agricultural Research in the Dry Areas (ICARDA) in Tel Hadya-Aleppo, Syria (36°01'N 37°20'E, elevation 300 m above sea level [masl], and 340 mm mean annual rainfall) and at a substation in Lattakia, Syria (35°31'N, 35°46'E, 7 masl, and 770 mm mean annual rainfall) during the 2003 to 2005 barley growing seasons. Barley hosts used in these experiments were cvs. Rihane (susceptible) and WI2291 (susceptible), line 9-26 F27 (resistant), a 1:1 mixture of Rihane and line 9-26 F27, and barley landraces Arabi Abiad (susceptible) and Arabi Aswad (moderately resistant) (34). In December 2003, the six treatments were planted in a randomized complete block design with three replications. Plots were 1.5 m (five rows) wide by 7.0 m in length. Each plot of barley was separated by an equal-sized wheat plot, a nonhost for *R. secalis*, to create a checkerboard pattern of wheat and barley plots. Pathogen-free barley seeds were sown in the fields, which had not been planted with barley or known hosts of *R. secalis* for 2 years at Tel Hadya and 1 year at Lattakia. No scald infection developed on plants originating from the same lot of seed in uninoculated control plots at Lattakia.

Spores from the eight isolates were mixed in equal proportions to a final concentration of 10^6 spores ml^{-1} . A surfactant (Tween) was added to the spore solution at a rate of 1 drop per 50 ml. Spores were applied to each plot at the seedling stage on 11 January 2004 with a motorized knapsack sprayer (Fox Motori

model F.320, Poviglio, Italy). Inoculation was done in the evening and was scheduled to coincide with cloud and fog cover lasting at least 48 h to ensure disease establishment. Samples of infected leaves were collected three times from all treatments at Tel Hadya in 2004 and twice in 2005. The first samples of infected leaf material were collected on 10 March 2004 (early tillering stage, hereafter referred to as early-season), 2 months after field inoculation. The second samples were collected on 15 April 2004 (stem elongation stage, hereafter referred to as middle-season), while the third sample was collected on 19 May 2004 (grain filling stage, hereafter referred to as late-season). Depending on the disease severity, at least 60 infected leaves were collected at regular intervals along transects within the inner three rows of each field plot in each collection and one isolation was made from each leaf.

The field plots were harvested at the end of the 2003–04 growing season on 15 June 2004. Stubble was retained in each field plot to allow *R. secalis* to complete its saprophytic phase. In December of the 2004–05 growing season, each plot was sown by hand with pathogen-free seed. The first visible natural scald infections were collected from all plots early in the growing season (8 March 2005) using the same sampling procedure as in the previous year. A second collection of infected leaf samples was made from each plot late in the season (4 May 2005).

The experimental design, inoculation procedure, and sampling strategy at Lattakia were identical to those in Tel Hadya. The six treatments were planted in December 2003 followed by inoculation on 11 January 2004. Two collections were made both in 2004 and in 2005. Early-season collection of scald-infected leaves was made on 2 March 2004, followed by a late-season collection on 27 April 2004. The field plots were harvested at the end of the 2003–04 growing season and stubble was also retained in each plot. In the 2004–05 season, the early-season collection of naturally infected barley leaves was made on 3 March 2005, followed by a late-season collection on 30 April 2005. The leaves were air-dried at room temperature followed by fungal isolations as described previously (3).

Greenhouse pathogenicity tests: aggressiveness on five hosts used in the field experiment. The percentage of leaf area covered by lesions for each barley host was used as a measure of aggressiveness for each of the eight isolates used in the field experiments. Cultures of the eight *R. secalis* isolates were used to produce inoculum for the pathogenicity tests as described by Bouajila et al. (3). The spore concentration was adjusted to 10^6 spores per ml with a hemacytometer.

For each barley host, five seeds were planted in 13 cm plastic pots containing a soil potting mix. There were four replicate pots per host per isolate, giving a total of 160 pots. Seedlings were grown in a greenhouse with a 14-h light/10-h dark photoperiod. The seedlings were inoculated at growth stage 13 (35), 14 to 16 days after planting. The seedlings were sprayed to run-off with a spore suspension (10^6 spores/ml) amended with Tween 20 at the rate of 1 drop per 100 ml, with a Mythos sprayer (model Thema, Di Martino spa, Mussolente, Italy). Inoculated plants were kept in a mist chamber at 12°C in darkness at a relative humidity of 100% for 48 h. After the mist period, the inoculated plants were moved back to the greenhouse with alternating temperatures of 17°C day and 10°C night. The plants were watered two or three times a week from the bottom to prevent cross contamination. Plants inoculated with sterile distilled water and Tween 20 served as controls.

