A Method of Inoculating Adult Wheat Plants with Urediospores of *Puccinia striiformis* to Measure Components of Resistance

L. H. M. BROERS and R. M. LOPEZ-ATILANO, Centro Internacional de Mejoramiento de Maiz Y Trigo (CIMMYT), Lisboa 27, Apdo Postal 6-641, Col. Juarez, Deleg. Cuauhtemoc, 06600 Mexico D.F., Mexico

ABSTRACT

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In preliminary experiments, six methods of inoculating a small section of wheat leaves with stripe rust urediospores were compared. The best method was to place small agar blocks preinoculated with urediospores of *Puccinia striiformis* on a leaf. Components of resistance could be easily measured using this inoculation method. High spore densities were needed to obtain good infection. For successful infection of 90% of the leaves, at least 200 spores per square centimeter should be applied. To achieve at least 15 infections per inoculated leaf, at least 485 spores per square centimeter of leaf were needed. Latency period, infection frequency, and length of infection were not significantly affected by the leaves or inoculation position on leaves. Latency period was the most precise component of resistance because of its low coefficient of variation. In a case where large numbers of genotypes have to be screened, infection length might be preferred because it was less time-consuming to measure than latency period.

Methods have been developed to inoculate wheat (*Triticum aestivum* L.) leaves or plants with rust spores for quantitative studies of monocycles. Most of them, including the settling tower (3) and the method of Andres and Wilcoxson (1), are suitable for rust fungi without extensive growth of individual infections, for which a scattered, approximately uniform distribution of urediospores is needed. Infections of those rust fungi will remain isolated throughout the infection cycle, provided spore densities are kept low and free moisture is not available on leaves for a second infection cycle.

Puccinia striiformis Westend f. sp. *tritici,* the causal agent of stripe (yellow) rust on wheat, has a typical unrestricted growth of individual infections. Infections grow longitudinally in the leaf, and subsequently, scattered individual infections may coalesce. Consequently, it is often impossible to distinguish individual infections on the leaf. Coalescence of infections interferes with the analysis of certain components of resistance for which assessment of number of infections over time, or final number of infections, is necessary. The problem of coalescing infections can be obviated by using a method in which only a very small part of the leaf (e.g., a 0.5-cm band across the leaf) is inoculated. Since the fungus only grows longitudinally and assuming that a single stripe originates from one infection (which is not necessarily the case), the number of stripes corresponds to the number of infections. These numbers can be used for subsequent analysis of latency period and/or infection frequency.

The objectives of the study were to develop a dependable and reproducible method of inoculating only a small part of wheat leaves with urediospores of *P. striiformis* and to evaluate the possibilities of measuring latency period, infection frequency, and length of infection.

MATERIALS AND METHODS

Flag (F) and second (F-1) leaves of cultivar Morocco were inoculated at stage DC50 (13). At this growth stage, flag leaves are young but fully developed. In some studies, the third leaves (F-2) were also used. Fresh urediospores of Mexican isolate 89009 of *P. s. tritici* (race 14E14 [4]) were used in all experiments. Four experiments were carried out.

A preliminary experiment (exp. A) was carried out to evaluate six methods of inoculating or incubating a small part of wheat leaves. For each method, five pots with five plants each were used. Incubation occurred for all plants in a dark room at 10 C for 16 hr.

I) Preinoculated agar pieces. Petri dishes containing a 2- to 3-mm layer of 0.8% water agar were inoculated with a 1:100 spore–talc mixture to give a spore density of 210 urediospores per square centimeter. Agar pieces measuring 0.5 × 2 cm containing the spores were placed on the adaxial surface of the leaf so that the entire width of the leaf was covered for 0.5 cm. To prevent the agar pieces from slipping off, a paper clip was put on the leaf. This also prevented the leaf from curling (Fig. 1). Incubation was done in a dew chamber with high relative humidity.

II) As I), but with incubation at 70% relative humidity.

III) Finger dip method. The adaxial surface of leaves was inoculated by pressing a marked area on leaves between thumb and forefinger after dipping the forefinger in a 1:100 spore–talc mixture. Incubation was done in a dew chamber with 100% relative humidity.

IV) Cotton tips with spore–talc mixture. Cotton tips were dipped in a spore–talc mixture (1:100) and rubbed gently over a marked area of the adaxial leaf surface. Incubation was in a dew chamber with 100% relative humidity.

