Virulence Associations in Oat Crown Rust

K. J. Leonard, Y. Anikster, and J. Manisterski

First author: formerly U.S. Department of Agriculture–Agricultural Research Service, Cereal Disease Laboratory, University of Minnesota, St. Paul 55108; and second and third authors: Institute for Cereal Crops Improvement, Tel Aviv University, Ramat Aviv 69978, Israel. Accepted for publication 14 July 2004.

ABSTRACT

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Isolates of *Puccinia coronata* obtained from natural populations of *Avena sterilis* in Israel, winter oat (*A. sativa*) cultivars in Texas, and spring oat cultivars in the Northern Plains states of Minnesota, North Dakota, and South Dakota were analyzed for significance of pairwise virulence associations. Isolates from all three regions were tested on 25 oat lines with single *P. coronata* (*Pc*) genes for crown rust resistance from *A. sterilis* and one line with a *Pc* gene from *A. sativa*. Isolates from Israel were tested also on 11 Iowa backcross lines with undesignated crown rust resistance genes from *A. sterilis*. Four associated virulence groups were identified from significant positive virulence associations that were consistent across all three regions. Group 38 included virulence to *Pc*-38,

Pc-39, *Pc*-55, *Pc*-63, and *Pc*-71; group 45 included virulence to *Pc*-45, *Pc*-46, *Pc*-48, *Pc*-52, *Pc*-54, and *Pc*-57; group 58 included virulence to *Pc*-35, *Pc*-40, *Pc*-58, and *Pc*-59; and group 61 included virulence to *Pc*-36, *Pc*-51, *Pc*-56, *Pc*-60, and *Pc*-61. Virulence to *Pc*-70 showed the strongest association to virulences in group 38 but also showed significant association with virulence to *Pc*-45, *Pc*-35, and *Pc*-58. Virulences in group 61 were consistently negatively associated with virulences in group 38 in each region. In Israel, virulences to five of the Iowa lines showed positive associations to virulences in group 61 and negative associations to virulences in groups 38 and 45. Close similarity of reactions of nearly all isolates to *Pc*-39, *Pc*-55, and *Pc*-71 suggest that these genes may be identical or nearly identical alleles.

Additional keyword: linkage disequilibrium.

Crown rust, caused by *Puccinia coronata*, is an important disease of cultivated oat (*Avena sativa*) in the United States and in many other parts of the world (18). Crown rust also occurs commonly on wild *A. fatua* in the United States (14) and on *A. sterilis* and other wild oat species in the Mediterranean regions of southern Europe, northern Africa, and the Middle East (30). Wild oat, particularly *A. sterilis*, from the Mediterranean regions has been an important source of genes for race-specific resistance to crown rust in the United States and Canada (14).

Recent surveys (15) show that isolates of *P. coronata* from Israel remain highly polymorphic for virulence or avirulence corresponding to resistance genes found in collections of *A. sterilis* made from Israel in the 1960s and 1970s. Such polymorphisms for virulence or avirulence to the resistance genes from *A. sterilis* also occur in populations of *P. coronata* collected mainly from cultivated oat in the United States, both in the north, where the sexual stage is an important part of the life cycle of *P. coronata*, and in the south, where the *P. coronata* population is asexual (14).

The presence of so much genetic diversity and so much unnecessary virulence in the pathogen population (14) makes it difficult to achieve more than ephemeral control of crown rust with the use of race-specific resistance alone. Vanderplank (21) and others (1,24,27) have suggested that certain virulence combinations in pathogens may occur much less frequently than expected from individual virulence frequencies because of reduced fitness associated with those combinations of virulence genes. Negative virulence associations, if identified, could indicate that the pathogen is especially vulnerable to certain combinations of resistance genes in the host. Thus, combining those resis-

tance genes in single cultivars (1,21) or in cultivar mixtures (27) could provide more durable protection than could be obtained by use of the same resistance genes separately. On the other hand, positive associations between pairs of virulences could indicate that the corresponding combinations of resistance genes might lose their effectiveness rather quickly (1,13,21).

It is quite clear, however, that the mere presence of positive or negative associations of specific pairs of virulences in a pathogen population does not necessarily imply that the association is due to some inherent effect on fitness of the pathogen. There are several factors that can lead to observed linkage disequilibria between pairs of virulence genes, including founder effects (genetic drift), directional selection imposed by growers' choices of resistant host cultivars, or blending of pathogen populations with different virulence frequencies through migration or inappropriate sampling procedures (9,16,23,25).

In this study, we analyzed virulence associations in three populations of *P. coronata* from different geographical regions that occur on three different types of host populations. The objective was to determine whether there are specific positive or negative virulence associations in pairwise comparisons that occur consistently in all three pathogen populations. Such consistent associations are likely to be due to fitness effects that are inherent in the specific virulence or avirulence gene combinations rather than due to host-imposed selection, pathogen migration, or genetic drift. The three populations of *P. coronata* included the sexual population on cultivated spring oat cultivars in the northern Great Plains of the United States, the asexual population on cultivated winter oat in Texas in the southern Great Plains, and the sexual population on wild oat in Israel where *A. sativa* is not grown commercially and where wild *A. sterilis* is abundant and commonly infected by *P. coronata* (5,22).

MATERIALS AND METHODS

Isolates. Virulence associations were determined for collections of isolates of *P. coronata* from Israel and from two regions

Corresponding author: K. J. Leonard; E-mail address: kurtl@umn.edu

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of the United States in 1991, 1992, 1993, 1994, and 1996. With the exception of Israel in 1996, each collection represented a different field of cultivated oat (*A. sativa*) in the United States or a single stand of wild oat (*A. sterilis* or *A. barbata*) in Israel. A single isolate was obtained from each collection. Thus, the sampling procedure produced a broad sample of genotypes of *P. coronata* from throughout the region in question. In the United States, 389 isolates of *P. coronata* were obtained from oat fields in Minnesota, North Dakota, and South Dakota of the Northern Plains, and 294 isolates were obtained from oat fields and breeders' nurseries in Texas in the Southern Plains. In Israel, 130 isolates of *P. coronata* were obtained in 1991–94; 122 were from *A. sterilis*, six were from *A. barbata*, and two were from an experimental planting of *A. sativa* near Tel Aviv. Isolates of *P. coronata* from Israel in 1996 were not used in the analyses of virulence associations, because the isolates in 1996 were collected from only two locations rather than as a random sample from throughout Israel. Also, 25 of the isolates collected in 1996 in Israel were from *A. sativa* in an experimental nursery at the Menashe Plateau. Total numbers of isolates per year for 1991, 1992, 1993, 1994, and 1996, respectively, were 43, 33, 120, 136, and 57 for the Northern Plains, 105, 101, 23, 47, and 18 for Texas, and 30, 42, 7, 51, and 48 for Israel. In addition, 73 aecial isolates of *P. coronata* were obtained from plants of *Rhamnus cathartica* and *R. palaestina* in the University of Minnesota buckthorn nursery in St. Paul in 1992. The aecial isolates were used to test for the persistence of certain virulence associations through a generation of random mating in the sexual cycle.

