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Originalarbeit

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Nature of mixed infection type 2(5) observed in rye (*Secale cereale* L.) plants carrying the *Pr1* leaf-rust resistance gene

Entstehung eines gemischten Infektionstyps 2(5) bei Trägern des Braunrost-Resistenzgens *Pr1* bei Roggen (*Secale cereale* L.)

Abstract

When challenged with a complex leaf-rust inoculum, rye plants carrying the dominant leaf-rust resistance gene *Pr1* predominantly react with a mixed infection type, IT 2(5). The present study investigates the nature of this mixed infection type. The results demonstrate, that IT 2(5) is not caused by partial dominance of *Pr1* nor by the genetic background. Rather, IT 2(5) reflects the occurrence of *Pr1*-virulent leaf-rust races among the inoculum in low frequency. A simple approach was followed to estimate the frequency of *Pr1*-virulent races in a leaf-rust population. This approach included comparative detached-leaf testing of *Pr1* carriers and *Pr1*-deficient genotypes and relating the numbers of pustules observed on both genotypes. For the present case we estimated a frequency of 3.19% of *Pr1*-virulent leaf-rust races among a local leaf-rust population sampled in the field. This estimate was verified in a validation experiment.

Key words: *Secale cereal*, *Puccinia recondite*, mixed infection type

Zusammenfassung

Bei der Inokulation von Trägern des dominanten Braunrostresistenzgens *Pr1* mit einer komplexen Braunrostpopulation kommt es vorwiegend zur Entwicklung eines gemischten Infektionstyps IT 2(5). In der hier vorgestellten Studie wurde die Entstehung dieses gemischten Infektionstyps untersucht. Die Ergebnisse belegen, dass IT 2(5) weder durch partielle Dominanz des Resistenzgens *Pr1*, noch durch Einflüsse des genetischen Hintergrundes verursacht wird. Die Entstehung von IT 2(5) liegt vielmehr in der Existenz einer *Pr1*-virulenten Braunrostrasse im verwendeten Inokulum begründet, die in einer niedrigen Frequenz auftritt. Mit Hilfe eines einfachen Versuchsansatzes wurde die Frequenz der *Pr1*-virulenten Braunrostrasse in der verwendeten Braunrostpopulation geschätzt. Hierbei wurden vergleichende Blattsegmenttests von *Pr1*-Trägern und Genotypen ohne *Pr1* durchgeführt und die bei beiden Genotypen erfassten Pustelanzahlen zueinander in Beziehung gesetzt. Im vorliegenden Fall wurde für die *Pr1*-virulente Braunrostrasse innerhalb einer im Feld gesammelten lokalen Braunrostpopulation eine Frequenz von 3,19% geschätzt. Durch ein Validierungsexperiment, in dem eine aus einer *Pr1*-virulenten und einer *Pr1*-avirulenten Braunrostrasse erstellte Rostpopulation eingesetzt wurde, konnte die geschätzte Frequenz bestätigt werden.

Stichwörter: *Secale cereale*, *Puccinia recondita*, gemischter Infektionstyp

Introduction

In rye (*Secale cereale* L.), leaf rust (LR) caused by *Puccinia recondita* f. sp. *secalis* is the economically most important

