Virulence Variability of *Puccinia coronata* f. sp. *avenae* Isolates Collected in Three Counties from Rio Grande do Sul State, Brazil

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ABSTRACT

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Using isolates collected in three counties of Rio Grande do Sul State, Brazil, the goals of this work were to determine (i) the pattern of virulence or avirulence of the isolates to 25 *Pc* resistance genes, (ii) the similarity in virulence among *Puccinia coronata* f. sp. *avenae* isolates considering their pattern of virulence or avirulence, (iii) the race code for each isolate by the North American system of nomenclature, and (iv) the supplemental *Pc* genes potentially useful as local differentials for *P. coronata* f. sp. *avenae* races. The results indicate that the southern Brazilian rust isolates presented a high level of virulence, because 66% of inoculations manifested the high infection type. Only the *Pc 68* gene was effective against all tested isolates. In general, each isolate presented a different pattern of virulence or avirulence, which indicates the high variability for virulence that the fungus presents at the sampled sites. However, the North American system of nomenclature was not completely sufficient in distinguishing southern Brazilian races. Thus, the genes *Pc 36, Pc 53, Pc 55,* and *Pc 63* represent a possible gene combination to be incorporated into the North American system of nomenclature.

Additional keywords: *Avena sativa* L., genetic resistance

In the southern region of Brazil, hexaploid oat (*Avena sativa* L.) is one of the most important winter cereals and is used for grain production (food and feed purposes) in rotation with wheat, as well as a cold-season pasture (1,5). Brazilian oat breeding programs started releasing cultivars in the 1980s, breaking a tradition in which most of oat cultivars recommended for commercial use in Brazil were bred in Uruguay and Argentina. Ever since, oat breeding in Brazil has achieved excellent results for high yield, seed size and weight, number of grains per panicle, plant stature, aluminum tolerance, and cold tolerance (1).

Despite the progress attained for adaptative characters and the industrial quality (milling yield) of white oat crops, few gains have been obtained relative to resistance to crown rust (caused by *Puccinia coronata* f. sp. *avenae*) in Brazil, and this is the most important disease affecting oat production (21). The pathogen limits yield potential and grain quality in susceptible genotypes in Brazil (2,4,9,19,21), as well as worldwide (11,13).

Despite being the most efficient type of disease control, vertical genetic resistance is considered to have low durability, such that genotypes remain resistant for relatively short time periods (7,15). In particular, the resistance determined by one or a few genes can be overcome by new pathogen races that arise by mutation or that migrate from other locations and increase in frequency due to strong selection pressure from the cultivation of uniformly resistant genotypes over large areas (12).

Studies performed in Brazil have indicated the existence of a great number of distinct physiological races of *P. coronata* f. sp. *avenae* as well as the presence of complex races ("super races") in southern Brazil. In 1997, Martinelli et al. (20) collected 53 fungal samples in Rio Grande do Sul (RS), Santa Catarina, and Paraná States and identified 53 distinct races, each carrying from 10 to 26 virulence genes, with an average of 20 genes per isolate. Cruz et al. (10) evaluated 28 oat differential near-isogenic lines for *P. coronata* (*Pc*) resistance genes in the field and all expressed the disease. Leonard and Martinelli (18) determined the virulence pattern for 27 *Pc* genes of 144 southern-Brazilian fungal isolates (collected between 1997 and 1999), 36 Uruguayan isolates (collected in1994 and in 1998), and 17 Russian isolates (collected in 1995). Within the southern Brazilian and the Uruguayan isolates, Leonard and Martinelli (18) determined that 70% were virulent for 30 to 70% of the differentials, that there were a large number of fungal races, and that there was no prevailing race. Moreover, Leonard and Martinelli (18) observed that the complexity in virulence of southern Brazilian and Uruguayan isolates was greater than the complexity in virulence of Russian isolates.

Previous works have provided valuable insights regarding the variability and the complexity of southern Brazilian *P. coronata* f. sp. *avenae* populations. However, it is essential to continuously survey *P. coronata* f. sp. *avenae* races occurring in this region in order to understand the diversity, complexity, and evolutionary rate of pathogen populations (arising from new races). Through such surveys, it will be possible to determine prevailing races in major oat cultivation regions, and to detect virulence phenotypes that pose a threat to currently grown oat cultivars. Surveys of virulence phenotypes in cereal rusts can provide information on the effectiveness of currently used resistance genes and the potential effectiveness of genes that have not yet been widely deployed in commonly grown cultivars and to give support to local breeding programs aiming to improve cultivar resistance levels.