Disease severity was assessed on the lamina of the second leaf from the base of each plant 17 days after inoculation, using the rating scale described by Salamati and Tronsmo (23). Briefly, a 0-to-5 scale was used, where 0 = symptom free, 1 = traces or small necrotic flecks, 2 = some chlorosis or necrosis along margins, 3 = necroses but less than 40% affected tissue, 4 = necroses on 40 to 80% of the lamina, and 5 = more than 80% and up to a fully wilted leaf.

Virulence on differential set of cultivars. The eight *R. secalis* isolates were also assayed for their virulence on 17 differential cultivars. Inoculation and scoring of diseased plants were as described by Bouajila et al. (3). Disease severity was assessed 17 days after inoculation, using the rating scale described by Ceoloni (5) with a slight modification: 0 = no visible symptoms; 1 = small lesions confined to leaf tips and brown to gray necrotic spots; 2 = somewhat larger lesions; 3 = larger and coalescing lesions with distinct margins; and 4 = total collapse of the leaf with no discrete lesions within the wilted area. Lesion types 0, 1, and 2 were considered incompatible (resistant) reactions and lesion types 3 and 4 were considered compatible (susceptible) reactions. A single observer made all disease assessments in both the aggressiveness and virulence tests and pots were assessed randomly with regard to treatments.

DNA extraction and microsatellite analysis. Fungal tissue was scraped off lima bean agar plates and freeze-dried. Approximately 0.05 g of the freeze-dried tissue was ground into a fine powder in a 2-ml microfuge tube with a mixer-mill. Total DNA from each fungal isolate was extracted by a modified hexadecyltrimethylammonium bromide (CTAB) extraction procedure (27). Extracted DNA was resuspended in Tris-EDTA buffer (10 mM/1 mM) and stored at -20°C . Eight microsatellite loci developed for *R. secalis* (17) were assayed in all individuals. These eight loci were amplified in multiplex polymerase chain reaction (PCR) with group I (*Rhyncho_1*, *Rhyncho_3*, *Rhyncho_4*, and *Rhyncho_5*) and II (*Rhyncho_2*, *Rhyncho_6*, *Rhyncho_7*, and *Rhyncho_8**) primers on either a Biometra T-gradient (Biometra GmbH, Goettingen, Germany) or an AB-GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) and subjected to capillary electrophoresis as described earlier (17). Allele assignments for each microsatellite locus were made using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) as described previously (17).

Selection coefficient estimation. Precipitation and temperature data were used to estimate the latent period between successive asexual generations and to estimate the dates of new epidemic cycles. A sprinkler irrigation system operating twice for 2 h each week was put in place to ensure the regular release and splash-dispersal of *R. secalis* spores during the course of the experiment at Tel Hadya. Based on rainfall data and this irrigation schedule, we estimated that the *R. secalis* populations underwent four cycles of asexual reproduction between the early- and the late-season collection at Tel Hadya in 2004 and 2005. We also estimated, based on rainfall distribution, that the pathogen underwent four cycles of asexual reproduction in both years at Lattakia.

The frequencies of the eight *R. secalis* isolates were measured for each time point and host. The relative reproductive fitness, as measured with selection coefficients, was estimated according to the methods described by Zhan et al. (38) based on the estimated number of asexual generations. Zhan's method is based on the standard theory of selection and is appropriate in cases of clonal competition among several strains of haploid organisms (38). Unlike simple measures of reproductive output or transmission rate, the selection coefficient is a parameter reflecting the overall fitness of any genotype/phenotype relative to the most fit isolate in the experimental population during the entire process of infection, colonization, reproduction, survival, and transmission. Thus, selection coefficients were considered to offer the most inclusive measure of the relative fitness for each genotype in an experimental field setting (38).

Statistical analyses. Analyses of variance for genotype frequencies, aggressiveness, and selection coefficients were performed using the general linear models of SAS Version 6.12 for Windows (SAS Institute, Cary, NC) or SPSS version 13.0 for Windows (SPSS Inc., Chicago). Bonferroni's correction was performed for multiple comparisons in SPSS, while Tukey's highly significant

difference was used for multiple comparisons in SAS. Pairwise comparisons were considered statistically significant if the *P* value was <0.05 in a two-sided test. Genotype frequencies were first transformed by natural logarithms to better satisfy the assumptions of analysis of variance. The standard errors of the selection coefficients were calculated for each of the isolates based on variation among 100 resamples of the frequency data using the Resampling Stats software package version 5.02, 2000 (Resampling Stats Inc., Arlington, VA). The genotype frequencies of fungal collections from different sampling times on the same hosts and from different hosts during the same sampling time were compared using contingency χ^2 tests (8) as described previously (38). Genotype diversities were calculated according to the method of Stoddart and Taylor (26) and compared using a *t* test as described by Chen et al. (6).