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windspread pathogen. The potential of epidemic incidence of this disease, with significant yield losses (Kobylanski and Solodukhina, 1983; Frauenstein, 1985; Miedaner and Sperling, 1995), as well as the biological profit cereal rusts are expected to realize with a warming climate (Jahn et al., 1995), emphasize the need for deploying natural genetic diversity to improve the resistance of rye to LR. To investigate approaches for a genetically based plant protection against LR, various studies on qualitative as well as quantitative LR resistance have been conducted (Parlevliet, 1977, 1989; Kobylanski and Solodukhina, 1983, 1996; Musa et al., 1984; Solodukhina, 1994, 2002; Miedaner et al., 2002; Wilde et al., 2006). In a long-term approach we started to systematically evaluate a world collection of genebank accessions to identify and characterize genes for LR resistance in rye (Ruge et al., 1999; Roux et al., 2000; Roux et al., 2007) and reported the genomic localization and effectiveness of the dominant LR resistance genes *Pr1* and *Pr2* (Wehling et al., 2003), as well as *Pr3*, *Pr4*, and *Pr5* (Roux et al., 2004). Like in other cereal-rust pathosystems (Stakman et al., 1962; McNeal et al., 1971; Roelfs and Bushnell, 1985; McIntosh et al., 1995) the reactions to rye LR observed in detached-leaf tests are scored by assigning discrete infection types (IT). For scoring the reactions to rye LR, we have used the scheme of Frauenstein and Reichel (1978), which basically comprises six infection types (IT 1 through IT 6). In some instances, though, "mixed" infection types show up when using a natural leaf-rust population as inoculum. These mixed types are characterized by a predominant "background" infection type (IT 2, 3, or 4) combined with ancillary incidence of fully developed pustules resembling IT 5 or even IT 6, and are designated as either IT 2(5), IT 3(5), or IT 4(5), respectively. For instance, genotypes carrying the resistance gene *Pr1* predominantly reacted with IT 2(5) when challenged with LR inoculum collected from rye plants in the trial field at the Groß Lüsewitz experimental station (Wehling et al., 2003; Roux et al., 2004). Mixed reaction patterns were observed also by others. Solodukhina (2002) reported heterogeneous LR resistance (IT X) in rye, which she observed in field trials at the stage of grain filling. Furthermore, occurrence of IT X was described for stem rust as well as leaf rust in wheat (Stakman et al., 1962; McIntosh et al., 1995; Felsenstein et al., 1998). The expression of LR resistance genes in wheat is known to be influenced by the genetic background, by suppressor and modifier genes (Kolmer, 1996) and by the prevailing temperature (Dyck and Samborski, 1982; Dyck and Johnson, 1983). Various conclusions have been drawn on the causes of "mixed" infection types with several wheat-rust systems. After inoculation of wheat genotypes with single pustule isolates of stem rust, Stakman et al. (1962) observed X-type reactions as a result of low temperature as well as of inadequate light conditions. For leaf-rust resistance genes *Lr14*, *Lr14a,* and *Lr14b* incomplete dominant inheritance was postulated to be responsible for the development of X-type reactions against leaf rust races (McIntosh et al., 1967; Dyck and Samborski, 1970). Rajaram et al. (1971), however, explained the occurrence of such ITs with the lack of modifier genes, i.e. the genetic background in the wheat varieties under investigation.

The present study addresses the nature of mixed infection type 2(5) on rye plants which carried the leaf-rust resistance gene *Pr1* and were inoculated with a natural leaf-rust population.

Material and Methods

Collection of single-pustule isolates (SPIs) from mixed infection types

Plants of a BC_5S_1 family segregating for the resistance gene *Pr1* were inoculated with a LR bulk sample originally collected in the trial field at Groß Lüsewitz experimental station (GL inoculum). After sampling, this bulk inoculum had been propagated for several years on rye plants of susceptible cvs. 'Pluto' and 'Ursus' under climate-chamber conditions. Pathogen inoculation and detached-leaf tests were carried out according to the method described by Wehling et al. (2003). By means of Pasteur pipettes, uredospores of 8 single, sporulating pustules (IT 5 or IT 6) were sampled from leaf segments exhibiting the mixed IT 2(5). The uredospores were directly dispersed onto detached leaves of the susceptible rye inbred line L-301, with the leaf segments lying on solid agar media in Petri dishes of 9 cm diameter. The susceptible cv. 'Ursus', which is characterized by broad leaf blades, was used for the subsequent maintenance and propagation of the 8 SPIs (*GL1* through *GL8*) by inoculating fresh detached leaves of cv. 'Ursus' every 14 days using the above mentioned method.