V) Cotton tips with 0.15% agar–spore mixture. As IV), but replacing the spore–talc mixture with a mixture of 90 mg of spores and 40 g of 0.15% water agar. Before application, the agar–spore mixture was stirred for 5 hr. Incubation was done in a dew chamber with 100% relative humidity.

VI) Duster method. A 1:200 spore–talc mixture was gently dusted over plants with an atomizer. Incubation was in small dew chambers fixed to the leaves with hairpins. The dew chambers were made of small rubber rings covered on one side with parafilm. Instead of a point inoculation, a point incubation was emphasized with this method. Incubation occurred in an environment with low (70%) relative humidity.

We used preinoculated agar pieces (method I, exp. A) to study the effects of four different incubation environments (exp. B). For each incubation treatment, seven pots with five plants each were used. The four environments were 1) dark, 10 C, 100% relative

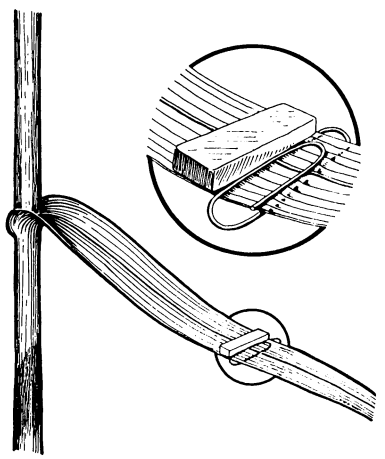

Fig. 1. Details of a method using preinoculated agar blocks to inoculate a small section of a wheat leaf with urediospores of stripe rust.

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humidity, for 16 hrs; 2) dark, 10 C, 70% relative humidity, for 16 hrs; 3) agar pieces covered with aluminum foil, plants left in a greenhouse, agar pieces removed from the leaves after 16 hr; and 4) agar pieces covered with aluminum foil, plants placed outside, agar pieces removed from the leaves after 16 hr (including one night).

In the third experiment (exp. C), four inoculation treatments were assessed. Three were based on preinoculated agar pieces in which different inoculum densities were applied on the agar in the petri dishes, where high density = ca 483 spores per square centimeter, intermediate density = ca 300 spores per square centimeter, and low density = ca 90 spores per square centimeter. In the fourth treatment, plants were inoculated by gently dusting spores over the plants, after which a part of the inoculated leaf was covered with an uninoculated agar piece. For all treatments, eight pots with five plants each were used. For each treatment, equal numbers of plants were incubated at 70 or 100% relative humidity, and for 16 or 48 hr, in a dark room at 10 C.

The fourth experiment (exp. D) was designed to investigate the size of the within-pot variation compared to the size of the between-pot variation with regard to latency period, infection frequency, and growth rate of the infections. The F, F-1, and F-2 leaves of 140 plants were inoculated with preinoculated agar pieces. There were five plants in each of 28 pots.

Infection frequency and leaf width were measured in all experiments. Latency period was assessed only in exp. C and exp. D. The length of infections was measured at two dates in exp. D only.

Both latency period and infection frequency were assessed in two different ways. A leaf could be divided into several longitudinal intervascular spaces using the large vessels as separators. Every day, the number of intervascular spaces with sporulation was counted until a maximum was reached. From these counts, the time at which 50% of the infected intervascular spaces contained sporulating infections was calculated by linear interpolation, as described for latency period assessment for leaf rust on barley (6). The time from incubation to 50% sporulating infections is the LP_{inter}. The infection frequency (IF_{inter}) in this case is equal to the final number of intervascular spaces with sporulation divided by the width of the leaf in centimeters (N/cm).

Instead of counting the number of intervascular spaces with sporulation, the number of sporulating stripes could be counted. One intervascular space could contain up to four sporulating stripes. A sporulating stripe is defined as a range of pustules that are arranged longitudinally on the leaf. One stripe may arise from different infections that at maximum are 0.5 cm apart (the width of the agar piece, in longitudinal direction of the leaves). Initially discrete infections coalesce rapidly and cannot be discriminated. Therefore, a stripe was considered as one infection. Each intervascular space could have up to four stripes, which do not coalesce because growth is only longitudinal. By counting the number of sporulating stripes each day and applying the same calculation as for LP_{inter}, the $LP_{stripes}$ is obtained. The infection frequency based on stripes ($IF_{stripes}$) is equal to the final number of sporulating stripes divided by the width of the leaf in centimeters (N/cm). The length of infection was measured to the nearest millimeter with a ruler at 350 hr and 422 hr after inoculation.