RESULTS

The incidence, severity, and progression of the epidemics were similar for individual host treatments in 2004 and 2005 at the two sites. In both years at Lattakia and Tel Hadya, disease severity was highest on highly susceptible cvs. Rihane and WI2291. Epidemic progression was similar in plots of both cultivars, and by the end of the growing season, there was 100% disease incidence and flag leaf infection was common on both cvs. Rihane and WI2291. Flag leaf infection was rare on susceptible cv. Arabi Abiad, which had a less severe epidemic compared with Rihane and WI2291. Disease severity and incidence were markedly lower on the moderately resistant landrace cv. Arabi Aswad compared with susceptible cvs. Rihane, WI2291, and Arabi Abiad. In contrast, no scald symptoms were observed on resistant line 9-26 F27, which remained immune throughout the experiments in Lattakia and Tel Hadya. In the mixture, all plants of line 9-26 F27 remained symptomless and disease incidence was about 50% of that observed on Rihane, which was the susceptible component of the mixture. Individual Rihane plants in the mixture showed high disease severity, some with flag leaf infection.

A total of 2,866 fungal isolates were assayed for multilocus haplotype at eight microsatellite loci: 1,135 from Tel Hadya in 2004, 1,073 from Tel Hadya in 2005, 366 from Lattakia in 2004, and 292 from Lattakia in 2005. Among these 2,866 isolates, 20 were "novel" isolates, i.e., they had multilocus haplotypes that did not match the inoculated isolates. These novel isolates arose through mutation, immigration, and recombination (M. M. Abang and B. A. McDonald, unpublished data) and were excluded from analyses presented in this paper.

Greenhouse pathogenicity assays. Aggressiveness tests. Significant differences ($P < 0.001$) in aggressiveness were observed among the eight inoculated isolates on each of the hosts used in the field experiment except line 9-26 F27 (Table 1). This line had no observable infections throughout the study. Isolates caused significantly ($P < 0.05$) less damage on cv. Arabi Aswad (mean severity = 2.65) compared with susceptible cvs. Rihane, Arabi Abiad, and WI2291 (Table 1). Although there was a significant cultivar-isolate interaction ($P < 0.001$), the interactions contributed less (8.6%) to overall variation relative to main effects due to cultivar (69.1%) and isolate (22.2%), providing weak evidence for host specialization in the greenhouse seedling assays. Isolates Sy33 and Sy21 infected significantly less leaf area than the other six isolates in the seedling assays, while isolates Sy56 and Sy40 had the highest overall aggressiveness on the barley hosts used in the field experiment.

Virulence assays. Each isolate had a unique virulence phenotype (pathotype) on the 17 barley differentials (Table 2). Differences were observed in virulence complexity, with isolates Sy15, Sy21, Sy34, and Sy40 being virulent on only four, six, three, and four differentials, respectively, while the other four isolates were virulent on between eight and nine differentials.

Change in frequencies of inoculated isolates. Data are presented for only five isolates because three of the eight inoculated isolates were never recovered in either of the two field experiments. With the exception of isolate Sy43, all isolates that were virulent on more than eight differentials were not found in any of the host treatments throughout the experiment at either location. In contrast, isolates Sy15, Sy21, Sy34, and Sy40, which were virulent on three to six differentials, were sampled from all host populations in both years at the two locations. Interestingly, isolate Sy34, which was virulent on the smallest number of differentials, also attained the highest frequency in all host populations in both years at Tel Hadya (Table 2; Fig. 1).

At Tel Hadya there were significant ($P < 0.001$) differences in inoculant frequencies among host populations in both the 2004 and 2005 growing seasons (Fig. 1). In 2004, the frequencies of all fungal isolates changed in a directional manner on cv. WI2291, except for isolate Sy43 (Fig. 1E). On cv. Rihane, Sy40 could not be detected in the late sampling of 2004 but appeared again in 2005. Similarly, on cv. Arabi Aswad, isolate Sy43 had initial frequencies of 0.06 and 0.04 in 2004 and 2005, respectively, but it was not detected at the end of either season (Fig. 1B).

In 2004, Sy15 had the highest relative fitness (i.e., the lowest selection coefficient) on most host populations, while Sy40 and Sy43 had the lowest fitness (Table 3). Sy21 had intermediate fitness on all host populations, while Sy34 had high fitness on cvs. Rihane and WI2291 but showed intermediate fitness on cvs. Arabi Abiad and Arabi Aswad. Overall, the largest selection coefficients at Tel Hadya in 2004 were on Arabi Aswad and the smallest selection coefficients were on WI2291 (Table 3).

