Low Appressorium Formation by Rust Fungi on *Hordeum chilense* Lines

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We gratefully acknowledge the financial support of the Spanish CICYT (project AGR89-0552).
We thank Mrs. R. G. Dekens for technical assistance and help in preparation of the manuscript.
Accepted for publication 22 May 1992.

ABSTRACT

Rubiales, D., and Niks, R. E. 1992. Low appressorium formation by rust fungi on *Hordeum chilense* lines. Phytopathology 82:1007-1012.

There were large differences among *Hordeum chilense* lines in appressorium formation by the wheat and barley leaf rust fungi. The percentage of germlings producing appressoria over stomata differed from three to more than 10-fold (depending on genotype and growth stage). The differences with cultivated wheat and barley and among lines of *H. chilense* were most pronounced in mature plants. The low appressorium formation may be considered a case of avoidance; it was due to poor stoma recognition not to poor spore germination. This avoidance was not expressed in *H. chilense* × wheat amphiploids. The rate of appressorium formation was not associated with stoma frequency or size, or with number or length of leaf hairs.

Additional keywords: cereal rusts, histology, *Puccinia hordei*, *Puccinia recondita*, resistance mechanism.

In the search for possible durable types of resistance to rust fungi, it is useful to investigate the different phases of the infection process and thus elucidate the resistance mechanisms (37). In principle, defense mechanisms may occur at any stage during pathogenesis. Bringing together different mechanisms in one plant genotype may be useful in obtaining durable and effective resistance.

Resistance mechanisms to rust fungi after stoma penetration are rather diverse (13). Relatively few cases in which mechanisms of host resistance inhibited spore germination, directional growth of germ tubes to the stomata, or appressorium formation have been reported. In almost all histological studies, the effects of plant genotype differences on germination percentage and/or appressorium formation are small, inconsistent, or absent (9,15, 16,18,25–27,30,32). Even on inappropiate plant species, the rate of germination and appressorium formation is normal, provided that the surface structure of the epidermis is very similar to that of the host species (12,25,33,36). However, Ferreira and Rijkenberg (11) reported that on a "resistant" *Gladiolus* species, the appressorium formation by the gladiolus rust (*Uromyces transversalis*) was hampered because of poor stoma recognition. Rust species from grasses produced appressoria on a wide range of gramineous species, including nonhosts, with the exception of *Puccinia melanocephala* from sugarcane, which produced few appressoria on some species (20).

Within a host species, a reduced appressorium formation may occur because of an aberrant epidermal structure (24) or reduced leaf wettability (4). The ability of triticale (17) and wheat (1) to inhibit formation of appressoria by *P. recondita* and *P. graminis* has been reported; however, quantitative data are not available.

Hordeum chilense Roem. & Schult. is not a host to the leaf rusts of wheat, rye, and barley (*Puccinia recondita* f. sp. *tritici*, *P. r. recondita*, and *P. hordei*, respectively). The species has a high level of resistance to the wheat stripe rust (*P. striiformis* f. sp. *tritici*) (28). During a study on resistance components of *H. chilense* to rust fungi, we found very low frequencies of appressorium formation on certain lines (accessions H7, H17, and H47); up to seven times fewer appressoria were formed on them than on the remaining lines (H1 and H12) (29). *H. chilense* is of interest because it is one of the parental species of "tritordeum", a new manmade potential crop. Aspects of the development and potentials of this crop have been discussed by Cubero et al (5) and Martín (21).

The purpose of the present study was to reconfirm the low level of appressorium formation on several *H. chilense* lines and

to determine if the differences among *H. chilense* lines for preappressorium resistance varied with rust species and plant development stage. We investigated whether reduced appressorium formation was also expressed in tritordeum by observing the amphiploids between *H. chilense* and wheat.

MATERIALS AND METHODS

Plant material and experiments. The five *H. chilense* lines used in the experiments were H1, H7, H12, H17, and H47. Also used were the tritordeum amphiploids designated HT22, HT27, and HT93; the wheats, Morocco and Little Club; the barleys, Topper and line L94; and the oat, Cebeco.

For seedling tests, the lines were sown in pots and after emergence were transplanted along the sides of plant boxes (37 × 39 cm). *H. chilense* line H12 was not included in the seedling experiments. The seedling experiments were conducted twice with *P. r. tritici* and *P. hordei*; there were eight replications (seedlings) in the first run and six replications in the second run of the experiment. Because of poor germination and/or germ tube growth of *P. coronata* and *P. s. tritici*, seedling experiments with these two rusts were run three times, with six replications each. The *H. chilense* lines were sown 10 days before the other lines because of their slower growth rate.

In the mature plant experiment, each line was represented by two pots (12 × 12 cm) each with two 14-wk-old plants. The *H. chilense* plants were in development stages 37–39 of the Zadoks' scale (35). Because of the short day length, the plants tillered extensively but did not start flowering. The cereal check cultivars were between stages 39 and 53. The tritordeum lines were not included in this experiment. The pots were placed randomly in the greenhouse compartment, and the experiment was run twice. Each rust-plant genotype combination was sampled as four leaf segments (the central part of the upmost expanded leaf of each plant) in each run.