Based on the analysis of 46 pathogen isolates collected in three counties of Rio Grande do Sul State, Brazil, the goals of this work were to determine (i) the pattern of virulence or avirulence of the isolates to 25 *Pc* resistance genes, (ii) the similarity in virulence among *P. coronata* f. sp. *avenae* isolates considering their pattern of virulence or avirulence, (iii) the race code for each isolate by the North American system of nomenclature for *P. coronata* f. sp. *avenae* (Pca code), and (iv) supplemental *Pc* genes potentially useful as local differentials for *P. coronata* f. sp. *avenae* races.

MATERIALS AND METHODS

Leaf samples with rust symptoms were collected from the Brazilian Recom-

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mended Oat Cultivars Field Trial (EBCRA), from 22 to 29 September 2003 (soon after plant flowering), in three locations (counties) of RS State: (i) Passo Fundo (PF), located at 28°15′46″ latitude south and 52°24′24″ longitude west at an altitude of 687 m; (ii) Capão do Leão (CL), located at 31°52′00″ latitude south and 52°21′24″ longitude west at an altitude of 13.24 m; and (iii) Eldorado do Sul (EL), situated at 30°05′22″ latitude south and 51°39′08″ longitude west at an altitude of 46 m. Leaf samples were air dried at room temperature for about 48 h and then stored at 4°C at the Wheat Leaf and Stem Rusts Laboratory of Brazilian National Research Center for Wheat (EMBRAPA) at Passo Fundo, RS. Cv. UFRGS 7, which has exhibited high susceptibility to crown rust in Brazil, was used as a susceptible check (SC) for multiplying field isolates. Seedlings of SC with a completely exposed first leaf (7 days after sowing) were sprayed with Tween 20 solution (10 µl 100 ml^{-1}); then, each seedling was inoculated with a single collected fungal sample by scratching the spores using a sterilized spatula. After inoculation, each seedling was isolated in a plastic cone to avoid cross contamination between fungal isolates and placed in a dark chamber at 100% relative humidity and 20°C for 18 h. Seedlings were maintained in a greenhouse between 20 and 24°C and 60 to 80% relative humidity.

After 15 days in the greenhouse, a single pustule was selected on each inoculated plant and spores collected from that single pustule were used to inoculate another seedling, repeating the procedures of the original inoculations. After 15 days, the procedures were repeated for each monopustular isolate. The strategy of two consecutive single-pustule isolations was chosen to guarantee the purity of obtained isolates and, therefore, increase the precision of race identification.

After being submitted to two consecutive single-pustule isolations, the spores of each of the 46 isolates were multiplied in five SC seedlings and 15 days later were collected with the help of an air pump and stored in a vacuum in glass tubes at 4°C. These spores later were resuspended in water at a concentration of 10^5 spores ml^{-1}, then sprayed onto seedlings of 25 *Pc* near-isogenic lines at the stage of completely exposed first leaf (7 days after sowing). Before inoculation, seedlings were sprayed with a water-Tween 20 solution (10 µl 100 ml^{-1}). After inoculation, they were protected by a plastic cone (to avoid cross-contamination among isolates) and placed in a 100% humidity dark chamber for 18 h at 20°C for spore germination and penetration. Afterward, seedlings were maintained in greenhouse conditions at between 20 and 24°C and 60 to 80% humidity. From the 25 *Pc* near-isogenic lines evaluated, 16 were from the North American System of Nomenclature (NASN), proposed by Chong et al. (8) (*Pc 40, Pc 45, Pc 46, Pc 50, Pc 38, Pc 39, Pc 48, Pc 68, Pc 51, Pc 52, Pc 58, Pc 59, Pc 54, Pc 56, Pc 62,* and *Pc 64*) and 9 were additional *Pc* isogenic lines (*Pc 14, Pc 35, Pc 36, Pc 53, Pc 55, Pc 57, Pc 60, Pc 61,* and *Pc 63*). The crown rust resistance genes evaluated were derived from collections of wild oat *A. sterilis*, except for the *Pc 14* gene, which is from Ascencao, a Brazilian cultivar of *A. sativa* (18).