In 2005, Sy43 had a low fitness on most host populations. Sy40 had the highest fitness on Arabi Abiad, Rihane, and the mixture but showed the lowest fitness on WI2291. Sy15 had the lowest fitness on Arabi Abiad but showed a high fitness on WI2291 and Arabi Aswad. Sy15 had the highest fitness on Arabi Aswad in both 2004 and 2005, and showed evidence for specialization on that host. Sy21 had intermediate fitness on all but one host population, while Sy34 had the lowest fitness on Rihane and the mixture with intermediate fitness on the other hosts. Overall, the largest selection coefficients at Tel Hadya in 2005 were on Arabi Aswad and the smallest selection coefficients were on Arabi Abiad and Rihane.

At Lattakia, only Rihane and the mixture had sufficient sample sizes in 2004 to allow a reliable estimation of changes in genotype frequencies. Other populations were therefore excluded from further statistical analyses. Differences in isolate frequencies were significant within each host population during the growing season (Fig. 2A and B; Table 3). The mean selection coefficient was significantly ($P < 0.05$) smaller on the mixture (Fig. 2B) compared with Rihane (Fig. 2A). Sy43 had the highest fitness on Rihane and the mixture, while Sy40 had the lowest fitness on both host treatments.

Relationship between aggressiveness and fitness in the field. There was little correlation between the aggressiveness of the isolates measured in a greenhouse seedling assay on the same five cultivars used in the field experiment and the measured selection coefficients in the field experiment. For example, Sy30 and Sy56 caused significantly ($P < 0.05$) greater damage on barley seedlings than Sy21 (Table 1), yet only Sy21 was detected in the field experiment (Fig. 1).

TABLE 1. Aggressiveness scores (based on leaf area infected) and their standard deviations (in parentheses) of eight isolates of *Rhynchosporium secalis* on each of four barley genotypes^z

Isolates	Arabi Abiad	Arabi Aswad	Rihane-03	WI2291	Overall isolate mean
Sy15	4.75 (0.50) b	3.75 (0.50) c	3.25 (0.50) b	4.75 (0.50) b	4.12 c
Sy21	3.75 (0.50) b	2.25 (0.50) b	4.25 (0.50) bc	4.50 (1.00) b	3.68 b
Sy30	5.00 (0.00) b	2.50 (1.00) bc	4.75 (0.50) cd	5.00 (0.00) b	4.31 cd
Sy33	0.75 (0.50) a	0.50 (0.57) a	1.00 (0.00) a	1.00 (0.00) a	0.81 a
Sy34	5.00 (0.00) b	2.75 (0.50) bc	5.00 (0.00) cd	4.75 (0.50) b	4.37 cd
Sy40	5.00 (0.00) b	3.50 (1.29) bc	5.00 (0.00) cd	4.75 (0.50) b	4.56 d
Sy43	5.00 (0.00) b	2.75 (0.50) bc	4.75 (0.50) cd	5.00 (0.00) b	4.37 cd
Sy56	4.75 (0.50) b	3.25 (0.50) bc	5.00 (0.00) cd	5.00 (0.00) b	4.50 d

^z Multiple mean comparisons were performed using the *t* test based on Tukey's highly significant difference test. Mean values followed by different letters within a column differ significantly at $P = 0.05$.

TABLE 2. Pathotype analysis of *Rhynchosporium secalis* isolates used in the field experiment

Cultivar ^x	Number ^y	Resistance genes	<i>Rhynchosporium secalis</i> isolate							
			Sy15	Sy21	Sy30	Sy33	Sy34	Sy40	Sy43	Sy56
Armelle		Rh, BRR1	0	0	1	0	0	0	0	0
Astrix		BRR2	0	2	0	0	0	0	0	0
Athene		BRR3	0	3	3	3	1	2	3	3
Igri		BRR4	3	3	4	3	2	3	4	0
La Mesita	7565	Rh4; Rh10; <i>Rh</i> at Rh-Rh3-Rh4, BRR5	2	3	3	4	3	2	4	4
Osiris	1622	Rh4; rh6; Rh10; (Rh3?) BRR6	0	2	4	4	2	2	3	3
Pirate		BRR7	1	0	2	0	0	1	1	1
Digger		Partial resistance	4	4	4	3	3	3	4	4
Trebi	936	Rh4; rh6 = <i>rh</i> ?; <i>Rh</i> at Rh-Rh3-Rh4	0	0	3	0	0	0	1	0
Jet	967	<i>rh</i> , rh6, rh7	4	1	4	3	1	3	4	3
Kitchin	1296	Rh9 (incomplete)	0	0	3	2	0	0	3	4
Steudelli	2266	<i>Rh/rh</i> , rh6; rh7	4	3	2	1	2	2	3	3
Bey	5581	Rh3 (?)	0	0	2	0	0	0	1	0
Atlas 46	7323	Rh; Rh2; Rh3	0	0	0	0	0	0	0	0
Modoc	7566	(Rh4); Rh2; rh6; (Rh3?); <i>Rh</i> at Rh-Rh3-Rh4	0	0	1	0	0	0	2	0
Forrajera	8158	Unknown	0	3	4	4	4	3	2	4
Abyssinia	668	(Rh1); Rh9	0	2	2	4	0	0	2	3
Total ^z			4	6	9	8	3	4	8	9