Rust inoculation. Urediospores of the rust species were multiplied on susceptible cultivars, harvested, dried in a desiccator, and mixed with *Lycopodium* spores (1:10, w/w). The rust isolates were *P. r. tritici* 'Flamingo' multiplied on 'Little Club'; *P. hordei* isolate 1-2-1 multiplied on line L98; *P. coronata* isolated from cultivated oat plots in Wageningen and multiplied on Cebeco; and *P. s. tritici* race 39E134 multiplied on Michigan Amber.

Fully expanded primary leaves of the seedlings were pinned to the soil in the flats in a horizontal position with their adaxial sides up. We added four petrolatum-greased slides per flat in the first run of the experiment to determine the distribution of inoculum. Six milligrams of urediospores per flat (20 mg for *P. striiformis*) was applied in a settling tower (8). Each milligram of inoculum resulted in deposition of about 65 spores per square centimeter. The flats with *P. r. tritici*, *P. hordei*, and *P. coronata* were transferred to a greenhouse compartment at about 16 C for incubation during the night. The urediospores were allowed to germinate and form appressoria under natural darkness, while the relative humidity was kept at the saturation point by means of an electric humidifier. In the morning, the flats were transferred to an illuminated greenhouse compartment at 20 C. The flats inoculated with *P. striiformis* were incubated 24 h in a dark chamber at 10 C and then transferred to an illuminated greenhouse compartment at about 15 C. Because the first run of the experiments with *P. coronata* gave poor germ tube growth and the second run gave low spore germination, a third experimental run was performed at the incubation temperature of 20 C.

The inoculum for the mature plants was applied by dusting the spore-*Lycopodium* mixture over the plants in a greenhouse compartment. The plants were placed around a glass panel, with the uppermost expanded leaves fixed in a horizontal position with iron weights. The incubation was as described for the seedlings.

Sampling, staining, and observation. Approximately 22 h after the onset of incubation (46 h for *P. striiformis*), a segment from the central part of each leaf was collected, fixed, and stained (3). The segments were laid, adaxial surface up, on filter paper, one end of which was dipped in fixative (3:1, absolute ethanol/glacial acetic acid, v/v). After about 48 h, the bleached segments were transferred to filter paper moistened with lactophenol-ethanol (1:2, v/v), which softened and cleared the tissue, for about 24 h and then were transferred onto filter paper moistened with Trypan blue stain (0.1% in lactophenol-ethanol) for at least 24 h.

About 100 rust spores per leaf segment were counted under ×100 magnification with a Nikon Optiphot light microscope (Nikon Inc., Melville, NY) and grouped into the following categories: nongerminated urediospores; germ tubes not forming appressoria; appressoria formed but not on a stoma; and appressoria formed on a stoma.

Because *P. striiformis* penetrates the stomata without forming appressoria, the categories for this rust fungus were nongerminated urediospores, germ tubes not ending on a stoma, and germ tubes ending on a stoma.

A urediospore was considered germinated when a germ tube at least as long as the diameter of the spore had been produced. Arcsine of the square root transformation of the proportions was applied for statistic analysis (31). The units for statistic analysis were the transformed proportions of germlings per leaf segment in each development stage. An analysis of variance was run, and the means were separated by the LSD test. Because the infection data for the two wheat and the two barley checks did not differ significantly in the seedling test, these data are presented as pooled figures for the wheat and barley, respectively.

Additionally, the surface of 16 seedling leaf segments per accession was measured (Table 1). The number of stomata and hairs per leaf were counted in one microscope field at ×100. Because the *H. chilense* leaves were narrower than the diameter of the microscope field, the counting was done in an approximately 2.5 mm^2 measured part of each segment. The length and width of five stomata and the length of five hairs per leaf were also determined.

RESULTS

Table 2 shows the averages per experimental run and the significance of the genotype effects. In this table, the percentage of germ tubes not ending on stomata includes two categories: germ tubes not forming appressoria and those forming appressoria but not on a stoma. Formation of appressoria away from stomata was relatively rare; on all genotypes this occurred for only 0–10% of the sporelings, without consistent differences between genotypes and species. The significant genotype effect on each rust for percentage of germ tubes not ending on stomata (Table 2) was due to germ tubes not forming appressoria at all. The effect was consistent over the experimental runs on all rust species except *P. coronata*.

There were significant differences between the runs for spore deposition on leaf and for spore germination. Effects of experimental runs on percentage of germ tubes not ending on stomata were only significant for *P. coronata*. Germination and germ tube growth in *P. striiformis* and *P. coronata* were inconsistent (Table 2). *P. coronata* (run 2) and *P. striiformis* had less than 60% germination, and of the germinated spores more than 70% did not end on a stoma. The *P. coronata* in run 1 germinated well but produced short germ tubes, of which 75% did not end on a stoma. Only the third run with *P. coronata* seemed as reliable as the *P. recondita* and *P. hordei* experiments. Further data on *P. striiformis* were based on the average of three runs; data on *P. coronata* were based on the third run only.

