Reduction in Pycnidial Coverage After Inoculation of Wheat with Mixtures of Isolates of *Septoria tritici*

NOGA ZELIKOVITCH and Z. EYAL, Department of Botany, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

ABSTRACT

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Inoculation of wheat seedlings with certain combinations of isolates of *Septoria tritici* resulted in marked reductions in pycnidial coverage of leaves compared with plants inoculated separately with individual components of the mixture. The effect was greater when the isolates were grown together than when grown separately and mixed before inoculation. The suppression of pycnidial formation did not depend on the ratio of the isolates in the mixtures. The addition of a culture filtrate from one isolate to the conidial preparation of another isolate resulted in marked symptom suppression. In conidial preparations where the growth medium was decanted and the spores were resuspended in fresh medium, marked reductions in pycnidial coverages were observed on all cultivars. These results suggest that *S. tritici* may produce substances that regulate the expression of symptoms on wheat leaves inoculated with isolate mixtures. Challenge inoculations of Kavkaz winter wheat with an isolate several days after the first inoculation with a different isolate also resulted in significant reductions in pycnidial coverage. It is possible that a resistant host response is triggered by the first inoculation.

Additional keywords: Septoria tritici blotch, *Triticum aestivum*

Septoria tritici blotch of wheat (*Triticum aestivum* L.), caused by the fungus *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn (anamorph = *Septoria tritici* Roberge in Desmaz.), is a major wheat disease in many parts of the world, causing severe losses in yield (10). Breeding for host resistance is considered the main defense against this disease but has not yielded adequate protection against it (4). Resistant germ plasm is not abundant and is often associated with undesirable late maturity and excessive plant height (5). Differentiation of host response to the pathogen is based on quantitative assessment of symptoms, and qualitative assessment of symptoms is rarely possible (10).

Marked differences in the geographic distribution of pathogenicity patterns of *S. tritici* within regions and countries were reported by several investigators (9,12). Significant cultivar-isolate interactions indicated the presence of specific virulences and specific genes for resistance in the interacting systems (7,12). Moreover, in these independent studies, a similar set of differential wheat cultivars was employed. In several programs where germ plasm and breeding materials are being evaluated under field conditions, Septoria tritici blotch epidemics are incited by artificial inoculation with a mixture of isolates of *S. tritici* of national origin (14). It is assumed that the expression of the various virulences present in the isolate mixture would be expressed and detected on proper wheat differentials included among the evaluated germ plasm. It was observed that the expression of specific virulences included in the isolate mixture is not readily exhibited as expected (14). The objectives of the present study were to assess the expression of pathogenicity after inoculation with conidial preparations or challenge inoculation using mixtures of isolates of *S. tritici* differing in their specific virulences.

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MATERIALS AND METHODS

Cultures of isolates of *S. tritici* were grown in liquid medium containing 30 g/L of sucrose and 10 g/L of yeast extract and shaken for 4 days on rotary shakers at 20 C. Four isolates of *S. tritici*, ISR398 (ATCC 148507), ISR7901, ISR8036, and ORG82076 (from Corvallis, OR), that differ in their virulence on a set of differential wheat cultivars (14) were used. The isolates were maintained on malt agar medium for 2–3 mo after routine culturing every 2 wk. Virulence of the cultures was maintained by reisolation of pycnidiospores from pycnidia produced on wheat leaf-extract medium (13).