Each collection of *P. coronata*, consisting of several rusted leaves, was brought to the U.S. Department of Agriculture–Agricultural Research Service Cereal Disease Laboratory at the University of Minnesota for testing. Urediniospores were collected from the sample with a cyclone spore collector, suspended in light mineral oil, and sprayed onto 7- to 9-day-old seedlings of oat cv. Marvellous, which is susceptible to all known races of oat crown rust. After the seedlings stood for 30 min to allow the oil to evaporate, they were placed overnight in a dew chamber at 18°C before being returned to the greenhouse at 18 to 28°C. In the greenhouse, the infected seedlings were maintained in polyethylene isolation cells to minimize cross contamination or escape of urediniospores. When uredinia appeared, a bulk sample of urediniospores was collected from each sample and stored at –55°C. Then, all but one of the seedlings were removed for each sample, and the remaining infected seedling was trimmed so that just one uredinium remained. The trimmed seedlings were placed in a dew chamber for 3 h to induce any urediniospores left scattered on the surface of the remaining leaf to begin germination. The seedlings then were removed from the dew chamber and allowed to dry, which killed the germinating urediniospores, and the seedlings then were returned to the isolation cells. After several days, the new urediniospores produced in the single uredinia were collected to establish single-uredinial isolates that were stored at –55°C until testing. Single-uredinial isolates from Israel also were placed in long-term storage in sealed glass vials in liquid nitrogen in cryostats in the rust culture collection of the Cereal Disease Laboratory. Single-uredinial isolates were obtained from seedlings of 'Marvellous' oat inoculated with aeciospores from a single aecial cluster using the same methods described for collections from infected oat leaves, and were stored and tested using the same procedures as for isolates obtained from oat.

Virulence tests. During months when there were no living oat plants in the field in Minnesota, the single uredinial isolates were tested for virulence in the greenhouse on sets of differential oat lines. Isolates from the United States were tested on two sets of oat lines. The first set consisted of 19 backcross lines with the following single *P. coronata* (*Pc*) genes for crown rust resistance: *Pc-35*, *Pc-38*, *Pc-39*, *Pc-40*, *Pc-45*, *Pc-46*, *Pc-48*, *Pc-54*, *Pc-55*, *Pc-56*, *Pc-58*, *Pc-59*, *Pc-60*, *Pc-61*, *Pc-62*, *Pc-63*, *Pc-64*, *Pc-67*,

and *Pc-68*. The second set consisted of nine backcross lines from the Iowa State University breeding program for multiline oat cultivars in which the following *Pc* genes had been backcrossed into *A. sativa* backgrounds: *Pc-14*, *Pc-36*, *Pc-50*, *Pc-51*, *Pc-52*, *Pc-53*, *Pc-57*, *Pc-70*, and *Pc-71*. The recurrent parents in these backcrosses contained some defeated genes for crown rust resistance for which virulence had become fixed in the U.S. populations of *P. coronata*. All 28 *Pc* genes, except *Pc-14*, were transferred to cultivated oat lines from collections of *A. sterilis* from the Mediterranean region of southern Europe, Asia Minor, or North Africa. Isolates of *P. coronata* from Israel were tested on these two sets of differentials, with the exception that the differentials with *Pc-50* and *Pc-53* were omitted due to insufficient seed. In addition, the isolates from Israel also were tested on a third set of 11 differential lines from the Iowa multiline breeding program with single unidentified crown rust resistance genes from *A. sterilis* backcrossed into cultivated oat lines that had some defeated resistance genes.

The single-uredinial isolates were tested for virulence on 7- to 9-day-old seedlings of the sets of differential oat lines by spraying them with a suspension of urediniospores and incubating the inoculated seedlings overnight in a dew chamber at 18°C. After 10 to 14 days in the greenhouse, the inoculated differentials were evaluated for infection type. Lines with abundant, large, heavily sporulating uredinia were scored as susceptible, and lines with no visible reaction, flecks, or small uredinia surrounded by chlorosis or necrosis and with sparse sporulation were scored as resistant.

Statistical analyses. Virulence associations were determined in 2-by-2 contingency tables for all possible paired combinations of differentials for isolates of *P. coronata* from Israel and from the Northern Plains and Texas in the United States. For each pair of differentials, the numbers of isolates virulent to both, avirulent to both, virulent to the first but avirulent to the second, or avirulent to the first but virulent to the second were determined. Expected frequencies of isolates in each category were calculated as the product of the observed frequencies of isolates virulent or avirulent on each differential. For example, the expected frequency of isolates virulent on both differentials of the pair was determined by the product of the frequency of isolates in the population virulent on the first differential multiplied by the frequency of isolates virulent on the second differential (24). Positive associations (coupling) were those in which the numbers of isolates virulent on both differentials or avirulent on both differentials exceeded the expected values. Negative associations (repulsion) were those in which the number of isolates virulent on one differential but avirulent on the other exceeded the expected values. *P* values for the statistical significance of the associations were calculated by Fisher's exact test (12).

The numbers of statistically significant ($P < 0.05$) positive and negative associations of virulence pairs were determined for each year in Israel, the Northern Plains, and Texas, and the mean values per year were compared between regions by a two-tailed *t* test. Similarly, the proportion of associations that were negative in the three regions were compared by two-tailed *t* test. The numbers of positive and negative virulence associations also were calculated for total isolates collected over all years in each region and numbers of associations of the same direction (positive or negative) that were common to two or three regions were determined. Paired virulences that showed either significant positive or significant negative associations in both regions of the United States and in Israel were considered likely to indicate combinations of genes that are favored by natural selection due to inherent epistatic interactions between alleles at the loci determining virulence and avirulence corresponding to the specific pair of *Pc* genes.