^x Table adapted from H. O. Pinnschmidt and Jakob Willas and available on the International Barley Scald Working Group website (<http://www.crpmb.org/scald/>).

^y Cultivar identification number.

^z Number of differentials on which an isolate produced a disease score of ≥ 3 .

Rate of pathogen evolution in different host populations during the parasitic phase. At Tel Hadya in 2004, the pathogen population evolved most slowly (i.e., the selection coefficients were smallest) on susceptible cvs. WI2291, Rihane, and Arabi

Abiad. Evolution was fastest on the moderately resistant landrace Arabi Aswad. This pattern was similar in 2005. A comparison between Tel Hadya and Lattakia was possible only for Rihane in 2004. On this cultivar, the genetic structure of the pathogen popu-

Fig. 1. Frequencies of five *Rhynchosporium secalis* isolates on different host treatments (Arabi Abiad, Arabi Aswad, Rihane, mixture of Rihane and Line 9-26 F27, and WI2291) at Tel Hadya over the 2004 and 2005 growing seasons. Differences in genotype frequency are significant ($P < 0.001$) for each treatment in both years. Numbers in parentheses indicate sample sizes for each collection. Tel-ES, Tel-MS, and Tel-LS are early-, mid-, and late-season collections.

lation (i.e., the genotype frequencies of the inoculants) in the early season was significantly different from that in the late season in Lattakia ($\chi^2 = 9.63$, 4 df, $P < 0.05$) but was unchanged in Tel Hadya ($\chi^2 = 4.52$, 4 df).

TABLE 3. Analysis of variance of selection coefficients in 2004 (at Tel Hadya and Lattakia) and 2005 (Tel Hadya)

Location and cultivar	Isolate	Mean selection coefficient 2004 ^w	Mean selection coefficient 2005
Tel Hadya			
Arabi Abiad	Sy15	0.06 (0.04–0.09)	0.36 (0.34–0.38)
	Sy21	0.23 (0.20–0.25)	0.18 (0.16–0.20)
	Sy34	0.17 (0.15–0.19)	0.20 (0.19–0.22)
	Sy40	0.37 (0.35–0.39)	0.02 (0.00–0.04)
	Sy43	0.14 (0.11–0.16)	0.26 (0.24–0.28)
Mean		0.19 b	0.21 d
Arabi Aswad	Sy15	0.02 (0.00–0.04)	0.00 (nd)
	Sy21	0.39 (0.36–0.41)	0.48 (0.46–0.50)
	Sy34	0.25 (0.22–0.27)	0.60 (0.58–0.62)
	Sy40	0.53 (0.50–0.55)	0.58 (0.56–0.59)
	Sy43	0.59 (0.57–0.62)	0.79 (0.77–0.81)
Mean		0.35 a	0.49 a
Rihane	Sy15	0.13 (0.11–0.16)	0.10 (0.08–0.13)
	Sy21	0.15 (0.13–0.17)	0.33 (0.30–0.35)
	Sy34	0.02 (0.00–0.04)	0.33 (0.31–0.35)
	Sy40	0.31 (0.29–0.33)	0.07 (0.05–0.09)
	Sy43	0.26 (0.24–0.29)	0.20 (0.18–0.22)
Mean		0.17 b	0.21 d
WI2291	Sy15	0.08 (0.06–0.10)	0.00 (nd)
	Sy21	0.17 (0.14–0.19)	0.47 (0.45–0.49)
	Sy34	0.07 (0.04–0.09)	0.57 (0.55–0.59)
	Sy40	0.14 (0.11–0.16)	0.61 (0.59–0.63)
	Sy43	0.20 (0.18–0.23)	0.57 (0.55–0.59)
Mean		0.13 c	0.44 b
Mixture	Sy15	nd	0.32 (0.29–0.34)
	Sy21	nd	0.35 (0.33–0.37)
	Sy34	nd	0.49 (0.47–0.51)
	Sy40	nd	0.01 (0–0.03)
	Sy43	nd	0.35 (0.33–0.37)
Mean		nd	0.30 c
Standard error ^x		0.01	0.01
Isolate × cultivar ^y		F value = 82.91 ($P < 0.0001$)	F value = 287.41 ($P < 0.0001$)
Isolate × cultivar × year ^z		F value = 169.57 ($P < 0.0001$)	
Lattakia			
Rihane	Sy15	0.14 (0.11–0.16)	nd
	Sy21	0.11 (0.09–0.13)	nd
	Sy34	0.35 (0.33–0.37)	nd
	Sy40	0.40 (0.38–0.43)	nd
	Sy43	0.06 (0.04–0.08)	nd
Mean		0.21 a	
Mixture	Sy15	0.16 (0.14–0.18)	nd
	Sy21	0.12 (0.10–0.15)	nd
	Sy34	0.14 (0.12–0.17)	nd
	Sy40	0.24 (0.22–0.26)	nd
	Sy43	0.10 (0.08–0.13)	nd
Mean		0.15 b	
Standard error		0.01	
Isolate × cultivar		F value = 51.88 ($P < 0.0001$)	
Tel Hadya vs. Lattakia			
Isolate × location (for Rihane in 2004)		F value = 184.08 ($P < 0.0001$)	