Ten-day-old seedlings (two-leaf stage) of susceptible semidwarf spring bread wheat cultivars Ceeon (Yt54A*3//N10/Bvr) and Shafir (Son64A-Tzpp/Nai60//FA) and the winter bread wheat Kavkaz (Lutescens 314H174/Bezostaya 1) were used in the study. Twenty plants of each cultivar were grown in soil in 30 × 30 × 7 cm containers (two containers per treatment). A conidial suspension of 40 ml of 1×10^7 spores per milliliter was sprayed onto 10-day-old wheat seedlings as described by Eyal and Scharen (8). Seedlings were sprayed with individual isolates or with a mixture of isolates. Isolate mixtures were prepared either by growing isolates together or by mixing individual cultures before inoculation in 1:1 or in alternating 8:2 or 2:8 ratios. In addition, centrifuged conidia of the two isolates were resuspended in fresh medium, and centrifuged conidia of one isolate were mixed with a culture filtrate of the other isolate. The effect of challenge inoculation was studied for isolates ISR398 and ISR8036 of *S. tritici*, whereas ISR398 produced low levels of pycnidia on Kavkaz. Challenge inoculations were done by inoculating 10-day-old seedlings of Kavkaz with one isolate, followed by another inoculation 7 days later with the second isolate.

The inoculated seedlings were placed in a humidity chamber within a temperature-controlled greenhouse (22 ± 1 C) where continuous mist was supplied by a humidifier for 48 hr. Plants were removed to a bench in the same greenhouse room at the same temperature.

The containers within the humidity chamber or on the greenhouse bench were completely randomized. Severity (estimation of percent pycnidial coverage on first leaf) was visually assessed 21 days after inoculation with standard drawings (6,10). Reductions in pycnidial coverage were calculated as the differences from the expected mean pycnidial coverage of each of the mixed isolates.

Analysis of variance (ANOVA) was used to evaluate the significance of differences in pycnidial coverage on Ceeon and Shafir for the different isolate preparations. Means were compared with Duncan's multiple range test. The expected pycnidial coverage for mixtures was calculated with the relative proportion of each of the two component isolates in the mixture. Reductions or increases in pycnidial coverage were calculated based on the differences of the recorded coverages from the expected values.

RESULTS

Effect of mixtures on pycnidial coverage. Inoculation of seedlings of the susceptible cultivar Shafir with mixtures

of three or four virulent isolates of *S. tritici* resulted in marked reductions in pycnidial coverages compared with coverages recorded for each of the isolates comprising the mixtures (Table 1). Inoculation with a mixture of three Israeli isolates (mixture 1) where the isolates were grown together (treatment A) resulted in reductions in pycnidial coverage greater than 58%. Inoculation with the same mixture, but where isolates were grown separately (adjusted to 1×10^7 spores per milliliter and mixed in equal proportion before inoculation) (treatment B), resulted in reductions ranging from 5.1 to 38.8%. Similarly, marked reductions in pycnidial coverages were obtained for each of the two treatments after inoculation with the mixture of the same three Israeli isolates to which the Oregon isolate (ORG82076) was added (mixture 2).

Table 1. Effect of isolates of *Septoria tritici* grown separately, in mixtures, or mixed before inoculation, on percent reduction in pycnidial coverage on seedlings of wheat cultivar Shafir

Isolate	Pycnidial coverage (%)	Mixture 1[v]		Mixture 2[w]	
		A[x]	B[y]	A	B
ISR398	45.0	75.9**[z]	38.8**	72.0**	51.0**
ISR7901	35.4	69.3**	22.0**	64.5**	37.7**
ISR8036	26.1	58.3**	5.1	52.0**	15.5*
ORG82076	20.9	...	...	40.0**	3.2
Difference between A and B		60.0**		43.0**	

[v] Mixture 1 = IRS398 + ISR7901 + ISR8036.
[w] Mixture 2 = ISR398 + ISR7901 + ISR8036 + ORG82076.
[x] Treatment A = isolates grown together in liquid sucrose + yeast extract medium.
[y] Treatment B = isolates grown separately adjusted to 1×10^7 spores per milliliter and mixed before inoculation in equal proportions.
[z] * = $P = 0.05$; ** = $P = 0.01$.