Six pairs of *Pc* genes were determined in earlier genetic analyses to be either closely linked or allelic. These included *Pc-35* and *Pc-54* (19), *Pc-38* and *Pc-62* (7), *Pc-38* and *Pc-63* (7), *Pc-39* and *Pc-55* (19), *Pc-46* and *Pc-50* (19), and *Pc-46* and *Pc-68* (28). In

addition, results of this study indicated that *Pc-39* and *Pc-71* and *Pc-55* and *Pc-71* may be alleles. Associations of virulence for the pairs of differentials with these genes were determined both for the uredinial isolates and for the aecial isolates. High levels of significance for positive associations of virulence that occurred in all three populations of uredinial isolates and that persisted through random mating in the sexual stage in the St. Paul buckthorn nursery was taken as evidence that the respective *Pc* genes may be alleles or even identical.

RESULTS

The first step in analyzing virulence associations in *P. coronata* was to consider virulences that correspond to *Pc* genes that have been reported to be closely linked or allelic (Table 1). Chong and Kolmer (4) presented evidence that *Pc-39* and *Pc-55* have the same race specificity and are likely to be the same gene obtained independently from two different accessions of *A. sterilis*, although Bonnett (2) found some differences in the temperature sensitivity of the resistance conferred by *Pc-39* and *Pc-55*. The very high degree of association of virulences to the *Pc-39* and *Pc-55* differentials, not only in all three uredinial populations of *P. coronata* isolates but also in the aecial population from the St. Paul buckthorn nursery (Table 1), support Chong and Kolmer's (4) conclusion. The same was true for the combinations of *Pc-39* and *Pc-71* and of *Pc-55* and *Pc-71*, thus indicating that *Pc-71* may be identical with *Pc-39* and *Pc-55*.

Other pairs of *Pc* genes reported to be allelic or closely linked did not show such high degrees of virulence associations. For example, virulences to *Pc-35* and *Pc-54* do not show significant association for any of the three uredinial populations or for the

aecial isolates. Virulences to *Pc-38* and *Pc-62* were not significantly associated in either the Northern Plains or Texas populations, or in the aecial isolates. Virulence to *Pc-38* and *Pc-63* were significantly associated in all collections of isolates, but the association was not as strong in the uredinial isolates from Israel or in the aecial isolates as the associations between virulences for the presumed identical genes *Pc-39*, *Pc-55*, and *Pc-71*. Thus, the virulence gene corresponding to *Pc-38* can be considered distinct from the virulence gene corresponding to *Pc-63*, even though the two virulences are significantly associated. Evidence for associations of virulences to *Pc-46* and *Pc-50* or *Pc-46* and *Pc-68* is incomplete, because isolates from Israel were not tested on the *Pc-50* differential and because no virulence to *Pc-68* was present among the isolates from Israel or among the aecial isolates from the St. Paul buckthorn nursery (Table 1). Nevertheless, the available data show that the race specificities of *Pc-46*, *Pc-50*, and *Pc-68* are all different.

In comparing the extent of virulence associations in Israel, Texas, and the Northern Plains of the United States, data for virulence to *Pc-55* and *Pc-71* were excluded from the analyses to avoid duplications for genes that are likely identical. For the 24 remaining differentials tested against isolates of *P. coronata* from both Israel and the United States, there were 276 possible paired combinations of corresponding virulences. Virulence associations were determined for each year in each region. On average over years of collection, there were 44 pairs of virulences with significant ($P < 0.05$, Fisher's exact test) positive (coupling) associations per year in Israel, 27 in the Northern Plains, and 33 in Texas. The average number of positive associations found per year in Israel was significantly greater than in the Northern Plains of the United States ($P = 0.032$, two-tailed t test), but the numbers of

TABLE 1. Associations of virulence toward pairs of linked oat crown rust resistance genes in populations of *Puccinia coronata* in Israel, Texas (TX), and the Northern Plains (NP) of the United States (US)^a

Virulence to	Significance of associations in indicated population ^b			
	Israel	US-NP ^c	US-TX	MN aecia ^d
<i>Pc-35/Pc-54</i>	0.531	0.381	0.066	0.496
<i>Pc-38/Pc-62</i>	3×10^{-5}	0.221	0.083	0.138
<i>Pc-38/Pc-63</i>	0.007	2×10^{-63}	2×10^{-26}	0.001
<i>Pc-39/Pc-55</i>	4×10^{-28}	7×10^{-101}	4×10^{-38}	4×10^{-10}
<i>Pc-39/Pc-71</i>	6×10^{-16}	7×10^{-86}	7×10^{-33}	8×10^{-9}
<i>Pc-55/Pc-71</i>	2×10^{-18}	8×10^{-86}	3×10^{-22}	2×10^{-8}
<i>Pc-46/Pc-50</i>	ND	0.089	0.075	0.212
<i>Pc-46/Pc-68</i>	...	0.326	0.044	...

^a Linkages of *Pc-35* to *Pc-54*, *Pc-39* to *Pc-55*, and *Pc-46* to *Pc-50* (19); linkage of *Pc-38* to *Pc-62* and *Pc-63* (7); linkage of *Pc-46* to *Pc-68* (28); allelism of *Pc-39* and *Pc-55* with *Pc-71* postulated from data showing strong positive virulence association. ND, virulence to *Pc-50* was not tested for isolates from Israel; ..., no isolates were virulent on *Pc-68* in these populations.

^b P values calculated by Fisher's exact test.

^c The Northern Plains population includes isolates from Minnesota, North Dakota, and South Dakota.

^d Aecial isolates were collected from *Rhamnus cathartica* and *R. palaestina* in St. Paul, MN in 1992.