RESULTS

The comparison of different point inoculation or incubation techniques (exp. A) showed preinoculated agar pieces to be the best method, resulting in a high percentage of infected leaves and high infection frequency. A spore density of 210 spores per square centimeter resulted in 91% of the leaves infected, with an average of 7.9 infections per centimeter (IF_{inter}). The other methods resulted in average percentages of infected leaves (APIL) of 0–17%, with an IF_{inter} of less than 1. The results suggested that humidity during incubation is not a critical factor, as similar percentages of infected leaves were obtained with preinoculated agar pieces in dry and in humid environments.

Variation in incubation conditions. The frequency of infected leaves, infection frequency, and applied spore density for exp. B are given in Table 1. The results indicate that for preinoculated agar pieces, the best environment for incubation is a dark room with controlled temperature (10 C). As in exp. B, humidity did not have any effect. If such a controlled environment is not available, agar pieces might be wrapped in aluminum foil and the plants left in a greenhouse or outside. By increasing spore density, the reduction of APIL and IF due to less favorable conditions for incubation can be compensated.

Effect of spore density. The highest probability of infection occurs with more than 300 spores per square centimeter (Table 2). The analysis of variance indicated that the effects on infection frequency of spore density and incubation time were significant. Moreover, a small, significant interaction between incubation time and spore density was found.

Table 1. Spore density, average percentage of infected leaves (APIL), and infection frequency (IF) for four incubation methods after point inoculation using agar pieces preinoculated with urediospores of *Puccinia striiformis* on the adaxial side of leaves of wheat cultivar Morocco (exp. B)

Method of incubation	Spore density (N/cm^2)	APIL (%)	IF_{inter}[x] (N/cm)	$IF_{stripes}$[y] (N/cm)
Humid	190	69	3.3 a[z]	7.9 a
Dry	175	86	3.4 a	8.6 a
Greenhouse	151	73	1.6 b	3.8 b
Outside	181	49	1.9 b	4.2 b

[x] Infection frequency measured as the number of intervascular spaces (marked by the large vessels) with sporulation per centimeter of leaf width.
[y] Infection frequency measured as the number of individual stripes per centimeter of leaf width.
[z] Numbers within a column with the same letter do not differ significantly at $P = 0.05$ (Tukey).

Table 2. Effect of inoculation density, incubation time, and humidity on infection frequency (IF), latency period (LP), and average percentage of infected leaves (APIL) (exp. C) using agar pieces preinoculated with urediospores of *Puccinia striiformis* to inoculate the adaxial side of leaves of wheat cultivar Morocco

Treatment	IF[x] (N/cm)	LP[y] (hr)	APIL (%)
Spore density ($spores/cm^2$)			
Atomizer	4 c[z]	332 a	81
High (483)	13 a	339 b	94
Inter (300)	9 b	329 a	96
Low (90)	6 c	345 b	87
Incubation time (hr)			
16	7 a	330 a	85
48	9 a	343 b	94
Humidity (%)			
70	8 a	339 a	88
100	8 a	334 a	91

[x] Infection frequency was measured as the number of individual stripes per centimeter of leaf width.
[y] Latency period was assessed based on individual stripes.
[z] Numbers within a column with the same letter do not differ significantly at $P = 0.05$ (Tukey).

The number of infections increased more at low densities than at high densities when incubation time was prolonged. Latency period was significantly affected by incubation time and spore density. The effect of incubation time was probably due to lower temperatures in the incubation room compared to the greenhouse. The effect of spore density is somewhat puzzling, as the intermediate spore density had a significantly shorter LP than both the low and the high spore densities. It was expected that the low spore density would have given the longest and the high spore density the shortest LP. For the F-1 leaf, this was the case.

Effect of leaf and position of inoculation on the leaf. By pooling the data of exp. C (Table 3), analyses of the leaf effect and position of inoculation on the leaf were possible. There was no detectable effect of leaf or position on leaf on LP and IF.

