Effect of Germ Tube Length on the Fate of Sporelings of *Puccinia hordei* in Susceptible and Resistant Barley

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ABSTRACT

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Within leaves of barley, there is great variation in fate among individual sporelings of *Puccinia hordei*. Some sporelings abort at one or another stage of the infection process; others establish colonies that vary in rate of growth and development. Histological examination of barley leaves 6 days after inoculation with *P. hordei* in three experimental series revealed a negative association between germ tube length of sporelings and the chance of successful establishment of a colony. This effect was found in a susceptible, a hypersensitive, and two partially resistant barley lines. Within these lines there was a significant negative correlation between germ tube length and size of the established colony. It is suggested that the formation of a long germ tube, necessary to reach a stoma, decreases the amount of energy available to the sporelings to infect the host.

Additional keywords: *Hordeum vulgare*, leaf rust.

In studies of the mechanisms of resistance of plants to pathogenic fungi, a useful procedure is to apply propagules to leaf tissue and determine the fate of germlings and plant response at infection sites. A common observation in such histological studies is that there are large differences in response among infection sites within leaves (2,4–11,19,21). Resistance genes seem to increase the frequency of certain types of responses dramatically, and the frequencies of other types of responses decrease accordingly. Most types of responses, however, are found on most plant genotypes to some extent.

Histological observations on the relationship between barley (*Hordeum vulgare* L.) and leaf rust fungus (*Puccinia hordei* Otth) provide an example. Sporelings of *P. hordei* may abort at several phases of development (13,17): after appressorium formation (nonpenetration), after substomatal vesicle formation (aborted substomatal vesicle), after formation of the first one to six haustorial mother cells without or with necrosis of plant cells (early abortion), or after the development of at least some branched hyphae (late abortion). Even in the extremely susceptible barley line L94, all these types of abortion may be found in one leaf segment, but the frequencies are low (13). The large majority of sporelings develop sporulating pustules although colonies show considerable variation in rate of growth and development (15).

In the partially resistant cultivar Vada, too, all types of abortion are found, but two types (early abortion without necrosis and late abortion) are significantly more frequent than in L94 (13,22). Accordingly, fewer sporelings develop sporulating pustules, again with large differences in rate of growth and development between the colonies.

Even the expression of major genes for hypersensitive resistance does not result in one type of response. The gene *Pa3* in an L94 background (L94-Pa3) increases the frequency of sporelings of an avirulent isolate that abort early with necrosis, but not all sporelings are arrested early. Many sporelings succeed in forming some branched hyphae, and some may even produce spores (17).

It is unknown why one sporeling on, for instance, Vada or L94-Pa3 aborts early, whereas a second sporeling at an infection site nearby develops into a sporulating colony. It also is unknown why, even in L94, some sporelings have a higher rate of growth and development than others in the same leaf.

Sporelings on a leaf differ markedly in the length of the germ tubes. A major cause is that urediospores are deposited at various distances from stomata. It is reasonable to assume that the longer the germ tube, the less energy remains for the sporeling to establish itself.

The aim of the present study was to investigate whether germ tube length affects the chance for successful establishment of the sporelings and whether there is a negative correlation between germ tube length and size of the established colonies.

MATERIALS AND METHODS

The experiment consisted of three consecutive series. The experiment was carried out in a greenhouse compartment in which the temperature fluctuated daily between 15 and 22 C.

Plant material. The first series contained barley lines L94, L94-Pa3, Julia, and Vada. Series 2 and 3 were performed with L94 and Vada. L94 is extremely susceptible to *P. hordei*. L94-Pa3 contains the early acting hypersensitive resistance gene *Pa3* from Rika × (Rika × Baladi) in a L94-like genetic background (17). Julia and Vada have an intermediate-to-high and a high level of partial resistance to *P. hordei*, respectively. Partial resistance is characterized by a reduced epidemic buildup, despite a susceptible infection type (18). The lines were planted in one 37 × 39 cm planting box per inoculation. Five (series 1), six (series 2), or seven (series 3) seedlings per line per box were raised. The lines were not randomized and replicated within the box because the study was focused on within-line effects.