TABLE 2. Numbers of significant ($P < 0.05$) associations of virulences to paired combinations of 24 oat crown rust resistance genes in populations of *Puccinia coronata* from Israel, Texas, and the Northern Plains of the United States^a

Associations in	Israel		Northern Plains ^b		Texas	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Single population	40	29	17	14	29	9
Israel and Northern Plains	12	6	12	6	...	...
Israel and Texas	21	2	...	...	21	2
Northern Plains and Texas	...	...	14	0	14	0
All three populations	24	1	24	1	24	1
Total ^c	97	38	67	21	88	12

^a Pos. = virulence to the first resistance gene of a pair is associated with virulence to the second, and avirulence to the first is associated with avirulence to the second. Neg. = virulence to the first resistance gene of a pair is associated with avirulence to the second, and avirulence to the first is associated with virulence to the second.

^b The Northern Plains population includes isolates of *P. coronata* from Minnesota, North Dakota, and South Dakota.

^c Total number of pairs of virulences that showed significant associations (determined by Fisher's exact test) in each population out of a total of 276 possible pairwise combinations (virulence to *Pc-55* and *Pc-71* were excluded from the analysis).

positive associations per year did not differ significantly between Israel and Texas or the Northern Plains and Texas. The average numbers of significant negative virulence associations ($P < 0.05$, Fisher's exact test) per year did not differ significantly in the three regions, and there were no significant differences in the proportions of total significant virulence associations that were negative in the three regions.

More significant virulence associations were found with calculations over the combined total isolates collected over the years 1991–94 for Israel and 1991–94 plus 1996 in the Northern Plains and Texas in the United States compared with calculations for any individual year (Table 2). Data from Israel in 1996 were not included in this comparison, because samples were collected from only two sites in Israel in 1996. There were 24 pairs of virulences with significant ($P < 0.05$) positive associations and 1 pair of virulences with significant ($P < 0.05$) negative associations that were consistent in all three regions. Fourteen pairs of virulences with significant positive associations were found in both regions of the United States but not in Israel. More of the positive virulence associations were common to Israel and Texas than to Israel and the Northern Plains, but there were more negative virulence associations common to Israel and the Northern Plains than to Israel and Texas (Table 2). In all, 49% of the 276 possible pairs of virulence showed either significant positive or significant negative association in Israel; 32 and 36% showed significant associations in the Northern Plains and Texas. The proportion of total significant virulence associations that were negative was 28% in Israel, 24% in the Northern Plains, and 12% in Texas.

Based on combined analyses over all years, most of the virulences could be assigned to one of four main association groups

that were consistent in all three populations from Israel, the Northern Plains, and Texas (Fig. 1). Group 38 includes virulences to *Pc*-38, *Pc*-39 (and by probable identity, *Pc*-55 and *Pc*-71), *Pc*-70, and *Pc*-63. The association of virulence to *Pc*-63 in this group was strong in the Northern Plains and Texas but less strong in Israel (Table 3). Group 45 consists of virulences to *Pc*-45, *Pc*-46, *Pc*-48, *Pc*-52, *Pc*-54, *Pc*-57, and *Pc*-70. The inclusion of *Pc*-70 in group 45 indicates a connection to group 38, for which *Pc*-70 shows a stronger association. Group 58 consists of virulences *Pc*-35, *Pc*-40, *Pc*-58, *Pc*-59, and *Pc*-70. Two members of group 58 virulences show a significant association with virulence to *Pc*-70, but neither of these group 58 virulences was significantly associated with other members of the group 38 virulences in all three populations of *P. coronata*, which is a reason for distinguishing groups 38 and 58 as separate virulence groups. Group 61, which is quite distinct from the other groups, is composed of virulences to *Pc*-36, *Pc*-51, *Pc*-56, *Pc*-60, and *Pc*-61. No members of virulence group 61 showed any consistent positive associations to virulences of any other group. Virulences to differentials with *Pc*-14, *Pc*-35, *Pc*-57, *Pc*-62, *Pc*-64, *Pc*-67, and *Pc*-68 did not have consistent positive or negative associations with virulences in any of the four virulence groups or with each other; therefore, these virulences were not assigned to any group.

Negative $\log_{10}$ values of the significance levels for virulence associations within the virulence groups are shown for each population in Tables 3 to 6 to indicate the relative strengths of those associations in the different regions. All virulences of group 38 showed significant positive associations in the Northern Plains and Texas, but the associations for *Pc*-39 and *Pc*-63, *Pc*-55 and *Pc*-63, *Pc*-63 and *Pc*-70, and *Pc*-63 and *Pc*-71 were not significant at $P < 0.05$ in Israel, probably because the frequency of virulence to *Pc*-63 was lower in Israel than in the United States (15). Virulence groups 45, 58, and 61 were less cohesive than group 38, but some strong associations were found to link members of each groups with at least two other virulences in the same group for the *P. coronata* population in each region. In group 45, the association between virulences to *Pc*-48 and *Pc*-52 was particularly strong in all three populations of *P. coronata* (Table 4), as was the association between virulences to *Pc*-36 and *Pc*-56 in group 61 (Table 6).

Three virulences of group 61 showed significant negative associations with virulence to *Pc*-39 and to the probably identical *Pc*-

Fig. 1. Virulence groups identified by significant positive virulence associations that were consistent for *Puccinia coronata* populations from Israel, the Northern Plains (Minnesota, North Dakota, and South Dakota), and Texas in the Southern Plains of the United States. Narrow lines indicate that the association was significant ($P < 0.05$) in all three populations. Thick lines indicate that the association was highly significant ($P < 0.0001$) in all three populations. Virulences are designated by number of the corresponding *P. coronata* (*Pc*) gene for resistance in oat. Virulence groups 38, 45, 58, and 61 are named for the virulence with the greatest number of positive associations within each group. Virulence to *Pc*-70 has the strongest association with group 38 but also has some positive association to one member of group 45 and two members of group 58. *Pc*-39, *Pc*-55, and *Pc*-71 may be independent identifications of the same resistance gene or alleles with identical or nearly identical race specificity.