Fifteen days after inoculation of the near-isogenic lines, reactions were scored according to a scale from 0 to 4, where 0 = absent uredia or other macroscopic infection symptoms, 1 = small uredia surrounded by chlorosis or necrosis, 2 = small to medium size uredia surrounded by chlorosis, 3 = medium size uredia in a chlorotic area, and 4 = large uredia without chlorosis or necrosis. Responses 0, 1, and 2 were considered indicative of host resistance (low infection type) and responses 3 and 4 were considered indicative of host susceptibility (high infection type) (22). Each isolate showed a particular combination of high and low infection types (virulence or avirulence) for the *Pc* genes of the differentials and received a letter code according to the NASN for *P. coronata* f. sp. *avenae* (8). The NASN allows the addition of new subgroups of four *Pc* genes and the addition of new letters to the right of the currently used letters. Thus, the additional *Pc* genes used in these tests were evaluated for their potential as local race differentials.

The virulence or avirulence patterns of isolates for each one of the studied *Pc* genes were transformed to a binary scale where 1 = high infection type or virulence and 0 = low infection type or avirulence. The similarity of virulence was estimated for each pair of isolates, through an index of simple coincidence (ISC) according to the equation ISC = C/N, where C is the number of differential lines on which a pair of isolates was either virulent or avirulent, and N is the total number of differential lines used (24). Based on the generated similarity matrix, a dendrogram was constructed using the unweighted pair group method with arithmetic means (25). To verify the adjustment between the similarity matrix and the obtained dendrogram, a cophenetic correlation coefficient (r) was calculated according to Sokal and Rolf (26), with the NTSYS pc 2.1 program (23).

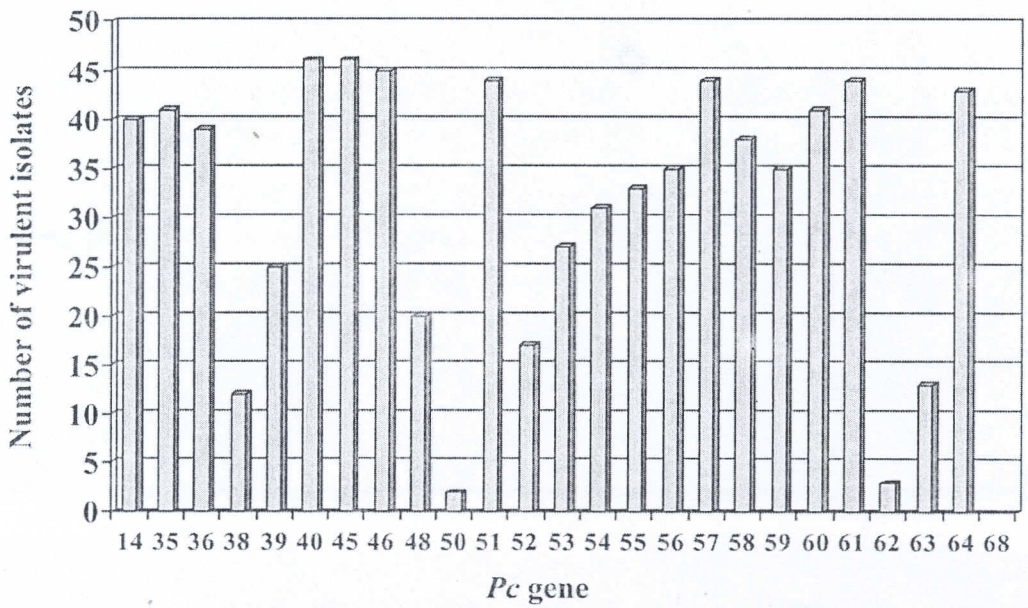

Fig. 1. Number of the 46 isolates of *Puccinia coronata* f. sp. *avenae* originated from Capão do Leão (CL), Passo Fundo (PF), and Eldorado do Sul (EL) counties, displaying virulence (high infection type) on each of the 25 *Pc* genes studied.