Inoculum. Inoculum of isolate 121 of *P. hordei* (avirulent to *Pa3*) was produced on adult plants of barley line L98 and was collected by means of a cyclone spore collector. Series 2 and 3 consisted of two plant boxes each. One of the boxes was inoculated with inoculum stored on a laboratory shelf for 6 days (series 2A and 3A); the other was inoculated with fresh inoculum (2B and 3B). One day before the collection of the inoculum, most of the old urediospores were removed by shaking (series 1) or by means of the cyclone spore collector (series 2 and 3). The inoculum, mixed (about 1:9) with *Lycopodium* spores, was applied to the adaxial sides of the seedling leaves in a settling tower. The amount of inoculum was about 1.8 mg (series 1), 2 mg (series 2A and 3A), and 1.2 mg (series 2B and 3B). The quantities of stored inoculum (series 2A and 3A) were this high to compensate for the expected lower germination percentage. Per milligram of applied inoculum, about 65 urediospores per square centimeter are deposited on the leaves. The procedures of inoculation and incubation were as described before (13). The resulting density

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of appressoria on the seedling leaves differed among the five inoculations (range 50 appressoria per square centimeter in 2B to 116 in 2A).

Sampling and staining. At 6 days after inoculation, segments of the central parts of the leaves were collected. Pale flecks (except on L94-Pa3 in series 1) indicated that the fungus had produced colonies.

The leaf segments were fixed in acetic acid:ethanol (1:3, v/v) for 45 min in petri dishes. The leaf segments were prepared as whole mounts for fluorescence microscopy (20) using Uvitex 2B (Ciba-Geigy Corp., Ardsley, NY) instead of Calcofluor. The leaf segments were simmered for 2 hr at about 75 C in lactophenol-ethanol, rather than boiled, because boiling would have caused the detachment of germ tubes. The leaf segments were mounted in glycerol.

Observations. The preparations were studied with a Nikon epifluorescence microscope (Fluophot) at ×100 or ×400. Thirty random infection sites were examined per leaf segment. The following data were recorded: length of germ tube, phase of development of the sporeling (13), and length (l) and width (w) of established colonies (that is, colonies having more than six haustorial mother cells). Colony size was calculated as the square root of $lw\pi/4$ (a geometric mean of length and width). The determination of germ tube length at ×400 was complicated by the often winding and branched growth habit. The eyepiece micrometer was turned to follow the windings. Branches shorter than 25 μm were ignored.

Infection sites were ignored if they were within five stomatal rows from the leaf edge, if the germ tube was broken (i.e., no urediospore connected to the germ tube), or if the germ tube and/or colony was intertwined with other germ tubes or colonies.

RESULTS

The effect of germ tube length on the development of sporelings was studied in three consecutive series, comprising two or four barley lines and one or two inoculum batches. This resulted in what can be seen as 12 trials. These trials should indicate how far possible effects are repeatable and generalizable over barley lines.

Germ tube length. The germ tube lengths ranged from zero, when an urediospore deposited on a stoma produced an appressorium without germ tube, to 950 μm. All the frequency distributions were skewed toward longer germ tube lengths. The median germ tube length differed somewhat among trials but was generally between 150 and 200 μm.

Effect of germ tube length on successful establishment. In Table 1, the proportions of established sporelings are presented per trial for various length classes of germ tubes. The data indicate consistently that the proportion of establishment is highest for the shortest germ tubes (class 0–99 μm, 10 out of 12 trials) and lowest for the longest germ tube class (length ≥300 or 400 μm, all trials). This means that aborted sporelings mainly were found to have developed from the longer germ tubes. Indeed, the median germ tube length of early aborted sporelings was 154 to 307% of that of established sporelings. The difference in germ tube length between early aborted and established sporelings was in all 12 trials highly significant (Kruskal-Wallis nonparametric test, $P < 0.01$). Germ tubes that produced nonpenetrating appressoria tended to be even longer than those associated with early aborted sporelings (data not presented).