TABLE 3. Significance levels ($-\log_{10} P$) of positive associations of virulences in virulence group 38 in populations of *Puccinia coronata* in Israel (1991–94) and in Texas and the Northern Plains of the United States (1991–96)^a

Location, virulence	Virulences				
	38	39	55	63	70
Israel					
39	12.32	...	...	...	...
55	12.01	27.36	...	...	...
63	2.15	...	...	...	...
70	4.39	11.92	13.01	...	...
71	7.87	16.25	17.68	...	12.10
Northern Plains					
39	21.95	...	...	...	...
55	18.70	100.18	...	...	...
63	62.63	32.62	30.01	...	...
70	6.34	29.03	29.58	10.21	...
71	20.89	85.17	85.09	33.45	25.66
Texas					
39	18.73	...	...	...	...
55	18.94	37.36	...	...	...
63	25.65	20.87	16.69	...	...
70	5.96	15.91	12.90	8.55	...
71	17.18	32.15	21.60	17.35	14.36

^a Significance was determined by Fisher's exact test. Data for $P > 0.1$ ($-\log_{10} P < 1.0$) are not given. Virulences are designated by the *P. coronata* (*Pc*) gene number of the corresponding resistance gene in oat.

55 and *Pc-71* (Table 7). These negative associations were more consistent in the Northern Plains and in Israel than in Texas, where virulence to *Pc-39*, *Pc-55*, and *Pc-71* occurred less frequently. Overall, negative associations were not found between virulences within any virulence group in any of the three *P. coronata* populations (Table 8). Negative virulence associations were observed most frequently between virulences of groups 61 and 38 in Israel and the Northern Plains. In Israel, negative virulence associations also were frequent between virulences of groups 61 and 45. There were several negative associations between virulences in groups 38 and 45 in Israel and the Northern Plains, which adds some support for keeping these groups separate despite the association with virulence to *Pc-70* in both groups. On the other hand, there were no significant negative associations between virulences in groups 38 and 58, which may indicate that these represent subgroups rather than two distinct groups.

TABLE 4. Significance levels ($-\log_{10} P$) of positive associations of virulences in virulence group 45 in populations of *Puccinia coronata* in Israel (1991–94) and in Texas and the Northern Plains of the United States (1991–96)^a

Location, virulence	Virulences					
	45	46	48	54	57	70
Israel						
46	5.74	...	...	...	...	...
48	7.89	7.52	...	...	...	...
54	9.57	4.93	5.59	...	...	...
57	20.76	8.20	8.53	11.54	...	...
70	3.13	1.48	6.98	...	3.62	...
52	4.55	3.19	13.07	...	...	...
Northern Plains						
46	1.95	...	...	...	...	...
48	6.96	...	...	...	...	...
54	4.04	...	4.99	...	...	...
57	2.07	9.38	1.36	...	...	...
70	1.93	...	...	...	...	...
52	...	...	8.93	...	...	...
Texas						
46	3.46	...	...	...	...	...
48	2.72	1.01	...	...	...	...
54	6.13	1.52	2.60	...	...	...
57	3.67	9.09	2.57	2.22	...	...
70	2.46	2.57	...	3.99	3.32	...
52	1.88	...	10.77	...	...	...

^a Significance was determined by Fisher's exact test. Data for $P > 0.1$ ($-\log_{10} P < 1.0$) are not given. Virulences are designated by the *P. coronata* (*Pc*) gene number of the corresponding resistance gene in oat.

TABLE 5. Significance levels ($-\log_{10} P$) of positive associations of virulences in virulence group 58 in populations of *Puccinia coronata* in Israel (1991–94) and in Texas and the Northern Plains of the United States (1991–96)^a

Location, virulence	Virulences			
	35	40	58	59
Israel				
40	2.39	...	...	...
58	5.90	1.53	...	...
59	6.86	...	3.42	...
70	4.84	2.60	7.83	...
Northern Plains				
40	...	...	...	...
58	1.56	2.25	...	...
59	12.32	...	6.19	...
70	6.14	...	2.81	...
Texas				
40	10.05	...	...	...
58	8.91	2.07	...	...
59	5.03	...	1.65	...
70	1.54	...	2.69	...

^a Significance was determined by Fisher's exact test. Data for $P > 0.1$ ($-\log_{10} P < 1.0$) are not given. Virulences are designated by the *P. coronata* (*Pc*) gene number of the corresponding resistance gene in oat.

Virulence to oat lines from the Iowa multiline breeding program that have unidentified crown rust resistance genes from *A. sterilis* were tested only with isolates of *P. coronata* from Israel. Therefore, virulences to these lines could not be assigned with confidence to any of the four identified virulence groups. Nevertheless, there are some patterns in the data from Israel that reveal some probable associations for these undesigned virulences. Virulences to lines X534, X715, and X721 compose a closely associated group based on the data from Israel, and virulences to lines Y346 and H548 show a distinct lack of affinity to the X534 group (Table 9). Affinities of virulences to the remaining six lines appear to overlap; however, some patterns are apparent when these virulences are compared with virulences in the four identified virulence groups (Table 10). Virulences to X534, X537, X716, X721, and X424-2 all showed significant positive associations with several members of group 61 and significant negative associations with members of group 45. These virulences also were consistent in showing significant negative association with at least one member of group 38. Thus, it appears that virulences to X534, X537, X716, X721, and X424-2 probably belong with group 61. Virulences to X465 and X475 show considerable association with group 58, but also show at least some significant

TABLE 6. Significance levels ($-\log_{10} P$) of positive associations of virulences in virulence group 61 in populations of *Puccinia coronata* in Israel (1991–94) and in Texas and the Northern Plains of the United States (1991–96)^a

Location, virulence	Virulences			
	36	56	60	61
Israel				
56	9.31	...	...	...
60	3.00	...	...	...
61	5.65	2.68	5.77	...
51	2.51	1.41	1.75	4.00
Northern Plains				
56	34.98	...	...	...
60	4.51	6.45	...	...
61	1.94	2.60	25.65	...
51	...	...	2.96	4.98
Texas				
56	15.85	...	...	...
60	2.54	1.87	...	...
61	3.32	5.18	13.01	...
51	2.22	...	1.11	11.51

^a Significance was determined by Fisher's exact test. Data for $P > 0.1$ ($-\log_{10} P < 1.0$) are not given. Virulences are designated by the *P. coronata* (*Pc*) gene number of the corresponding resistance gene in oat.