Mixture combinations. Mixing isolates ISR398 and ISR8036 in a 1:1 ratio after adjustment to 1×10^7 spores per milliliter caused marked reduction in pycnidial coverage on seedlings of the two wheat cultivars (Table 2). Changing the ratios (8:2 or 2:8) of the two isolates in the mixture resulted in similar reductions to that of the 1:1 ratio. Suspension of conidial precipitate of the two isolates in fresh sucrose + yeast medium in a 1:1 ratio resulted in reductions of 58.3% on Ceeon and 69.9% on Shafir. Adding culture filtrate of ISR8036 to resuspended conidia of isolate ISR398 increased pycnidial coverage on Ceeon. Reductions in pycnidial coverage of 12.2% on Ceeon and 16.2% on Shafir occurred when the filtrate of ISR398 was mixed with resuspended conidial precipitate of ISR8036, although these were not

Table 2. Effect of mixture ratios of isolates ISR398 and ISR8036 and culture conditions on percent pycnidial coverage on susceptible spring wheat cultivars Ceeon and Shafir

Isolates and treatment[v]	Ratio[w]	Ceeon			Shafir		
		Observed	Expected[x]	Reduction[y] (%)	Observed	Expected	Reduction (%)
ISR398 (cc)		37.2 a[z]	...	...	71.1 a	...	...
ISR8036 (cc)		7.3 cd	...	...	45.1 c	...	...
ISR398 (cm)		21.5 b	...	...	54.9 b	...	...
ISR8036 (cm)		4.9 cd	...	...	42.7 cd	...	...
ISR398 (cc) + ISR8036 (cc)	1:1	6.5 cd	22.2	70.7	34.8 e	58.1	40.1
ISR398 (cc) + ISR8036 (cc)	8:2	9.4 c	31.2	69.8	19.6 fg	65.9	70.2
ISR398 (cc) + ISR8036 (cc)	2:8	3.0 d	13.3	77.2	22.8 f	50.3	54.7
ISR398 (cm) + ISR8036 (cm)	1:1	5.5 cd	13.2	58.3	14.7 g	48.8	69.9
ISR398 (cm) + ISR8036 (m)	...	34.1 a	21.5	−58.6	59.8 b	54.9	−8.9
ISR398 (m) + ISR8036 (cm)	...	4.3 cd	4.9	12.2	35.8 de	42.7	16.2

[v] cc = Conidial culture in liquid sucrose + yeast extract in which it grew, cm = centrifugation of conidial culture and resuspension of conidial precipitate in fresh sucrose + yeast medium, and m = culture filtrate after removing conidia by centrifugation.
[w] Adjusted to 1×10^7 spores per milliliter and mixed before inoculation.
[x] (Isolate A + Isolate B)/relative proportion of isolates in mixture.
[y] [(Expected − Observed)/Expected] × 100.
[z] Values followed by the same letter are not significantly ($P = 0.01$) different by Duncan's multiple range test.

Table 3. Effect of mixture ratios of isolates ISR398 and ISR7901 and different treatments on percent pycnidial coverage on susceptible spring wheat cultivars Ceeon and Shafir

Isolates and treatment[v]	Ratio[w]	Ceeon			Shafir		
		Observed	Expected[x]	Reduction[y] (%)	Observed	Expected	Reduction (%)
ISR398 (cc)		37.2 a[z]	...	...	71.1 a	...	...
ISR7901 (cc)		14.6 c	...	...	37.4 de	...	...
ISR398 (cm)		21.5 b	...	...	54.9 b	...	...
ISR7901 (cm)		16.3 c	...	...	44.2 cd	...	...
ISR398 (cc) + ISR7901 (cc)	1:1	2.6 e	25.9	89.9	33.6 e	54.3	38.0
ISR398 (cc) + ISR7901 (cc)	8:2	4.3 de	32.6	86.7	60.5 b	64.4	6.0
ISR398 (cc) + ISR7901 (cc)	2:8	5.5 de	19.1	70.9	44.6 cd	44.2	0.0
ISR398 (cm) + ISR7901 (cm)	1:1	1.7 e	18.9	91.0	36.7 de	49.6	26.0
ISR398 (cm) + ISR7901 (m)	...	33.3 a	21.5	−54.9	57.6 b	54.9	−4.9
ISR398 (m) + ISR7901 (cm)	...	8.8 d	16.3	46.0	45.6 c	44.2	3.2