Effect of germ tube length on colony size. The data in Table 2 indicate that sporelings with longer germ tubes tended to give rise to smaller colonies. The shortest germ tubes (class 0–99 μm) produced the biggest colonies in 10 out of 12 trials. Also the rank correlation of the germ tube length and colony size was significant (and negative) in all trials (Table 2). These rank correlations were calculated on the bulked data over the five to seven leaf segments per trial. The rank correlations for the individual leaf segments were of about the same magnitude and also were almost always significant. Figure 1 illustrates the relationship between germ tube length and colony size for one of the trials (Julia, series 1). It seems that the maximum attainable colony size depends on germ tube length, but other factors may

TABLE 1. Proportion of establishment of sporelings of *Puccinia hordei* on four barley lines in relation to the length of the germ tube

Length class of germ tube (μm)	L94 (highly susceptible) series					L94-Pa3 (hyper-sensitive) series	Julia (partially resistant) series	Vada (partially resistant) series					Average number of sporelings
	1	2A	2B	3A	3B	1	1	1	2A	2B	3A	3B	
0–99	0.93	0.86	0.87	0.98	0.97	0.81	0.83	0.70	0.58	0.81	0.56	0.59	61.3
100–199	0.86	0.75	0.86	0.95	0.92	0.79	0.95	0.55	0.49	0.73	0.39	0.44	51.6
200–299	0.87	0.74	0.94	0.86	0.68	0.71	0.87	0.56	0.46	0.59	0.24	0.20	33.5
300–399	0.81	0.56	0.85	0.31	0.50	0.44	0.28	0.37	0.33	0.61	0.12	0.08	19.5
≥ 400	0.38	0.44	0.63	...[a]	...[a]	0.17	0.19	0.06	0.26	0.31	...[a]	...[a]	20.1

[a] Data on the relatively few sporelings from germ tubes longer than 400 μm have been included in the figure for length class 300–399 μm.

TABLE 2. Average size (μm) of 6-day-old colonies of *Puccinia hordei* on four barley lines in relation to germ tube length

Length class of germ tube (μm)	L94 (highly susceptible) series					L94-Pa3 (hyper-sensitive) series	Julia (partially resistant) series	Vada (partially resistant) series				
	1	2A	2B	3A	3B	1	1	1	2A	2B	3A	3B
0–99	755	550	603	474	472	317	664	434	255	357	225	254
100–199	702	539	542	413	384	316	594	399	292	330	222	197
200–299	639	461	604	330	344	271	529	366	338	316	172	170
300–399	617	352	561	300	324	237	497	301	147	243	156	151
≥ 400	421	289	476	...[a]	...[a]	193	517	169	159	145	...[a]	...[a]
Correlation between colony size and germ tube length[b]	−0.38**	−0.38**	−0.20**	−0.37**	−0.33**	−0.12*	−0.36**	−0.26**	−0.13*	−0.24**	−0.22**	−0.29**

[a] Data on the few colonies from germ tubes longer than 400 μm have been included in the figure for length class 300–399 μm.
[b] Kendall's coefficient of rank correlation: * = significant at the 5% level; ** = significant at levels lower than 1%.

cause individual colonies to not reach the maximum size that corresponds to their germ tube length. Scattergrams of 10 out of 12 trials showed a similar picture. In two cases (Vada series 1 and 3A), the scattergram did not show such a colony size limit in relation to germ tube length.

DISCUSSION

Ellingboe (4) proposed that during the infection process of a powdery mildew fungus on a host plant the sporelings have to overcome a series of "hurdles"—barriers at which the infection may be stopped. A certain proportion of the sporelings gets over each hurdle. The effectiveness of each hurdle to arrest sporelings depends on the genes for incompatibility in the host.