TABLE 7. Significance levels ($-\log_{10} P$) of negative associations of virulences in populations of *Puccinia coronata* in Israel (1991–94) and in Texas and the Northern Plains of the United States (1991–96)^a

Location, virulence	Virulences		
	39	55	71
Israel			
51	(2.45)	(2.57)	(1.43)
56	(1.36)	(1.63)	...
61	(1.90)	(3.26)	(3.18)
Northern Plains			
51	(1.48)	(1.71)	(1.51)
56	(2.33)	(2.79)	(2.50)
61	(6.11)	(7.59)	(5.53)
Texas			
51	(1.48)	...	(3.21)
56	(2.33)	...	(1.38)
61	(6.11)	(1.21)	...

^a Significance was determined by Fisher's exact test. Values in parentheses indicate a negative association (i.e., virulence to one resistance gene is associated with avirulence to the other gene in the pair); values of $P > 0.1$ ($-\log_{10} P < 1.0$) are not shown. Virulences are designated by the *P. coronata* (*Pc*) gene number of the corresponding resistance gene in oat.

positive associations with members of two or three other groups; therefore, they cannot be assigned with confidence to any identified group. Virulences to X117-1964-15-4 and Y347 also show no strong association with any identified group. Virulence to Y346 clearly is not associated with group 61, but associations to the other three groups cannot be ruled out. Virulence to H548 shows greatest affinity to groups 45 and 38 and significant negative associations with members of group 61 (Table 10).

DISCUSSION

The proportions of virulence pairs that showed significant associations in populations of *P. coronata* in the United States and Israel were greater in this study than in an earlier study of Canadian populations of *P. coronata* by Kolmer and Chong (11). Kolmer and Chong found an average of only 9% of the virulence pairs that they analyzed showed significant associations in any given year between 1974 and 1990 in collections from the Prairie Provinces and only 1% showed significant associations in collections from Eastern Canada. Kolmer and Chong analyzed virulence to just 10 *Pc* genes: *Pc-35*, *Pc-38*, *Pc-39*, *Pc-40*, *Pc-45*, *Pc-46*, *Pc-48*, *Pc-50*, *Pc-54*, and *Pc-56*. The frequency of virulence to *Pc-48* remained low throughout their study (<1% in both regions of Canada for 12 of the 17 years) (4); therefore, associations of other virulences with virulence to *Pc-48* would be unlikely to be detected in Kolmer and Chong's populations. Among the other nine virulences that Kolmer and Chong (11) analyzed, only five combinations in their study (*Pc-38* and *Pc-39*, *Pc-45* and *Pc-46*, *Pc-45* and *Pc-54*, *Pc-46* and *Pc-54*, and *Pc-35* and *Pc-40*) corre-

spond to pairs that were consistently associated in the United States and Israel in our study. This may account for the low proportion of virulence pairs that showed significant associations in Kolmer and Chong's (11) study.

In the U.S. populations, there was an average of 10% of the 276 possible pairs of virulence that showed significant positive associations and 3% that showed significant negative associations per year from 1990 to 1996 in the Northern Plains; in Texas, there was an average of 12% with significant positive associations and 4% with significant negative associations. In Israel, the proportion with significant positive associations was 16% for the years from 1991 to 1994 and the proportion with significant negative associations was 5%. The data reported by Kolmer and Chong (11) indicate only the total proportions of significant associations and do not distinguish between positive and negative associations.

Linkage disequilibria that account for either positive or negative associations between pairs of virulences may occur in several different ways (1,9,23,25). Linkage disequilibria may arise from founder effects as new populations of the pathogen are established or from genetic drift where population crashes regularly occur and relatively few genotypes survive from one year to the next. For pathogens that lack sexual reproduction with genetic recombination, the disequilibria produced in this way may persist over long periods of time. Linkage disequilibria also may occur by admixture between populations by pathogen migration. Mixtures of populations also may be produced inadvertently in studies of pathogen populations by inappropriate sampling procedures that

TABLE 8. Numbers of significant ($P < 0.05$) negative associations between pairs of virulences between and within virulence groups in populations of *Puccinia coronata* in Israel, Texas, and the Northern Plains of the United States^a

Virulence groups	Population		
	Israel	Northern Plains ^b	Texas
38 and 45	4	6	1
38 and 58	0	0	0
38 and 61	15	17	2
45 and 58	1	0	2
45 and 61	14	2	5
58 and 61	4	2	0
Within ^c	0	0	0
Other ^d	6	5	3

^a Negative association indicates that virulence in a member of the first indicated group is associated with avirulence in the second indicated group. Significance was determined by Fisher's exact test.

^b The Northern Plains population includes isolates of *P. coronata* from Minnesota, North Dakota, and South Dakota.

^c Comparisons made between virulences within the same virulence group.

^d Negative associations involving virulences that have not been assigned to any virulence group.

TABLE 10. Net number of significant associations between virulence to oat lines from the Iowa multiline program and virulences of identified groups among isolates of *Puccinia coronata* from Israel in 1991–94^a

Iowa line	Virulence group ^b			
	Group 38	Group 45	Group 58	Group 61
X534	-2	-5	2	5
X537	-2	-5	-1	3
X716	-3	-5	1	5
X721	-1	-6	-1	4
X424-2	-4	-3	-4	4
X465	2	-2	4	2
X475	3	2	4	2
X117-1964-15-4	0	0	1	2
Y347	1	0	2	2
Y346	4	3	4	0
H548	5	7	3	-3

^a Number of positive associations between virulence to the Iowa line and virulences in the indicated group ($P < 0.05$, Fisher's exact test) minus the number of significant negative associations between virulence to the Iowa line and virulences in the indicated group.

^b Group 38 includes virulence to *Pc-38*, *Pc-39*, *Pc-55*, *Pc-63*, *Pc-70*, and *Pc-71*; group 45 includes virulence to *Pc-45*, *Pc-46*, *Pc-48*, *Pc-52*, *Pc-54*, *Pc-57*, and *Pc-70*; group 58 includes virulence to *Pc-58*, *Pc-35*, *Pc-59*, and *Pc-70*; and group 61 includes virulence to *Pc-61*, *Pc-36*, *Pc-51*, *Pc-56*, and *Pc-60*.