[v] cc = Conidial culture in liquid sucrose + yeast extract in which it grew, cm = centrifugation of conidial culture and resuspension of conidial precipitate in fresh sucrose + yeast medium, and m = culture filtrate after removing conidia by centrifugation.
[w] Adjusted to 1×10^7 spores per milliliter and mixed before inoculation.
[x] (Isolate A + Isolate B)/relative proportion of isolates in mixture.
[y] [(Expected − Observed)/Expected] × 100.
[z] Values followed by the same letter are not significantly ($P = 0.01$) different by Duncan's multiple range test.

Table 4. Effect of mixture ratios of isolates ISR7901 and ISR8036 and culture conditions on percent pycnidial coverage on susceptible spring wheat cultivars Ceeon and Shafir

Isolates and treatment[v]	Ratio[w]	Ceeon			Shafir		
		Observed	Expected[x]	Reduction[y] (%)	Observed	Expected	Reduction (%)
ISR7901 (cc)		14.6 a[z]	...	...	37.4 bc	...	...
ISR8036 (cc)		7.3 b	...	...	45.1 a	...	...
ISR7901 (cm)		16.3 a	...	...	44.2 ab	...	...
ISR8036 (cm)		4.9 bc	...	...	42.7 ab	...	...
ISR7901 (cc) + ISR8036 (cc)	1:1	6.7 b	10.9	38.4	19.3 d	41.3	53.3
ISR7901 (cc) + ISR8036 (cc)	8:2	8.2 b	13.1	37.4	19.3 d	39.0	50.5
ISR7901 (cc) + ISR8036 (cc)	2:8	4.4 bc	8.7	49.9	25.7 d	43.6	41.1
ISR7901 (cm) + ISR8036 (cm)	1:1	2.0 c	10.6	80.7	20.3 d	43.5	53.2
ISR7901 (cm) + ISR8036 (m)	...	4.1 bc	16.3	74.8	11.9 e	44.2	73.1
ISR7901 (m) + ISR8036 (cm)	...	5.9 bc	4.9	−20.4	33.2 c	42.7	22.2

[v] cc = Conidial culture in liquid sucrose + yeast extract in which it grew, cm = centrifugation of conidial culture and resuspension of conidial precipitate in fresh sucrose + yeast medium, and m = culture filtrate after removing conidia by centrifugation.
[w] Adjusted to 1×10^7 spores per milliliter and mixed before inoculation.
[x] (Isolate A + Isolate B)/relative proportion of isolates in mixture.
[y] [(Expected − Observed)/Expected] × 100.
[z] Values followed by the same letter are not significantly ($P = 0.01$) different by Duncan's multiple range test.

Table 5. Effects of challenge inoculations with different isolates of *Septoria tritici* on percent reduction in pycnidial coverage on Kavkaz wheat seedlings

Isolate	Seedling age (days)	Pycnidial coverage (%)	ISR398[x] + ISR8036	ISR398[y] + ISR8036	ISR8036[y] + ISR398
ISR8036	10	53.1	77.2*[z]	...	78.3*
ISR398	10	5.9	2.1	1.5	...
ISR8036	17	26.1	...	74.6*	...
ISR398	17	0.1	...	...	2.9

[x] Isolates mixed and/inoculated together using 1×10^7 spores per milliliter.
[y] First isolate inoculated on 10-day-old seedlings and challenged 7 days later with second isolate.
[z] Asterisk indicates significant at $P = 0.05$.

significant. Mixing isolates ISR398 and ISR7901 of *S. tritici* (Table 3) or isolates ISR7901 and ISR8036 (Table 4) in 1:1, 8:2, or 2:8 ratios resulted in marked reductions in pycnidial coverages. Mixing suspensions of conidial precipitates with fresh medium also resulted in marked reductions. Pycnidial coverage was increased when a resuspended preparation of ISR398 was mixed with filtrate of ISR7901 (Table 3) or with filtrate of ISR8036 (Table 2). In the reciprocal combination where resuspended preparations of ISR7901 or ISR8036 were mixed with filtrate of ISR398, pycnidial coverages were lessened (Tables 2 and 3). When conidia of ISR8036 were mixed with filtrate of ISR7901, an increase in pycnidial coverage was recorded on Ceeon (Table 3).