Comparative resistance tests with GL inoculum vs. SPIs

Sixteen individuals of each the two near-isogenic lines (NIL) *JKI-04-840-9* and *JKI-04-840-22* (BC_5S_2 families), both of which were homozygous for the leaf-rust resistance gene *Pr1* (*Pr1*-NILs), were used in comparative resistance tests. Firstly, a detached-leaf test was conducted and two leaf segments of the first and the second true leaf of 14-15 and 21-23-day old plantlets, respectively, were inoculated with the GL inoculum in two consecutive inoculation experiments. Secondly, two leaf segments of the third true leaf of 4 of these plantlets (28-30-day old) were inoculated with either of the eight SPIs, *GL1*-*GL8*. This inoculation was carried out by a small-sized infection tower, which allows for inoculating 1-5 detached leaves with uredospores of a single SPI. Uredospores of the SPIs were directly released into the infection tower by means of Pasteur pipettes. The subsequent incubation of inoculated leaf segments followed the common method. In both experiments host-pathogen reactions were scored using the scale of IT1 through IT6 according to Frauenstein and Reichel (1978), supplemented by the recently defined mixed IT 2(5) (Roux et al., 2004).

Main Experiment: Quantification of the frequency of IT 2(5)-inducing uredospores among the GL inoculum

In this experiment, 98 individuals of the *Pr1*-NIL *JKI-04-840-22* and 96 individuals of the recipient sus-

ceptible inbred line L-301 were jointly inoculated with the GL inoculum in detached-leaf tests using a normal-sized infection tower according to Wehling et al. (2003). Digital photographs were shot using a stereomicroscope with a fixed eight-fold blow-up to document the reactions of each leaf segment to the rust inoculum 8-10 days post infection (dpi). Using a standard office software application, graphical frames of defined size were laid over the photographs to enable the counting of pustules across a standard leaf area of 23.0 x 3.8 mm. The frequency ρ_1 of IT 2(5)-inducing uredospores among the GL leaf-rust population was estimated as the ratio of mean numbers ($\hat{\rho}_1 = R_1 = \bar{x}_1/\bar{y}_1$) of fully developed leaf-rust pustules per leaf area observed on the *Pr1*-NIL and L-301 individuals, respectively. To compare the means a one-tailed Welch *t*-test for equality of means from distributions of different variances was applied. A Wald confidence interval (C.I.) for ρ_1 was calculated using the Poisson log-linear model according to Price and Bonett (2000).

Validation Experiment

In a validation experiment the *Pr1*-avirulent SPI6 (Roux et al., 2007) and the *Pr1*-virulent SPI *GL1* were mixed at a ratio of 96.81 to 3.19 (w/w) to generate a mixed spore population with defined virulence frequencies. Following the method described in the previous section 120 individuals of the *Pr1*-NIL *JKI-04-840-22* and 120 individuals of the inbred line L-301 were inoculated with this artificially generated mixed spore population. Pustules were counted across a standard leaf area and a notional frequency ρ_2 of *Pr1*-virulent uredospores within the artificial mixed spore population was calculated as the ratio of the mean numbers ($\hat{\rho}_2 = R_2 = \bar{x}_2/\bar{y}_2$) of fully developed leaf-rust pustules which were observed on the *Pr1*-NIL and L-301 individuals, respectively. Rates of IT 2(5)-inducing uredospores observed with the GL inoculum in the main experiment (R_1) and with the artificial mixed spore population in the validation experiment (R_2), respectively, were tested for equality as the difference of double ratios according to Kish (1995), with $H_0 : R_2 - R_1 = 0$; $H_a : R_2 - R_1 \neq 0$, and assuming non-correlated samples. Briefly, the variances of R1 and R2, respectively, were calculated as $var(R_i) = R_i^2(var(\bar{x}_i)/\bar{x}_i^2 + var(\bar{y}_i)/\bar{y}_i^2)$ and the variance of the difference as $var(R_2\text{-}R_1) = var(R_2) + var(R_1)$. The 95% confidence interval for the difference was calculated from the standard error of the difference.