TABLE 9. Significance levels ($-\log_{10} P$) of associations of virulences to pairs of oat lines from the Iowa multiline program in the population of *Puccinia coronata* in Israel in 1991–94^a

Line	X534	X716	X721	X537	X424-	X117-	X465	X475	Y347	Y346
X716	13.46	...	...	...	...	...	...	...	...	...
X721	6.81	4.18	...	...	...	...	...	...	...	...
X537	5.61	...	...	...	...	...	...	...	...	...
X424-	1.50	...	1.82	4.01	...	...	...	...	...	...
X465	1.92	1.92	6.08	5.68	...	...	...	...	...	...
X475	5.13	2.14	1.23	3.47	...	...	4.68	...	...	...
X117-	1.63	2.12	4.18	1.24	...	...	...	...	...	...
Y347	1.60	2.10	1.40	...	...	1.33	2.71	3.22	...	...
Y346	...	...	...	(1.29)	...	...	5.43	2.77	3.44	...
H548	(3.89)	(2.37)	(2.53)	(1.42)	(3.03)	...	...	...	1.46	3.35

^a Significance was determined by Fisher's exact test. Data for $P > 0.1$ ($-\log_{10} P < 1.0$) are not given. Values in parentheses indicate negative associations (i.e., virulence to one resistance is associated with avirulence to the other resistance in the pair).

combine isolates from disparate populations where frequencies of virulence to specific resistance genes are greatly different. Selection imposed by host populations also may produce linkage disequilibria in relation to virulence genes. When two or more genes for resistance are combined in prevalent host cultivars, natural selection likely will result in positive associations of the corresponding virulence genes in the pathogen population (24). On the other hand, the use of two resistance genes exclusively in separate prevalent cultivars leads to negative associations between the corresponding virulences in the pathogen population (8,9,16).

In practical terms for control of plant diseases by race-specific resistance, the most interesting cases of linkage disequilibrium for pairs of virulences are those that arise from pleiotropic or epistatic effects in which a gene for virulence to a cultivar with one gene for resistance alters the fitness of the pathogen on another cultivar with a different gene for resistance (1). If the pleiotropic or epistatic effect increases fitness for pathogen isolates with virulence genes to both host cultivars, a positive association between the two virulence genes is likely to occur. If the pleiotropic or epistatic effect decreases fitness for isolates with the second virulence gene, a negative association is likely to be produced. Of course, positive associations of virulence genes also can be expressed as positive associations of avirulence genes, and it may be due to pleiotropic or epistatic effects of one avirulence gene relative to another. Thus, the net effect of pleiotrophy or epistasis that produces a positive association may be considered to result in selection that increases combined virulence by favoring the combination of two virulence genes or against combined virulence by favoring the combination of two avirulence genes.

Vanderplank (21) emphasized the potential value of breeding cultivars with combinations of resistance genes for which the corresponding virulences are negatively associated in the pathogen population. Wolfe and Schwarzback (27) extended this concept to the use of mixtures of cultivars or lines in which the components are selected to take advantage of negative associations of virulences in the pathogen population. By the same reasoning, combinations of resistance genes for which the corresponding virulences are positively associated in the pathogen population should be avoided, because selection for one virulence of an associated pair will tend to increase the frequency of the associated virulence (1,13,23). The increase in frequency of virulence to *Pc-70* in Texas and the Northern Plains of the United States in the absence of documented use of *Pc-70* commercially in either region (14) may be an example of this effect. Virulence to *Pc-70* was associated with virulence to both *Pc-38* and *Pc-39*, which were used extensively in oat cultivars grown in the Northern Plains in the 1990s (4,14).

Positive virulence associations were found more frequently than negative associations in Israel and the United States in this study, in contrast to the Kolmer's (10) report of approximately equal numbers of significant positive and negative associations in wheat leaf rust, *P. tritricina*, in Canada. When he passed the collected isolates through one generation of random mating on the alternate host, Kolmer found an increased number of significant virulence associations, with the majority being positive. The sexual stage of *P. tritricina* does not occur in nature in North America. Welz (23) found that most virulences that he tested in barley powdery mildew, *Blumeria graminis* f. sp. *hordei*, were in linkage equilibrium, but he found four positive virulence associations and four negative virulence associations in collections of isolates from conidia in Germany, and he found two positive and seven negative virulence associations in collections of ascospore isolates. Hovmøller and Østergård (8) reported that five pairs of virulences were positively associated and six were negatively associated in most of their 14 samples of *B. graminis* f. sp. *hordei* from Denmark.

Other studies have reported few or no negative associations of virulences in pathogen populations. Royer et al. (17) found three

positive virulence associations but no negative associations in wheat powdery mildew, *B. graminis* f. sp. *tritici*. In lettuce downy mildew, *Bremia lactucae*, Lebeda (13) reported 20 significant positive virulence associations out of 55 possible pairs of virulence and no significant negative associations in the Czech Republic. Hanson and Magnus (6) tested 15 pairs of virulences in *Rhynchosporium secalis* in Norway and found that all were in linkage equilibrium. On the other hand, Zhang et al. (29) found that most of the virulence combinations that they tested in *R. secalis* in California showed significant positive associations between pairs of virulences that were consistent over 3 years of sampling. Wolfe and McDermott (26) compared data for virulence associations in collections of *B. graminis* f. sp. *hordei* from six countries in Europe and concluded that linkage disequilibrium generally occurs more commonly in countries that deploy a larger diversity of resistant cultivars. This is consistent with the greater number of virulence associations found in our collections of *P. coronata* in wild populations of *A. sterilis* in Israel, where a greater diversity of resistance genes have been found (2,30) than in the United States, where a smaller subset of those resistance genes have been used in commercial oat production (14).

Because linkage disequilibrium in pathogen populations can be due to a variety of causes, it is difficult to know from most studies whether the observed virulence associations have any general significance or whether they are coincidental to a particular place or time. For example, Vanderplank (20) concluded from virulence associations observed in *P. graminis* f. sp. *tritici* populations in the United States and Canada that spring wheat in the Prairie Provinces of Canada was saved from stem rust by the inability of the pathogen to combine virulence to resistance gene *Sr6* with virulence to *Sr9* in a phenotype fit to survive in Canada. However, Wolfe and Knott (25) showed that Canadian spring wheat cultivars were protected by other resistance genes for which virulence was lacking in the southern Great Plains of the United States, which is the source area of stem rust inoculum for the spring wheat areas of the Northern Plains of the United States and the Prairie Provinces of Canada. On the other hand, several of the other negative virulence associations in *P. graminis* f. sp. *tritici* cited by Vanderplank (21) also were found by Alexander et al. (1) to be present not just in asexual populations in the Great Plains but also in sexual populations of *P. graminis* f. sp. *tritici* in Washington and Idaho. Thus, certain combinations of virulence genes may cause some reduction of fitness in *P. graminis* f. sp. *tritici*, but the extent of any such possible fitness reduction has not been quantified.