RESULTS

From all samples collected and purified, 46 isolates of *P. coronata* f. sp. *avenae* were used in this study, with 15 from PF, 15 from EL, and 16 from CL. Inoculation of the 46 isolates on each of the 25 *Pc* near-isogenic lines resulted in 1,150 infection sites, of which 764 (66%) showed a virulence response (high infection type) and 386 (34%) showed an avirulence reaction response (low infection type). Seven of the differentials (*Pc 40, Pc 45, Pc 46, Pc 51, Pc 57, Pc 61.* and *Pc 64*) were susceptible to more than 90% of the isolates tested (Fig. 1). Only *Pc 38, Pc 48, Pc 50, Pc 52, Pc 62, Pc 63,* and *Pc 68* were resistant to more than 50% of the isolates in our tests (Fig. 1). The gene *Pc 68* was the most resistant differential because none of our isolates was virulent on it (Fig. 1).

Based only on the *Pc* genes from the NASN for the fungus *P. coronata* f. sp. *avenae*, it was possible to classify the 46 isolates in 30 distinct races (Table 1). The most frequent race was SBPH, which occurred six times (13%), but was not detected in CL. The second most frequent race was SDTH, which occurred four times (8.7%) but was not detected in PF. Another two races (SJTR and SJRR) occurred three times (6.5%), whereas four races occurred two times (4.3%; SSSR, SQNM, SDPH, and SGPM). Twenty-two races were detected only once (Table 1).

With the goal of quantifying the efficiency of the NASN for identifying races of the fungus *P. coronata* f. sp. *avenae* in southern Brazil, a comparison was made with the isolate virulence similarity, considering all 25 genes used in the present work. Only the isolates that presented the same virulence and avirulence pattern for all 25 *Pc* genes revealed an index of simple coincidence of 100% (i.e., were completely similar). Of the six isolates classified as race SBPH according to the NASN of fungus *P. coronata* f. sp. *avenae*, five had a similarity of 100% when the data from all 25 *Pc* genes were used, indicating that such isolates probably represent the same race (Fig. 2). The only isolate that did not present 100% similarity was EL3, which has one extra virulence gene (Fig. 2). For the second most frequent race, SDTH, three isolates were identical, but the fourth had an extra virulence gene (Table 1; Fig. 2). Races SJTR and SJRR each had two identical isolates and one distinct isolate when all 25 differentials were considered. Races SSSR and SQNM had two identical isolates each, but races SDPH and SGPM had two distinct isolates each based on all 25 differentials. Considering the three collecting locations, there was no clear pattern of virulence variability distribution (Fig. 2), because the isolates did not cluster according to the collecting location and a high number of overlaps were detected among the isolates from different locations.

The use of all 25 *Pc* genes increased the number of isolates with different virulence or avirulence patterns from 30 (NASN only) to 36 (Fig. 2). Among the sampled isolates, those which presented the highest number of virulence genes were CL6 and EL4, with 21 genes, whereas isolates that revealed the lowest number of virulence genes were CL16 and PF2, with 12 virulence genes each. The most frequently collected isolate, SBPH, was observed to have 15 virulence genes and the average number of virulence genes per isolate was 17 genes (Table 1).

Table 1. *Puccinia coronata* f. sp. *avenae* isolates collected in Capão do Leão (CL), Passo Fundo (PF), and Eldorado do Sul (EL) Counties, nomenclature according to the North American System (NASN) only, nomenclature including a new subset of *Pc* genes (*Pc 36, Pc 53, Pc 55,* and *Pc 63*) as local differentials, and number of virulence (*vir*) or avirulence (*avr*) genes for each isolate for the 25 *Pc* genes used

Isolates	NASN	NASN plus *Pc* genes subset	No. of *vir/avr* genes
EL3	SBPH	SBPHS	16/9
EL8, PF1, PF6, PF11 and PF12	SBPH	SBPHQ	15/10
EL2	SDTH	SDTHS	18/7
EL9, EL10 and CL15	SDTH	SDTHQ	17/8
EL5, EL13	SJTR	SJTRN	19/6
EL11	SJTR	SJTRS	20/5
EL15	SJRR	SJRRS	19/6
CL13 and CL14	SJRR	SJRRN	18/7
CL10 and CL11	SSSR	SSSRP	20/5
CL5 and PF5	SQNM	SQNMP	16/9
PF10	SDPH	SDPHS	17/8
PF14	SDPH	SDPHG	13/12
CL1	SGPM	SGPMJ	16/9
PF3	SGPM	SGPMS	16/9
EL6	QGTT	QGTTS	19/6
CL4	SBNR	SBNRN	15/10
PF7	SBPG	SBPGQ	14/11
PF15	SBPM	SBPMS	15/10
EL7	SBPR	SBPRQ	16/9
EL1	SDMH	SDMHS	15/10
CL2	SDPR	SDPRG	15/10
CL16	SDTG	SDTGQ	12/13
EL12	SJSR	SJSRN	18/7
PF13	SGFM	SGFMK	15/10
CL12	SGLR	SGLRN	14/11
CL3	SGNQ	SGNQN	14/11
EL4	SGTR	SGTRN	17/8
PF2	SLBM	SLBMK	12/13
CL9	SLMR	SLMRF	16/9
CL8	SQMM	SQMMP	17/8
PF4	SQPR	SQPRP	19/6
PF9	SQPT	SQPTF	17/8
PF8	SSNM	SSNMP	18/7
EL14	SSTM	SSTMT	21/4
CL7	TGNM	TGNMS	17/8
CL6	TQPT	TQPTP	21/4
Average[a]	...	...	17/8