In exp. D, the findings for leaf effects were confirmed (Table 4). Moreover, no effect on the length or growth rate of infection could be detected. In the case of Morocco (highly susceptible to stripe rust), all leaves and positions on the same leaf gave similar reactions with respect to the components studied. This may not be the case for cultivars that possess resistance.

Relation among inoculum density, infection chance, and $IF_{stripes}$ on the flag leaf. Data of experiments for which $IF_{stripes}$, spore density, and APIL were assessed, were combined and graphed. Correlation coefficients were calculated after pooling the data of different experiments. The relation between infection frequency and APIL (Fig. 2) can be described by the following nonlinear function: $APIL = -119.3 \times \exp^{-0.49 \times IF} + 94.5$, ($r^2 = 0.92$) (equation 1).

The relationship between applied spore density (SD) and IF (Fig. 3) can be described by linear regression: $IF = 0.91 + 0.029 \times SD$, ($r^2 = 0.81$) (equation 2).

From equations 1 and 2, it can be calculated that to obtain an APIL of over 90%, a spore density of at least 200 spores per square centimeter should be applied. This will give an IF of 6.7 stripes per centimeter, which is low for a reliable LP assessment. To measure LP, more than 15 stripes per centimeter should be present. According to equation 2, a spore density of more than 485 spores per square centimeter should be applied.

Variation in components. In exp. D, three variance components were measured: between-pot variance (p_i), within-pot between-plants variance ($q[p]_{ij}$), and within-plants between-leaves variance ($l[q(p)]_{ijk}$). The mean squares of the variance components are given in Table 5 for all components of resistance measured in exp. D, based on the following model: $Y_{ijk} = \mu + p_i + q(p)_{ij} + l[q(p)]_{ijk} + \epsilon_{ijk}$, in which Y is one of the components of resistance measured.

In all cases, the pot effect was highly significant, and in only one case (LP_{inter}) was the plant-within-pot component significant (Table 5). The coefficient of variation (CV) is a mean-corrected measure of error variance which can compare different experiments, independent of the unit of measurement. CVs for the components ranged from 6.1 to 31.9%. The lowest CV was obtained for LP, indicating that this component of resistance is most appropriate for comparing genotypes for differences in resistance to stripe rust. Second lowest CV was found for length of infections. Since this is a much less time-consuming assessment than LP, it might be preferred when large numbers of genotypes have to be screened in the greenhouse. The infection frequency had the largest CV.

Table 3. Effect of leaf and position on leaf on latency period (LP) and infection frequency (IF) of *Puccinia striiformis* on leaves of wheat cultivar Morocco inoculated with preinoculated agar pieces (exp. C)

Leaf position	LP_{inter}[v] (hr)	$LP_{stripes}$[w] (hr)	IF_{inter}[x] (N/cm)	$IF_{stripes}$[y] (N/cm)
Flag				
Top	318 a[z]	333 a	3 a	8 ab
Base	316 a	332 a	3 a	9 ab
F-1				
Top	324 a	338 a	2 a	7 ab
Base	323 a	336 a	2 a	5 a
F-2				
Top	326 a	340 a	3 a	12 b
Base	324 a	338 a	3 a	9 ab

[v] Latency period based on intervascular spaces (marked by the large vessels) with sporulation.
[w] Latency period based on individual stripes.
[x] Infection frequency measured as the number of intervascular spaces (marked by the large vessels) with sporulation per centimeter of leaf width.
[y] Infection frequency measured as the number of individual stripes per centimeter of leaf width.
[z] Numbers within a column with the same letter do not differ significantly at $P = 0.05$ (Tukey).

Table 4. Latency period (LP), infection frequency (IF), length of infection at 350 and 422 hr after inoculation, and average percentage of infected leaves (APIL) (flag, F-1, and F-2) of wheat cultivar Morocco plants after inoculation with agar pieces preinoculated with urediospores of *Puccinia striiformis* (exp. D)

Leaf	LP_{inter}[v]	$LP_{stripes}$[w]	IF_{inter}[x]	$IF_{stripes}$[y]	Length (cm) 350 hr	Length (cm) 422 hr	APIL (%)
Flag	292 a[z]	310 a	4 a	16 a	2.9 a	4.2 a	96
F-1	293 a	305 a	5 a	17 a	2.8 a	3.9 a	94
F-2	290 a	305 a	5 a	19 a	2.6 a	3.7 a	93

[v] Latency period based on intervascular spaces (marked by the large vessels) with sporulation.
[w] Latency period based on individual stripes.
[x] Infection frequency measured as the number of intervascular spaces (marked by the large vessels) with sporulation per centimeter of leaf width.
[y] Infection frequency measured as the number of individual stripes per centimeter of leaf width.
[z] Numbers within a column with the same letter do not differ significantly at $P = 0.05$ (Tukey).