Challenge inoculations. Inoculation of Kavkaz with the mixture of ISR398 and ISR8036 resulted in significant reduction in pycnidial coverage compared with inoculation with the virulent isolate ISR8036 (Table 5). Almost no change was recorded on Kavkaz when compared with avirulent isolate ISR398. Inoculation of 10-day-old seedlings of Kavkaz with ISR398 and reinoculation of the same leaves 7 days later with ISR8036 resulted in a nonsignificant reduction compared with ISR398 but a significant reduction when compared with ISR8036. Reversing the order of inoculation, i.e., challenging with ISR398 after inoculation with ISR8036, resulted in a reduction when compared with ISR8036 but no significant change when compared with ISR398 (Table 5).

DISCUSSION

The response of wheat cultivars to *S. tritici* is evaluated by assessing the level of chlorosis, necrosis, or pycnidial density (10). Under optimal environmental conditions during 15–21 days postinoculation (18–25 C, high relative humidity, and illumination of 400 $\mu E \cdot s^{-1} \cdot m^{-1}$ pycnidial formation is usually induced on most wheat cultivars (2,10). Chlorosis or necrosis with no pycnidial formation is expressed in suboptimal environmental conditions, with highly resistant cultivars, or upon inoculation across graminaceous genera and species with isolates of *S. tritici* (3,10). It is highly probable that under proper environmental conditions, susceptible bread wheat cultivars inoculated with a single virulent isolate of *S. tritici* will consistently express high pycnidial production.

Suppressed pycnidial production after inoculation of wheat seedlings with isolate mixtures is a rather unique phenomenon. Marked reductions in symptom expression (percent pycnidial coverage) on susceptible wheat cultivars were observed by inoculation with mixed combinations of two to four isolates of *S. tritici* grown together or separately and mixed before inoculation.

Symptom suppression by mixtures of *S. tritici* was neither isolate nor cultivar specific. Reductions in pycnidial coverages resulted when reciprocal mixtures of conidial precipitates were resuspended in fresh medium. These findings strongly suggest that isolates of *S. tritici* produce inhibitory substances in vitro but do interfere with postinoculation processes leading to the suppression of pycnidial production on wheat seedlings. The suppression of symptoms after mixtures between centrifuged spores of isolate pairs resuspended in fresh medium may suggest that the substances may be associated with the spore surface.

When centrifuged conidia of isolate ISR398 were mixed with a culture filtrate of either ISR7901 or ISR8036, higher than expected pycnidial coverages were recorded on seedlings of Ceeon. *S. tritici* may produce substances that have the ability to suppress or promote pycnidial coverage in certain isolate combinations. The ability of fungi to produce germination-promoting substances as well as germination inhibitors was reported by Allen (1). Symptom suppression in challenge inoculations where virulent/avirulent isolates were employed suggests that host-pathogen interaction was rather small, because similar levels of reduction occur whether the avirulent was inoculated first or used as a challenge, and the phenomenon is rather pathogen dependent. An antagonistic effect between two races of *Melampsora medusae* Thuem. on *Populus* spp. was partly attributed to host-mediated interaction (11). The results here do not rule out the possibility that a host response is triggered by the first inoculation.

It is possible that symptom suppression also occurs after inoculation with a single isolate, yet isolates may express differential sensitivity to the formed substances. The production of such substances by *S. tritici* may be involved

in regulating the composition of isolates in the population on or within host tissue regardless of, and in addition to, their virulence. This may have considerable evolutionary and epidemiological significance. Moreover, inoculation of germ plasm or breeding material with isolate mixtures may result in altered severity and different virulence spectra than expected from that of the initial isolate composition.

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