Results

Reaction of Pr1-NILs to the GL inoculum and the SPIs GL1 to GL8

In inoculation experiments with the GL bulk inoculum, all of the tested plants of two *Pr1*-NILs showed non-compatible host-pathogen background reactions, which were of either pure IT 2 or mixed IT 2(5) type (Tab. 1). The predominant IT 2(5) was observed with 25 of 32 individuals and was characterized by an IT 2 background reaction (chlorotic hypersensitivity response) associated with a small number of pustules per leaf which in respect to their size resembled IT 5 or IT 6 (Fig. 1).

Reaction patterns were profoundly different when leaves of the same individuals were challenged with either of the SPIs, *GL1* to *GL8*, rather than with the GL inoculum. In all cases, the SPIs gave rise to compatible host-pathogen reactions, i.e., IT 5 or IT 6 (Tab. 1).

Frequency of Pr1-virulent races among the GL inoculum

The *Pr1*-NIL *JKI-04-840-22* and its recipient parent, i.e., susceptible inbred line L-301, were analyzed with 98 and 96 detached leaves, respectively, each taken from a different plant. Detached-leaf tests were carried out with

Tab. 1. Reaction of 32 individuals from two *Pr1*-NILs to the GL inoculum (GL) and to 8 SPIs (*GL1* to *GL8*) isolated from mixed-IT 2(5) pustules of *Pr1* carriers

Pr1-NILs	Reaction (IT) to inoculum															
Individuals/NIL	GL	**GL1**	GL	**GL2**	GL	**GL3**	GL	**GL4**	GL	**GL5**	GL	**GL6**	GL	**GL7**	GL	**GL8**
JKI-04-840-9																
Individual 1 of 16 [a]	2 (5) [b]	**5** [c]	2 (5)	**6**	2 (5)	**6**	2	**5**	2	**5**	2 (5)	**5**	2 (5)	**5**	2 (5)	**5**
Individual 2 of 16 [a]	2 (5)	**6**	2 (5)	**6**	2	**6**	2 (5)	**6**	2 (5)	**5**	2 (5)	**5**	2	**6**	2 (5)	**5**
JKI-04-840-22																
Individual 1 of 16 [a]	2 (5)	**5**	2 (5)	**5**	2 (5)	**5**	2 (5)	**5**	2 (5)	**6**	2 (5)	**5**	2	**5**	2	**5**
Individual 2 of 16 [a]	2 (5)	**5**	2 (5)	**6**	2 (5)	**5**	2 (5)	**5**	2 (5)	**6**	2 (5)	**5**	2	**5**	2 (5)	**5**

[a] From each *Pr1*-NIL, two of 16 individuals (#1 & 2, #3 & 4, etc.) were inoculated with one of the 8 SPIs, *GL1* to *GL8*. Each of the 16 individuals/NIL was challenged with the GL inoculum.
[b] Each score given in GL columns corresponds to the highest of four IT scores recorded in two experiments, each carried out with two repetitions.
[c] Each score given in SPI columns corresponds to the highest of two IT scores recorded in two independent repetitions; values in bold indicate a compatible host-pathogen reaction.