In our studies with *P. coronata*, we found several pairs of virulences that showed strong positive associations ($P < 0.0001$), not only in the sexual population on spring oat in the Northern Plains and the asexual population on winter oat in Texas but also on the sexual population on wild *A. sterilis* in natural habitats in Israel. These consistently highly significant positive associations occurred in four virulence groups that accounted for 19 of the 24 distinct virulences tested (virulences to *Pc-55* and *Pc-71* were excluded because they may be the same as virulence to *Pc-39*). These consistent positive associations are almost certainly due to pleiotropic or epistatic effects inherent in *P. coronata*, because it is not conceivable that genetic drift, population mixing, or directional selection imposed by resistance in the host could have produced the same patterns of associations in three separate populations of *P. coronata* growing under such different conditions.

The virulence groups distinguished by significant positive associations within each group may help to explain the large amount of apparently unnecessary virulence observed in populations of *P. coronata* on cultivated oat in the United States (14). Widespread use of a resistance gene corresponding to one member of a virulence group will cause strong directional selection favoring the corresponding virulence gene, but it also may increase the fitness of *P. coronata* genotypes with other virulence genes in the

same virulence group. The combination of *Pc-38* and *Pc-39*, which was used widely in oat cultivars in the Prairie Provinces of Canada and in the Northern Plains of the United States, provided good protection from oat crown rust for several years, even though virulence to *Pc-38* and *Pc-39* occur in the same virulence group. This protection can be attributed to the initial very low frequencies of virulence to either *Pc-38* or *Pc-39* in North America at the time the combination was first used commercially. Now that frequencies of virulence to *Pc-38* and *Pc-39* are high, virulence to *Pc-70*, a third member of virulence group 38, is increasing in frequency in the United States without any documented use of *Pc-70* in commercial oat production in the United States (14). Similarly, the greater frequency of virulence to *Pc-40* in the United States (14) than in Canada (4) may be due to the use of resistance genes *Pc-58* and *Pc-59* in the United States. Virulence to *Pc-40* is in the same virulence group as virulence to *Pc-58* and *Pc-59*. Also, virulences to *Pc-45*, *Pc-46*, and *Pc-54* in virulence group 45 have been shown to occur together at greater frequency in California than in the rest of the United States and at greater frequency in countries of southern Europe than in northern Europe (14). These virulences may become more common in the Northern Plains, because of the expanding use of *Pc-48* in Canadian oat cultivars, of which the corresponding virulence is in virulence group 45.

The only negative virulence associations that were consistently significant in the Northern Plains, Texas, and Israel were those involving virulence to *Pc-39* (and *Pc-55* and *Pc-71*, which may be the same as *Pc-39*) and virulence to *Pc-61* as well as *Pc-51* and *Pc-56*, which are also members of virulence group 61. To some extent, the relative lack of consistent negative virulence associations may be an artifact, because larger sample sizes are needed for statistical significance of negative associations than for positive associations (3). Thus, with larger samples, more negative associations between virulences of group 61 and virulences of other groups may have been detected. Because combinations of resistance genes corresponding to virulences with negative associations may give more durable protection than combinations corresponding to virulences with positive associations, it might seem reasonable to promote combinations of *Pc-36*, *Pc-51*, *Pc-56*, *Pc-60*, or *Pc-61* with *Pc-39* or other resistance genes that correspond to virulences in groups other than group 61. Unfortunately, all of the known virulences in group 61 already occur at moderate to high frequencies in the United States (14). Virulences to several of the Iowa oat lines (e.g., X424-2, X534, X537, X716, and X721) also may belong to virulence group 61 based on data from the *P. coronata* population in Israel. Frequencies of virulence to these lines in the United States are not known. These Iowa lines should be included in future crown rust virulence surveys to test whether they might be useful as parents to develop favorable combinations of crown rust resistance genes that would be more difficult for *P. coronata* to overcome.

Virulences to *Pc-62* and *Pc-68*, which are rare in the United States, were not found to be consistently associated with any of the four identified virulence groups. This may be a consequence of the difficulty in detecting associations when individual frequencies are close to 0 (25). Recently released oat cultivars in Canada use *Pc-68* in combination with other *Pc* genes, and the resistance of these cultivars is still highly effective. It is interesting that none of the isolates of *P. coronata* from Israel that we tested from 1991 through 1996 were virulent to *Pc-68*. This may indicate that virulence to *Pc-68* is difficult to maintain in natural populations of *P. coronata*. On the other hand, *Pc-68* was obtained from an accession of *A. sterilis* from Algeria (28); therefore, it is not clear whether *Pc-68* occurs in the *A. sterilis* populations of Israel. Still, virulence to *Pc-38*, *Pc-56*, *Pc-63*, and *Pc-67* occurred in easily detectable frequencies in Israel even though those genes also were obtained from accessions of *A. sterilis* from Algeria (7,19,28).

The approach used in this study differed from earlier studies of virulence associations by comparing associations over a range of different pathogen populations to determine which associations were consistent and likely due to pleomorphic or epistatic effects inherent to the pathogen rather than to extraneous influences. Among previously published studies, the work of Alexander et al. (1) with *P. graminis* f. sp. *tritici* provides the best evidence of virulence associations due to pleomorphic or epistatic effects, because they analyzed virulence data for sexual and asexual populations of pathogens from different geographical regions. The availability of isolates of *P. coronata* from the wild populations on *A. sterilis* in Israel was particularly valuable for our study of virulence associations, because it added the dimension of virulence associations in the pathogen population in a natural, genetically heterogeneous host population. Similar studies also could be done with pathogen populations on wild relatives of other crops, such as *B. graminis* f. sp. *hordei* or *P. hordei* on *Hordeum spontaneum* in Israel.

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