^w Values in brackets are the lower and upper bounds of the 95% confidence interval. Overall cultivar means followed by different letters are significantly different ($P < 0.05$).

^x Standard error of the mean selection coefficient of the isolates. Standard error and confidence intervals were not estimated for Sy15 on Arabi Aswad and WI2291 because the mean was 0 in all replications.

^y Cultivar × isolate interaction as determined by univariate analysis of variance using SPSS software (Chicago).

^z Fungal populations sampled from the mixture were excluded from this analysis. nd = not determined due to small sample size.

Pathogen isolates had the highest mean selection coefficients on Arabi Aswad and the lowest mean selection coefficients on WI2291 in 2004 (Table 3). Selection was significantly higher ($P < 0.05$) on Arabi Aswad than on all other hosts and this was consistent across years at Tel Hadya. In contrast, the selection coefficients on WI2291 changed significantly ($P < 0.05$) over time, being the lowest in 2004 and second only to that of Arabi Aswad in 2005. In 2005 at Tel Hadya, the overall selection coefficient of fungal isolates on the mixture (0.30) was intermediate between those on Rihane (0.21) and Arabi Abiad (0.21) on the one hand, and those on WI2291 (0.45) and Arabi Aswad (0.49) on the other. The overall selection coefficient of fungal isolates on the mixture in 2004 at Lattakia (0.15) was significantly lower ($P < 0.05$) than that on the pure stand of Rihane (0.21).

Genotype diversity in the pathogen collections across host populations. Although not significant, genotype diversity in pathogen collections from all five host treatments at Tel Hadya decreased from early to late season in 2004 (Table 4). The fungal collection from Arabi Aswad in 2004 consistently had the lowest genotype diversity, while that from WI2291 had the highest diver-

Fig. 2. Frequencies of five *Rhynchosporium secalis* isolates on two host treatments (Rihane and a mixture of Rihane and Line 9-26 F27) at Lattakia during the 2004 growing season. Differences in genotype frequency are significant ($P < 0.001$) for each treatment. Numbers in parentheses indicate sample sizes for each collection. Lat-ES and Lat-LS are early- and late-season collections.

sity. During the 2005 growing season at Tel Hadya, there was an increase in genotype diversity in pathogen collections from all host treatments except WI2291. The genotype diversity in the fungal collection from the mixture increased significantly ($P < 0.05$) during the 2005 growing season at Tel Hadya, while those of other host populations increased only slightly in the course of the season. In the early season of 2005 at Tel Hadya, the genotype diversity of the fungal collection from the mixture was significantly ($P < 0.05$) lower in comparison to the collection from the susceptible component Rihane. Comparison between Tel Hadya and Lattakia showed that genotype diversities decreased during 2004 at Tel Hadya while increasing over the season at Lattakia. In 2004, the genotype diversities of the pathogen populations from the mixture and Rihane at Tel Hadya were significantly ($P < 0.05$) lower than the genotype diversities on the same hosts at Lattakia (Table 4).

Temporal and spatial variation in fitness and local adaptation of pathogen strains. No isolate consistently increased in frequency on Rihane across years and locations while decreasing in frequency on all other host populations. The fungal isolates varied in their degrees of adaptation to particular hosts at Tel Hadya as indicated by a highly significant host-pathogen interaction ($P < 0.0001$) in the analysis of variance of selection coefficients in both 2004 and 2005 (Table 3). When data were pooled across years, there was a highly significant ($P < 0.0001$) cultivar-isolate-year interaction (Table 3). For example, Sy40 and Sy43 decreased in frequency and had significantly higher selection coefficients on Rihane at Tel Hadya in 2004 but increased in frequency and had significantly lower selection coefficients on Rihane in 2005. Comparison between Tel Hadya and Lattakia in 2004 showed that, in the early season, the inoculant frequencies from Rihane and the mixture were similar at Tel Hadya but significantly different in Lattakia ($\chi^2 = 9.7$, 4 df, $P < 0.05$).