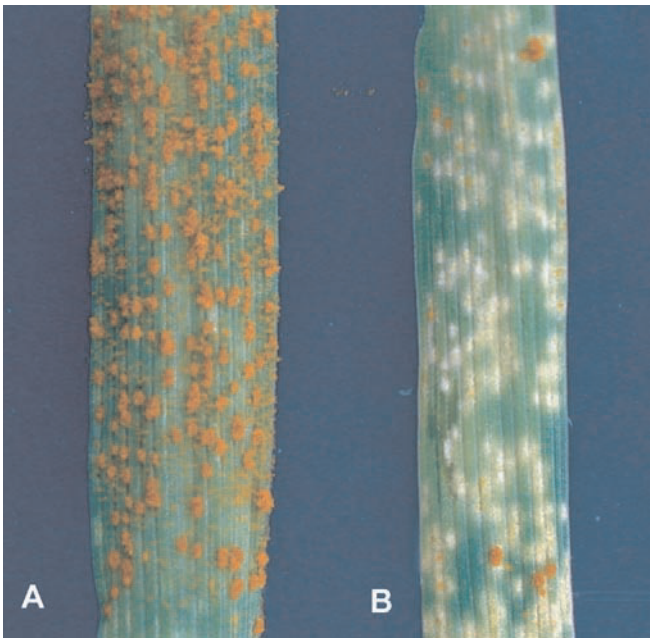

Fig. 1. Reaction of rye genotypes to GL inoculum. **A**, susceptible inbred line L-301 reacting with IT 6; **B**, *Pr1*-NIL reacting with mixed infection type IT 2(5).

the GL inoculum and the formation of pustules indicating compatible reaction between an uredospore and the host tissue was recorded across a defined leaf area. Among the *Pr1*-NIL individuals which reacted as either IT 2 or IT 2(5), a mean number of 2.34 fully developed pustules per leaf area was observed, with a range of 0-5. In comparison, the range of pustule numbers counted on the fully susceptible L-301 individuals was 48-104 per leaf area with a mean value of 73.41 (Fig. 2, Tab. 2). The comparison of means conducted by a Welch *t*-test confirmed the means as being different with high significance ($t = 57.76$; $t_{0.05;\,192} < 1.658$).

Assuming that each counted pustule stemmed from a single compatible uredospore, the frequency ρ_1 among the GL leaf-rust population of those uredospores which could specifically overcome the resistance gene *Pr1* was estimated at $\hat{\rho}_1 = 0.0319$ (Tab. 2), with a 95% C.I. of [0.028; 0.036].

Validation experiment

A total of 120 detached leaves, each taken from a single plant of the *Pr1*-NIL *JKI-04-840-22* and the susceptible inbred line L-301, respectively, were inoculated in a validation experiment with an artificially generated mixed-spore population consisting of 96.81% of the *Pr1*-avirulent SPI6 and 3.19% of the *Pr1*-virulent SPI *GL1*. Fully developed pustules per standard leaf area were registered with a mean number of 1.75, covering a range of 0-6 among *Pr1*-NIL plants (Tab. 3). In contrast, susceptible individuals of L-301 revealed a mean number of pustules of 53.18, with a range of 29–98. Following the procedure of the main experiment, the frequency ρ_2 of uredospores which showed a compatible reaction to the resistance gene *Pr1* among the GL leaf-rust population was estimated at $\hat{\rho}_2 = 0.0329$, with a 95% C.I. of [0.029; 0.038]. The difference $\hat{\rho}_2 - \hat{\rho}_1 = 0.001$ of the estimated frequencies in the main and the validation experiment was confirmed as being not divergent from zero, with a 95% C.I. of (−0.0046; 0.0066).

Discussion

In previous studies mixed infection types were sometimes observed when rye plants carrying resistance genes *Pr1, Pr3, Pr4,* or *Pr5* were challenged with complex LR inoculum, and the emergence of virulent LR races in initially low frequency was discussed as a hypothetical explanation (Wehling et al., 2003; Roux et al., 2004). The present study concludes that this assumption is appropriate. The results demonstrate that when individual plants carrying the *Pr1* resistance gene were challenged either with the GL leaf-rust population or with SPIs sampled from single IT 2(5) pustules, these plants invariably reacted very differently depending on the inoculum, i.e., either partly resistant (IT 2(5)) or fully susceptible (IT 5 or 6), respec-