[a] Average of virulence/avirulence genes.

DISCUSSION

The inoculation of the 46 southern Brazilian *P. coronata* f. sp. *avenae* isolates on the 25 *Pc* near-isogenic lines resulted in 66% virulence response and 34% avirulence reaction response, which indicates generally high virulence of these isolates. Similar result was reported by Leonard et al. (17) with Israeli *P. coronata* f. sp. *avenae* isolates.

The differentials *Pc 40, Pc 45, Pc 46, Pc 51, Pc 57, Pc 61,* and *Pc 64* were susceptible to more than 90% of the isolates tested. Leonard and Martinelli (18) found that *Pc 45, Pc 46, Pc 57, Pc 60*, and *Pc 61* were susceptible to more than 90% of the isolates that they collected in southern Brazil in 1997, but these differentials were susceptible to somewhat lower proportions (18 to 87%) of the isolates collected in 1998 and 1999. Only *Pc 38, Pc 48, Pc 50, Pc 52, Pc 62, Pc 63,* and *Pc 68* were resistant to more than 50% of the isolates in the present work, and this is consistent with the low frequencies of virulence to these differentials among the isolates Leonard and Martinelli (18) evaluated from 1997 to 1999.

None of our isolates was virulent on *Pc 68*, showing that the *Pc 68* gene was the most resistant differential, which also is similar to observations by Leonard and Martinelli (18), who found (in 1997 to 1999) only 1 of 144 isolates from southern Brazil that was virulent on *Pc 68*. This gene is widely recognized as one of the most effective genes against this fungus (6,14). Despite the fact that the present work did not detect any *Pc 68*-virulent

isolate, this result is not conclusive evidence of the nonexistence of virulent isolates to this gene in southern Brazil, because the studied isolates were collected in the EBCRA, where none of the cultivars present this gene. In case one isolate would contain the *Pc 68* avirulence gene, this isolate would have a reduced fitness due to an unnecessary gene for virulence, possibly resulting in a competitive disadvantage against other non-*Pc 68*-containing isolates. Thus, an isolate containing this unnecessary gene will tend to occur in lower frequency. Because the number of isolates herein studied was relatively small, it is possible that, because of a sampling error, this particular *Pc 68* isolate has not been collected. Nevertheless, *Pc 68* is a potential source of resistance to the pathogen, but this only can be proven after its use in large areas for long periods of time.

Crown rust isolates collected on the California Islands in the United States were not virulent to *Pc 38*, *Pc 39*, *Pc 50*, *Pc 52*, *Pc 56*, *Pc 62*, and *Pc 68* and were not avirulent to *Pc 14*, *Pc 45*, *Pc 46*, and *Pc 54* (14). These results partially agree with the results herein described because, among the southern Brazilian isolates (i) virulence to *Pc 68* was not detected, (ii) the *Pc* genes *Pc 38*, *Pc 50*, and *Pc 62* were effective against a large number of isolates, (iii) the gene *Pc 45* was not effective against any isolate, (iv) the *Pc 46* gene was effective against only one isolate, and (v) the *Pc 54* gene was effective against only 33% of the isolates. However, the results disagree regarding the genes *Pc 39*, *Pc 52*, and *Pc 56*, which presented virulent isolates in the present work. Leonard (16) also found high frequencies of virulence to *Pc 14*, *Pc 45*, *Pc 46*, and *Pc 54* in isolates collected in California from 1990 to 2000, and a few isolates virulent to *Pc 68* in the same period. Leonard (16) indicated that the virulence to *Pc 45*, *Pc 46*, and *Pc 54* occurred at high frequency in southern Europe but not in northern Europe or in the United States, except for California. It is also interesting that van Niekerk et al. (27) found that virulence to *Pc 45*, *Pc 46*, and *Pc 54* occurred at very high frequency in South Africa, and that Leonard and Martinelli (18) detected relatively high frequencies of virulence to *Pc 45*, *Pc 46*, and *Pc 54* in Brazilian and Uruguayan isolates. The reason for this apparent association of greater virulence in warmer climates is yet not clear.