The infection process of *P. hordei* in barley also may be regarded as a hurdle course. Hurdles that should be overcome are stoma penetration, substomatal vesicle germination, haustorium formation, and the effect of a possible effective gene for hypersensitive resistance after haustorium formation (14).

The present study suggests that the ability of a sporeling to overcome the series of hurdles successfully not only depends on the "height" of the hurdles (determined by the host genotype) but also on the vitality of the sporeling. Sporelings that are almost exhausted by having made a long germ tube are less likely to complete the infection cycle successfully. The within-leaf variation in success of infection among sporelings may be attributed to some extent to the distance a sporeling had to grow from the site of deposition to the nearest stoma. The fact that even some sporelings with very short germ tubes do not establish colonies (Table 1) suggests that other factors also may play a role. Such factors also may reduce the growth rate of established colonies, leading to smaller colony size than maximally attainable at a certain germ tube length (Fig. 1). One factor may be the physiological quality of the spores in the inoculum batch. Ellingboe (4) stressed that, in powdery mildew, even conidia formed in a controlled environment during a period of 2–4 hr still may differ physiologically. Another factor that may contribute to within-leaf variation of a genetically uniform population of sporelings is the host tissue attacked by the sporeling. This effect has been demonstrated in *Erysiphe graminis* DC. (3,9,10) and also in *P. hordei* in partially resistant host plants (15). Sporelings of *P. hordei* that happen to direct their first attempts at haustorium formation to the mesophyll side of the epidermis probably have a better chance to establish than sporelings attacking only mesophyll cells. It seems that, in partially resistant lines, epidermal cells are less able to prevent haustorium formation by *P. hordei* than mesophyll cells (15). It also is conceivable that the host tissue is a genetic mosaic, having various levels of resistance distributed randomly over the leaf. This possibility is difficult to investigate.

One could argue that the negative association between length of germ tube and chance to establish may be explained by some kind of induced resistance. Spores deposited on or next to a stoma will have formed short germ tubes that may start the infection process some hours before the sporelings with longer germ tubes. The latter group of sporelings may have to cope with host tissue in which the defense mechanism has been switched on by the former group. In a recent study, however, no evidence for induced resistance at the hurdle of haustorium formation has been obtained (16).

It may seem odd that 6 days after inoculation there is still a significant effect of the germ tube length on colony size. Evidence from recent studies (1,15) suggests, however, that differences in size among colonies are mainly due to differences in degree and duration of growth stagnation immediately after establishment. The extent of exhaustion by building the germ tube may determine partially how serious this stagnation is for a certain sporeling in a certain leaf.

The high proportion of early abortion in Vada observed in this and earlier studies is not necessarily due to longer germ tubes