Changes in genotype frequencies during the saprophytic phase. Changes in isolate frequencies between years. At Tel Hadya, the frequencies of isolates sampled from Arabi Aswad changed significantly ($\chi^2 = 15.72$, 4 df, $P < 0.01$) between growing seasons. Sy15 decreased in frequency from 0.13 to 0.00 and Sy40 increased in frequency from 0.03 to 0.12. A significant ($\chi^2 = 16.18$, 4 df, $P < 0.01$) change in isolate frequencies between years was also observed in WI2291, where the frequency of Sy15 decreased from 0.17 to 0.01 while Sy43 increased from 0.06 to 0.13. Also, the frequency of Sy21 increased from 0.23 to 0.36 while that of Sy34 fell slightly from 0.50 to 0.46. These results suggest

that Arabi Aswad and WI2291 had a significant effect on the fitness of particular fungal strains during the saprophytic phase of the disease cycle (Fig. 1).

DISCUSSION

Although the eight pathogen isolates used in this study originated from the same field and host, they possessed rare alleles for at least one of the eight characterized microsatellite loci. Our null hypothesis was that the fungal strains would possess the same level of virulence and that all strains would have equal fitness on all host treatments. The alternative hypothesis was that the strains would possess different levels of virulence and aggressiveness, and that the strains would differ in fitness on all host treatments. Significant differences were observed in aggressiveness (as measured with traditional greenhouse seedling assays) and in selection coefficients on different hosts (as measured in the field experiment). Virulence tests using 17 barley differentials revealed that each of the eight isolates was a unique pathotype (Table 2). This finding is consistent with a recent study in Tunisia that found 93 *R. secalis* pathotypes among 100 isolates tested using the same set of host differentials (3), and with other studies using neutral markers that found high genetic diversity within pathogen populations sampled from individual barley fields (14,24,28).

Spatial and temporal variation in selection coefficients during the parasitic phase. We found that individual *R. secalis* genotypes differed in fitness on the different hosts at Tel Hadya in 2004 compared with 2005, and on the same host at Tel Hadya and Lattakia (Fig. 1; Table 3). Considering that each location or year represented a different environment, these results indicate that physical environment also plays an important role in shaping the composition of local *R. secalis* populations (11,13). The starting point in isolate frequencies in Tel Hadya was very different in 2004 and 2005. For instance, Sy34 had the highest initial frequencies in 2004 and increased in frequency on all hosts during the year, but its frequency decreased on these hosts in 2005. On the other hand, Sy21 had lower initial frequencies and decreased in frequency in 2004, but its frequency consistently increased in 2005 on all treatments except Rihane. Temporal variation in selection coefficients may have been affected by frequency dependent selection which favored the minority genotype Sy21 in 2005 relative to the majority genotype Sy34. It is also possible that different strains were favored in different physical environments. Pathogen fitness depends on the susceptibility of the host, the reproduction ability of the pathogen, and the suitability of the physical environment, including factors such as temperature, light intensity, humidity, and dew period (15). The spatial and temporal dynamics of host selection in the *R. secalis*-barley plant pathosystem is likely to be affected by all of these factors.

Selection during the saprophytic phase of the disease cycle. The genetic structure of the pathogen populations on Arabi Aswad and WI2291 changed significantly ($P < 0.01$) during the saprophytic phase of the disease cycle. Differences in survival abilities on barley stubble affected the frequencies of particular *R. secalis* genotypes (e.g., Sy15 and Sy40) in some host populations (e.g., Arabi Aswad and WI2291) between growing seasons (Fig. 1). The intense competition that occurs among *R. secalis* genotypes and with other fast-growing saprophytes on stubble is likely to favor isolates that have good saprophytic abilities. This may have a major impact on pathogen genetic structure over time. For example, Sy21 decreased in frequency on Rihane and WI2291 during the parasitic phase but its frequency increased during the saprophytic phase (Fig. 1E). In contrast, Sy15 increased significantly in frequency during the parasitic phase of growth on Arabi Aswad, Rihane, and WI2291 but decreased in frequency on all three hosts during the saprophytic phase. Competitive ability on stubble during the saprophytic phase and aggressiveness on living hosts during the epidemic phase represent two separate but com-

TABLE 4. Genotype diversities and their standard errors (in parenthesis) in *Rhynchosporium secalis* collections made from different host treatments and sampling times in Tel Hadya in 2004 and 2005, and Lattakia in 2004