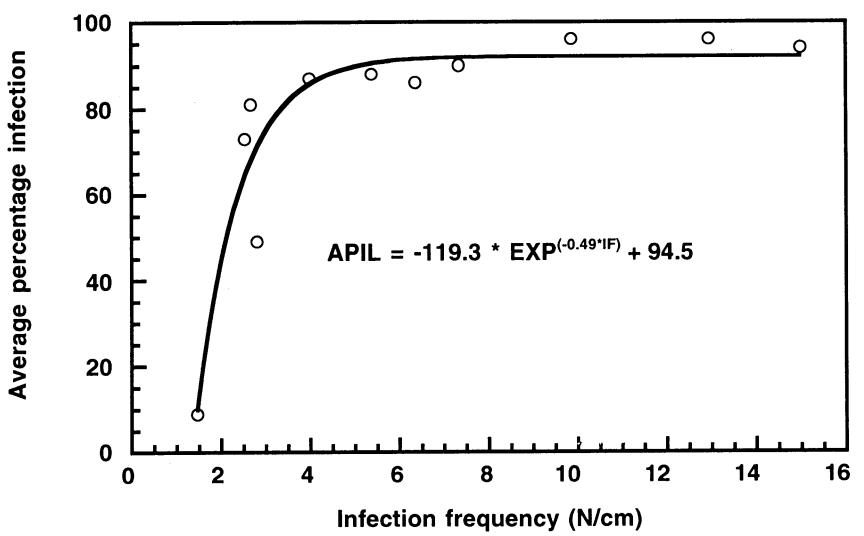

Fig. 2. Relationship between infection frequency and average percentage of infected leaves on wheat cultivar Morocco after inoculation with stripe rust urediospores.

DISCUSSION

This study shows that the agar method for inoculation of adult wheat plants (cv. Morocco) with stripe rust urediospores is reliable and reproducible. The agar method works best in a dark room with temperatures between 10 and 15 C. If these facilities are not available, a cool greenhouse is a good alternative. In this case, higher spore densities should be used, and agar pieces should be wrapped in aluminum foil.

Incubation time had little effect on IF. With high spore densities, 16 and 48 hr of incubation gave similar results. The effect of humidity was negligible in the described experiments.

The spore density should be very high (more than 485 spores per square centimeter) to obtain high APIL and a relatively high IF. For other wheat rusts (*Puccinia recondita* f. sp. *tritici* and *P. graminis* f. sp. *tritici*), much lower spore densities obtain a similar IF. For wheat leaf rust, 100 urediospores per square centimeter resulted in ca 30 pustules on a susceptible wheat genotype (2). Because of the relatively low germination rate and the undirected growth of germ tubes of stripe rust spores, the chance of infection is reduced considerably. In the described experiments, germination never exceeded 60%, although fresh spores were used throughout the experiments. Other researchers also found low germination rates for urediospores of stripe rust (11). Spore germination can easily exceed 85% for other rusts (10,14).

Germ tubes of *P. striiformis* do not seem to have any directional growth. Observations on germinating stripe rust spores on 0.8% agar showed that germ tubes grew away from the agar, bent, and returned to the agar again. On seedling leaves, the germ tubes showed a similar behavior. A germ tube can cross four stomates without entering any of them (L. H. M. Broers, *unpublished*). These observations suggest that germ tubes have difficulty finding and penetrating a stoma. This considerably reduces the chance of stoma penetration and subsequent infection of the leaf.

The large-pot effect indicates that small genotypic differences for components of resistance will be difficult to detect unless the variation among pots can be reduced. One source of between-pot variance might be the difference in average plant development stage and leaf age of plants in different pots. Covariance analysis with development stage or days since leaf emergence as covariable on the one hand and reduction of variation in development stage on the other might increase the efficiency of the experiment and increase the chance of finding genotypic differences for components of resistance.