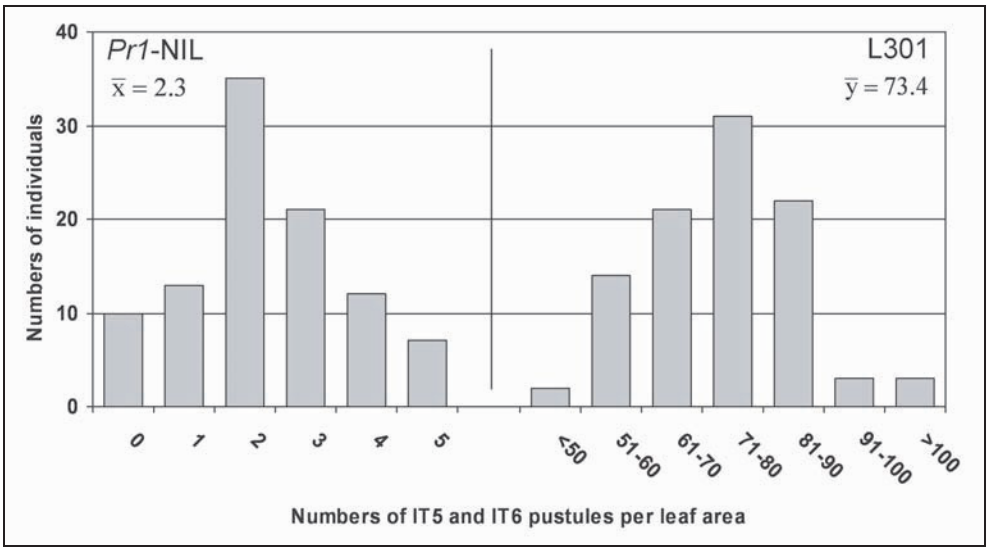

Fig. 2. Distribution of the numbers of fully developed pustules (IT5 or IT6) across a leaf area of 87.4 mm² on *Pr1*-NIL and L-301 individuals challenged with the GL inoculum in detached-leaf tests of the main experiment.

Tab. 2. Main experiment: Compatible host-pathogen reactions of individuals of *Pr1*-NIL (*JKI-04-840-22*) and of L-301 to the GL inoculum in detached-leaf tests

	No. plants*	No. fully developed pustules			Estimated frequency ($\hat{\rho}_1$) of *Pr1*-virulent uredospores# and its 95% C.I.
		abs.	per leaf area		
			mean	sd	
Pr1-NIL	98	229	2.34	1.3	0.0319 [0.028; 0.036]
L-301	96	7047	73.41	12.0	

* Each plant was represented by one detached leaf; sd, standard deviation.
# Calculated from the ratio of mean numbers of fully developed pustules/leaf area.

Tab. 3. Validation experiment: Compatible host-pathogen reactions of individuals of *Pr1*-NIL (*JKI-04-840-22*) and of L-301 to the artificial mixed-spore population in detached-leaf tests

	No. plants*	No. fully developed pustules			Reestimated frequency ($\hat{\rho}_2$) of *Pr1*-virulent uredospores# and its 95% C.I.
		abs.	per leaf area		
			mean	sd	
Pr1-NIL	120	210	1.75	1.2	0.0329 [0.029; 0.038]
L-301	120	6382	53.18	10.8	

* Each plant was represented by one detached leaf; sd, standard deviation.
# Calculated from the ratio of mean numbers of fully developed pustules/leaf area.

tively. Since the same *Pr1*-homozygous plants were tested with the GL inoculum and a given SPI, partial dominance of *Pr1* in a heterozygous state as well as genetic-background effects such as modifier genes can be ruled out as potential causes for these differences. Also, the mixed infection type may not be confused with partial resistance caused by quantitative inheritance of resistance. Rather, it may be inferred from the results that in the present case, mixed infection type 2(5) indicates the presence of one or several virulent leaf-rust races among the GL inoculum which are able to overcome the *Pr1* resistance gene. When these races constitute the entire inoculum, as in the case of SPIs *GL1* through *GL8*, then *Pr1*-NILs turned out to be fully susceptible to this inoculum.