The use of all 25 *Pc* genes (16 from NASN and 9 others) increased the number of isolates with different virulence or avirulence patterns from 30 (NASN only) to 36. Such results indicate the presence of high variability in the population and the need for including new *Pc* genes in the NASN, for a better discrimination of Brazilian crown rust isolates. Therefore, we propose the inclusion of a new subgroup of four *Pc* genes (*Pc 36*, *Pc 53*, *Pc 55*, and *Pc 63*, in that order) to the NASN for the fungus *P. coronata* f. sp. *avenae*. The *Pc 53*, *Pc 55*, and *Pc 63* genes candidates are to be added to the NASN as a new subgroup, because they presented a relative balance between the number of virulent and avirulent isolates. The *Pc 36* gene, which presented the lowest percentage of virulent isolates among the remainder genes, also is proposed as part of this addition. Chong et al. (8) chose not to include the *Pc 55* and *Pc 63* genes in the NASN differentials, because these genes presented identical reactions to those of the *Pc 39* and *Pc 38* genes, respectively, when tested against Canadian isolates of the fungus. Nevertheless, in the present work, we suggest the inclusion of these genes in the nomenclature system, because these genes presented different reactions toward the Brazilan isolates herein evaluated, which also is in agreement with results reported by Martinelli et al. (20), Cruz et al. (10), and Leonard and Martinelli (18). The inclusion of four additional herein-proposed genes in the NASN differential set was effective, because it divided the 46 isolates into 36 distinct races, as well as the use of all 25 *Pc* genes here evaluated.

Both the NASN and our proposed set of 20 differentials indicated the presence of high virulence variability among the southern Brazilian isolates and the nonexistence of a predominant race. These results are in agreement with those of Martinelli et al. (20), who detected 53 distinct races among 53 southern Brazilian isolates. Nevertheless, the results reported by