Treatments	n^y	Early-season ^z	Late-season ^z
Tel Hadya 2004			
Arabi Abiad	130 (56)	3.006 (0.219) ab	2.835 (0.387) a
Arabi Aswad	63 (32)	2.747 (0.257) a	2.317 (0.382) a
Mixture	127	2.946 (0.237) ab	nd
Rihane	127 (36)	2.856 (0.239) ab	2.571 (0.418) a
WI2291	131 (48)	3.461 (0.279) bc	2.977 (0.436) a
Tel Hadya 2005			
Arabi Abiad	112 (111)	2.924 (0.246) c	3.057 (0.229) ab
Arabi Aswad	100 (87)	2.344 (0.222) b	2.776 (0.222) ab
Mixture	91 (97)	1.793 (0.184) a	3.826 (0.336) c
Rihane	110 (128)	2.722 (0.226) bc	3.374 (0.318) bc
WI2291	123 (111)	2.820 (0.185) c	2.670 (0.203) a
Lattakia 2004			
Mixture	59 (43)	4.379 (0.339) a	4.455 (0.351) a
Rihane-03	70 (54)	3.852 (0.366) a	4.445 (0.341) a

^y n = sample size; values within and outside parentheses represent the sample size of the early- and late-season collection, respectively.

^z For each environment, values within columns followed by different letters are significantly different ($P < 0.05$). nd = not determined; small sample size in late-season did not allow meaningful estimation.

plementary processes that are vital to the evolution of *R. secalis* (25). The existence of differential selection during parasitic and saprophytic phases suggests that mutant pathotypes able to infect newly released resistant cultivars may emerge more slowly than expected if the increase in frequency of the favored pathotypes during the parasitic phase is offset by the decrease in frequency during the saprophytic phase of the disease cycle.

Evidence for a trade-off between virulence complexity and pathogen fitness. There was a strong negative correlation between the pathotype complexity exhibited by the *R. secalis* isolates on the barley differentials in greenhouse tests and their competitive fitness in the field. Four of the eight *R. secalis* isolates were virulent on three to six differentials. The other four isolates were virulent on between eight to nine differentials. Of the four isolates with complex virulence, only Sy43 was found in the host populations at Tel Hadya. Our finding that virulence complexity was negatively correlated with fitness suggests that directional selection against unnecessary virulence operates in this pathosystem. Unnecessary genes for virulence have also been found in *R. secalis* populations from Idaho and Oregon (11), Norway (23), Denmark (13), and Canada (31). A shift in the *R. secalis* population from complex races toward simple races was also reported by Jackson and Webster (12) and others (29,41).

Effect of host resistance on pathogen fitness and evolution. The selection coefficients were distributed over a narrower range and with a significantly ($P < 0.05$) smaller mean selection coefficient on the mixture treatment compared with the pure stand of Rihane at Lattakia in 2004 (Table 3, Fig. 2), suggesting that disruptive selection might have been operating in the mixture. However, there was a highly significant ($P < 0.001$) change in genotype frequencies on the mixture at Tel Hadya in 2005 (Table 3, Fig. 1D), which argues against the expectation that a cultivar mixture would slow down the rate of evolution in pathogen populations (38). Host mixtures have been proposed as an alternative method to retard pathogen evolution in agricultural ecosystems (19), but our findings indicate that the rate-retarding effect of mixtures on pathogen evolution may be affected by the environment. Environmental conditions can influence host diversity effects on disease through their effect on factors such as host density and epidemic length and intensity (10).

Line 9-26 F27 has consistently shown a highly resistant scald reaction in the field (34) and was not infected by any of the eight isolates applied individually in the greenhouse or as a mixture in the field. Pathogen isolates had the lowest overall fitness and showed the most rapid evolution on moderately resistant landrace Arabi Aswad. The generally lower genotype diversity of isolates on Arabi Aswad, the wide range over which selection coefficients were distributed, and the significantly greater change in isolate frequencies in both years compared with susceptible hosts raise questions about the durability of the resistance operating in Arabi Aswad. For instance, though Sy15 occurred at relatively low frequency in the field, this isolate was slightly more virulent on Arabi Aswad than on susceptible cv. Rihane (Table 1). Also, Sy15 increased in frequency on this host in both years (Fig. 1; Table 3). The black seed color, palatable stem, excellent tillering, and drought tolerance of Arabi Aswad have made this landrace the favorite and most widely cultivated cultivar in most parts of drought-prone northern Syria where the fungal isolates were sampled. Although Arabi Aswad is a landrace cultivar with supposedly quantitative resistance, the presence of isolates such as Sy15 suggests its large-scale deployment in monoculture could select for specifically adapted pathogen isolates and lead to the erosion of the scald resistance (9,19).

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