Leaf and position of inoculation on the same leaf of Morocco did not affect the LP or IF. It should be kept in mind that the variation among pots was large and did not allow for detection of small differences. Furthermore, it is questionable whether different leaves and leaf positions will react similarly on genotypes that have resistance to stripe rust, as leaf effects have been reported for other rusts (5,6,8,9).

Of the three components studied, LP seems to be the best for precise detection of small genotypic differences in resistance to stripe rust, because its CV was lower than the CV of the other components. However, when screening large numbers of genotypes, length of infection might be preferred because its assessment is less time-consuming than assessing LP. IF is not a very sensitive component for measuring genotypic differences in resistance, as has been shown for other rusts (2,7).

Both LP_{inter} and $LP_{stripes}$ showed a highly significant pot effect. This large-pot effect might hinder the detection of small genotypic differences. The $LP_{stripes}$ is probably more sensitive than LP_{inter}. The mean square of the pot effect of $LP_{stripes}$ is about 22% smaller than the mean square of the pot effect of LP_{inter}. Therefore, the chance of identifying small genotypic differences is larger, because the pot effect will act as experimental error when different treatments (genotypes) are used (12). Moreover, LP_{inter} has a significant plant-within-pot effect, which is also indicative of a large error variance.

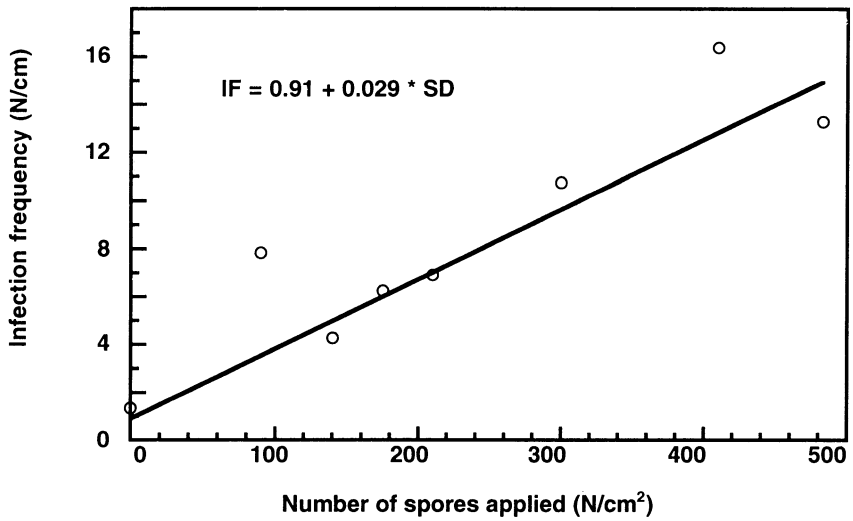

Fig. 3. Relationship between number of stripe rust urediospores applied and the infection frequency on wheat cultivar Morocco.

Table 5. Mean squares (MS) for three sources of variance components (pot, plant within pot, and leaves within plant), coefficient of variation, and mean for latency period (LP), infection frequency (IF), length of infection at 350 and 422 hr after inoculation, and length increase (exp. D) for stripe rust on leaves of wheat cultivar Morocco using preinoculated agar pieces for inoculation

Source of variance	LP		IF		Length of infection		Length increase[y]
	inter[u]	stripes[v]	inter[w]	stripes[x]	350 hr	422 hr	
Pot	8721.6*[z]	6813.0*	28.0*	586.3*	335.2*	604.0*	119.5*
Plant within pot	2337.3*	497.9	3.4	60.7	17.8	28.4	11.2
Leaf within plant	316.5	552.7	3.1	55.6	18.6	27.1	11.6
CV	6.1	7.7	28.0	31.9	15.5	13.4	30.6
Mean	291.9	307.0	6.3	23.4	27.9	39.0	11.1

[u] Latency period based on intervascular spaces (marked by the large vessels) with sporulation.
[v] Latency period based on individual stripes.
[w] Infection frequency measured as the number of intervascular spaces (marked by the large vessels) with sporulation per centimeter of leaf width.
[x] Infection frequency measured as the number of individual stripes per centimeter of leaf width.
[y] Length 422 hr after inoculation minus length 350 hr after inoculation.
[z] * = Significant at $P = 0.05$.

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