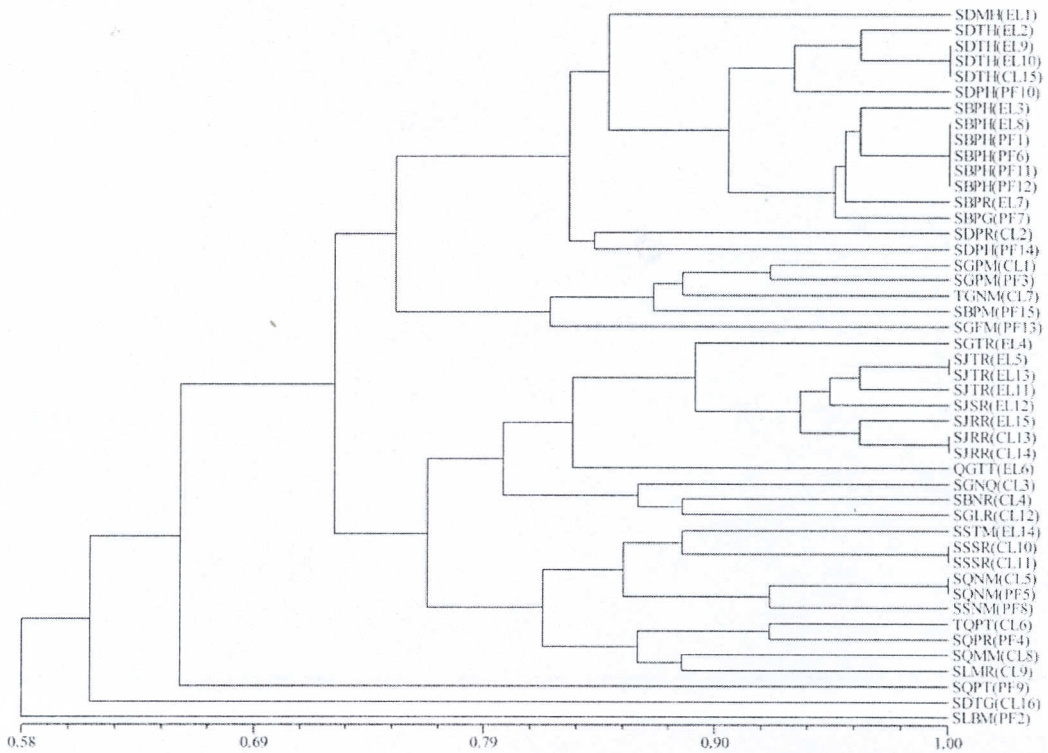

Fig. 2. Dendrogram of 46 *Puccinia coronata* f. sp. *avenae* isolates, collected in three Rio Grande do Sul counties, Passo Fundo (PF), Capão do Leão (CL), and Eldorado do Sul (EL), with their respective nomenclatures according to the North American System for Nomenclature (8). The dendrogram was obtained through the unweighted pair group method with arithmetic means clustering method from the similarity index of simple coincidence, based on the patterns of virulence and avirulence of the 46 isolates to the 25 *Pc* genes employed in this study. The value of the cophenetic correlation coefficient is 0.82.

van Niekerk et al. (27) show less diversity in *P. coronata* in South Africa than in Brazil. Van Niekerk et al. (27) evaluated isolates collected from eight locations over 2 years and found only five races (SBLL, SGLL, PBBB, SDQL, and JBBM) based on the NASN differentials. Among these races, only SBLL occurred at high frequency. We did not find any of the South African races in Brazil.

The isolates CL6 and EL4 presented the highest number of virulence genes (21 genes), whereas the isolates CL16 and PF2 presented the lowest (12 genes). SBPH was the most frequent isolate, presenting 15 virulence genes. With 17 genes as the average number of virulence genes per isolate, results indicate high pathogen race variability and a high number of super races. Thus, in Brazil, these two phenomena could explain the great difficulties faced by Brazilian oat breeders when selecting for crown rust resistance. The occurrence of super races in such high frequency occurs neither in the pathogen's center of origin in Israel (17) nor in the California Islands, where the isolates present six virulence genes on average (14).

Among the possible causes for such high variability in populations of the fungus causing crown rust, the most accepted are (i) recombination of virulence genes via sexual crossings, (ii) asexual recombination via anastomosis (hyphal fusion), and (iii) mutation stockpiling (7). In Brazil, there are no reports of a sexual cycle for this fungus, making asexual recombination or mutation stockpiling the major contributory factors of such high variability. Such evolutionary mechanisms probably are potentialized in Brazil due to the fact that pathogens are broadly spread by wind (3), and that the oat is cultivated year-round in the southernmost countries of South America. It is expected that asexual recombination and mutations do occur year round and are widespread in this region. Another possible explanation is the fact that the isolates were collected in the EBCRA, where there is a high concentration of elite genotypes presenting a large number of resistance genes. Such a collection would generate strong selection pressure, leading to the establishment of a high number of virulence genes.

Apparently, there is no clear pattern of virulence variability distribution among the three collecting locations herein analyzed, because the isolates did not cluster according to the collecting location. There are two possible explanations for the absence of local clustering tendency: (i) the ease of dispersal allows all isolates to be uniformly distributed throughout different locations or (ii) the isolates were collected on the EBCRA, which is represented by the same cultivars in the three locations.

The results of the present work corroborate the idea that the southern Brazilian *P. coronata* f. sp. *avenae* populations have a high variability for virulence. The inclusion of one subset of *Pc* genes in the NASN was sufficient to distinguish the local isolates and, therefore, the permanent addition of this subset is recommended to improve the efficiency of this system. Furthermore, we have shown that the isolates analyzed in this study have a high level of virulence and did not show a local grouping tendency, indicating that they are distributed uniformly among the three locations of sampling. Moreover, the present studies demonstrate that some few genes still maintain low virulence frequencies and that the great diversity and high levels of virulence among isolates of *P. coronata* f. sp. *avenae* in Brazil will make it unlikely that long-lasting control of crown rust can be obtained with race-specific resistance.

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