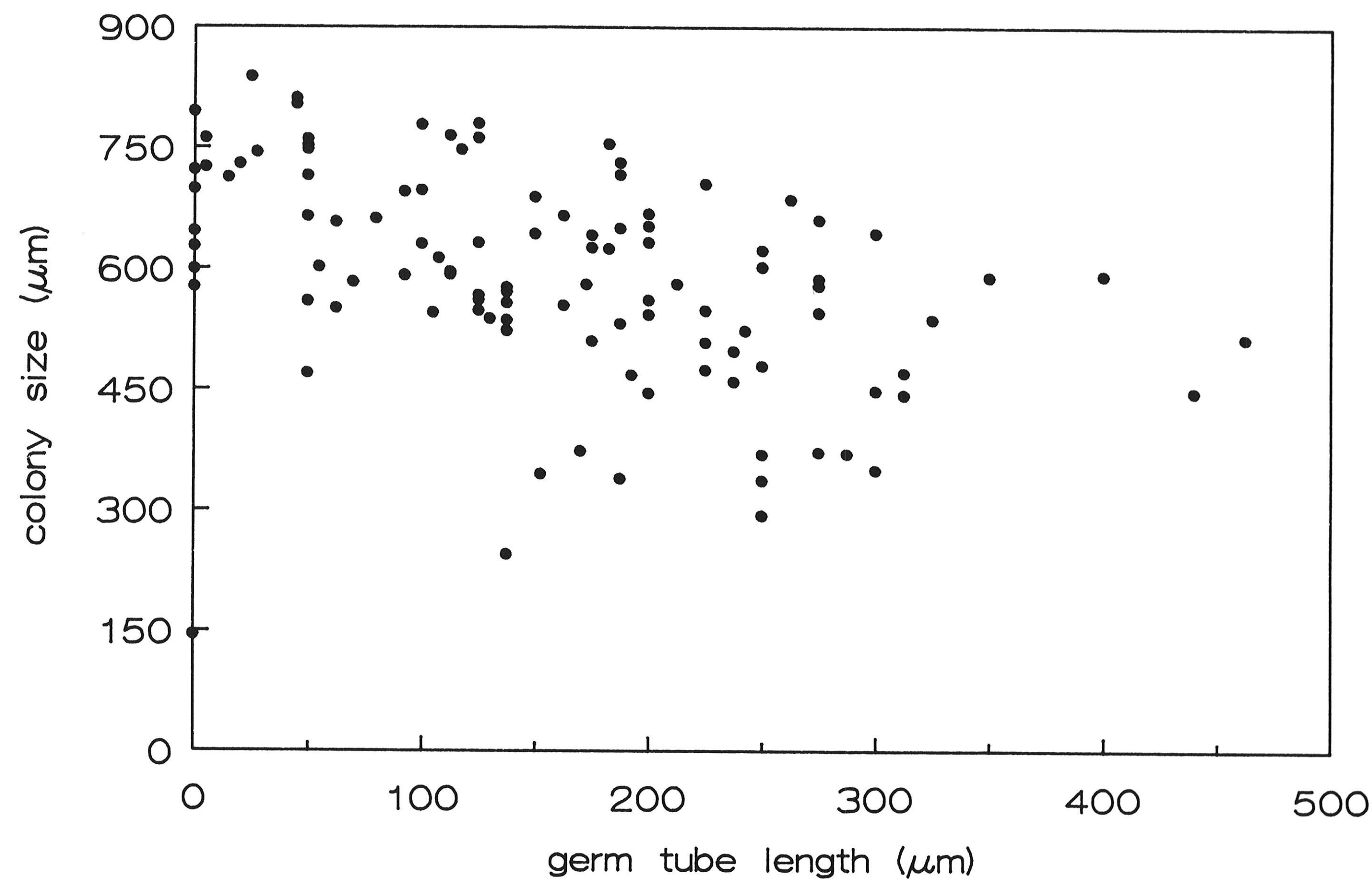

Fig. 1. Scattergram of germ tube length and colony size of 107 colonies of *Puccinia hordei* in seedling leaves of barley line Julia, 6 days after inoculation. Kendall's coefficient of rank correlation is −0.36.

on Vada than on L94. Average germ tube length on Vada did not differ consistently from that on L94 in the present study. It rather appears that in Vada a sporeling needs more energy to take the hurdle of haustorium formation than in L94. The present observations pertain to variation in destiny of sporelings within rather than between barley lines. It is conceivable, however, that a barley genotype with few stomata per square centimeter would require a longer average length of germ tubes than a genotype with a high stomatal frequency. The former barley line would appear more resistant than the latter if the "height" of the hurdles (the number and type of genes for resistance) is the same. The "resistance" would rest on a more serious exhaustion of the sporelings due to the longer germ tubes. This mechanism probably would enhance effects of incomplete resistance genes and would be durable and effective against several pathogen species. A second effect of fewer stomata per square centimeter would be that fewer colonies would develop because of the fewer potential sites of entry into the leaf. It remains to be investigated whether intraspecific variation in stomatal frequency (12) is large enough to contribute to differences in level of resistance.

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