The experimental approach applied in the present study represents, a simple way to clarify the nature of a mixed infection type and to approximately determine the frequency of pathogen races which can overcome a specific resistance gene. This approach should be applicable also with other instances of mixed infection types in race-specific host-pathogen systems.

The simple approach, as applied in the main experiment, to estimate the frequency of virulent races among a complex pathogen population may result in an overestimation in cases where the doses of applied inoculum exceed the saturation level for the given unit leaf area. Following the multiple infection transformation (Gregory, 1948), there is not a linear relationship between numbers of pustules that develop on inoculated leaves vs. the numbers of spores applied per leaf. Thus, unless the appropriate inoculum dose is determined in elaborate saturation experiments, frequency estimates should be confirmed by a validation experiment. In the present study, the nearly perfect conformity of the two frequencies estimated in the main and the validation experiment is a clear indication that the dose of inoculum applied during the main experiment did not exceed the saturation level for the rye-leaf rust pathosystem. Otherwise, a significantly higher frequency of the virulent race would have been expected in the validation experiment as compared to the main experiment.

A second potential cause which may lead to overestimating the frequency might be given when a susceptible host line carries other, yet unknown, race-specific resistance genes to the same pathogen. Any resistance gene in the susceptible host genotype which matches an avirulence gene present among the pathogen population may limit the number of countable spore pustules per leaf area on the susceptible line, thus increasing the ratio of mixed-IT pustules to the total number of pustules. It is, thus, advisable to use a susceptible genotype which has been proven susceptible to a range of different inocula, as was the case for rye inbred line L-301 in the present study. Furthermore, L-301 was found to be fully susceptible also in other inoculation experiments (data not shown) with 15 SPIs which had been selected out of 1200 SPIs in an effort to assemble a SPI core collection (Klocke, 2004). Finally, in the present case we did not observe any hypersensitive response (HR) on inoculated leaf segments of L-301, which was reassuring that L-301 was indeed fully susceptible to the inoculum used.

We have determined the frequency of virulent LR races by relating the number of IT 2(5) pustules counted on a *Pr1* genotype of rye to the total number of pustules occurring on a *Pr1*-deficient genotype, the latter of which served a comparison basis. We also tested the feasibility of a more direct approach by omitting the use of a susceptible genotype, namely, to relate the number of 2(5) pustules to the summed numbers of these pustules *plus* the chlorotic spots which are caused by *Pr1*-mediated HR of the host tissue (Fig. 1) to non-virulent uredospores on the same leaf segment of a *Pr1* genotype. However, since the HR-mediated chloroses tend to quickly converge after the onset of the HR response, we reckon this approach to be not sufficiently precise and practicable.

The appearance of a heterogeneous or mesothetic infection type X (IT X) is reported for various host-pathogen systems, e.g., leaf rust and stem rust in wheat (Stakman et al., 1962; McIntosh et al., 1967; Dyck and Samborski, 1970; Rajaram et al., 1971; McIntosh et al., 1995; Felsenstein et al., 1998; Singh et al., 2001) and leaf rust in rye (Solodukhina, 2002). The IT X is characterized by heterogeneous symptoms, which are evenly or randomly distributed over the leaves and sometimes include the whole range of infection types (Stakman et al., 1962; Roelfs and Bushnell, 1985; McIntosh et al., 1995). Individuals displaying IT X are mostly assigned a resistant host response and various causes for the formation of IT X have been reported for several wheat-rust systems (Stakman et al., 1962; McIntosh et al., 1967; Dyck and Samborski, 1970; Rajaram et al., 1971). The present study may serve as example of demonstrating that in the case of IT 2(5), the occurrence of a virulent race in low frequency among a leaf-rust population is the causal event.

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