Genotype effects. With regard to *P. recondita* and *P. hordei*, the *H. chilense* accessions tended to allow fewer germ tubes to end on the stomata than did the wheat and barley (Figs. 1,2). Among the *H. chilense* lines, there were significant and large differences (Figs. 1,2); the differences could be as large as a factor of 10 or more. The accessions H7, H17, and H47 allowed a much lower appressorium formation than H1 and H12. The leaf morphology of the accessions differed significantly (Table 1). The *H. chilense* accessions had significantly more but smaller stomata than the tritordeums and cereal checks. The number of hairs

per square centimeter also differed significantly among accessions within a species. In general, the hairs on *H. chilense* were longer than those on the other species.

Plant stage effects. In mature plants, fewer *P. recondita* and *P. hordei* germlings ended on a stoma than in seedlings (Figs. 1,2). The differences within *H. chilense* were more pronounced in mature plants (Fig. 2) than in seedlings (Fig. 1).

Rust species specificity. The data of the third run with *P. coronata* indicated that fewer germ tubes ended on a stoma on *H. chilense* than on the nonhost wheat and barley checks (Fig. 1). The host oat check, however, did not allow a significantly higher appressorium formation than the *H. chilense* accessions. There was no tendency of infraspecific differences in *H. chilense* as with *P. recondita* and *P. hordei*.

The results with *P. striiformis* (Fig. 1) were inconclusive. The percentage of germlings ending on a stoma was lower than 40%

TABLE 1. Stoma density and size (length × width) and hair density and length on upper epidermis of seedling leaves of the lines used in this study[w]

Accessions	Leaf morphology			
	Stomata		Hairs	
	Number[x] (per cm^2)	Size[y] (μm)	Number[x] (per cm^2)	Length[y] (μm)
Hordeum chilense				
H1	4,372 b[z]	177 e × 45 f	992 a	221 b
H7	6,266 a	178 e × 51 e	480 c	294 ab
H17	7,097 a	179 de × 47 f	700 b	260 ab
H47	6,360 a	186 d × 50 e	253 c	240 ab
×*Tritordeum*				
HT22 (H1 × Cocorit)	2,912 d	328 a × 73 ab	398 c	53 d
HT27 (H7 × Cocorit)	2,823 d	342 a × 73 ab	198 cd	32 de
HT93 (H47 × TB487)	2,595 d	310 b × 76 a	1,458 a	220 b
Triticum aestivum				
Morocco	2,607 d	319 ab × 70 b	571 c	109 c
Little Club	2,798 d	315 b × 68 bc	333 c	76 c
Hordeum vulgare				
L94	2,850 d	225 b × 55 d	65 cd	28 de
Topper	3,390 c	204 c × 55 d	200 cd	23 de
Avena sativa				
Cebeco	2,965 d	249 b × 67 bc	246 cd	29 de

[w] Data were based on 16 leaves per accession.
[x] Countings were done on about 0.025 cm^2 per leaf.
[y] Measurements were based on five stomata and hairs per leaf.
[z] The same letters within columns indicate that differences are not statistically significant ($P < 0.05$).

TABLE 2. Spore density, spore germination, and percentage of germ tubes of wheat leaf rust (*Puccinia recondita*), barley leaf rust (*P. hordei*), oat crown rust (*P. coronata*), and wheat yellow rust (*P. striiformis*) not ending on stomata of seedlings and mature plants of *Hordeum chilense* and other gramineous species

Rust	Experimental run[u]	Spores applied (per cm^2)	Total number of spores/cm^2 on leaf	Percentage of germination	Percentage of germ tubes not ending on stomata[v]	
					Genotype effect	Effect of experimental run
Seedlings						
P. recondita	1	319	282*[w]	85.5*	61.1***[x]	...[x]
	2	ND[y]	355	94.3*	52.2***	
P. hordei	1	260	129	83.2**	58.6***	...
	2	ND	364**	95.7	53.9***	
P. coronata	1	501	298**	88.4	75.2*	***
	2	ND	272	52.9***	86.4	
	3	383	300	80.9	59.3***	
P. striiformis	1	900	475**	53.0*	72.7***	...
	2	ND	201	18.1	73.3*	
	3	ND	ND	...[z]	71.7*	
Mature plants						
P. recondita	1	ND	44	61.4	73.0***	...
	2	ND	150***	78.9**	76.6***	
P. hordei	1	ND	153*	80.5**	70.3***	...
	2	ND	135*	91.1*	66.3***	

[u] Number of germ tubes not forming appressoria plus those that formed appressoria but not on a stoma relative to the total amount of germinated urediospores per leaf.
[v] Eight leaves were studied per line and leaf rust combination in run 1. Six were studied in all other cases.
[w] *, **, *** = Significance of genotype effects within experimental run (0.05, 0.01, 0.001 level, respectively).
[x] Significance of differences among experimental runs (*** = 0.001 level; ... = no significant difference).
[y] ND = not determined.
[z] Only categories after spore germination were recorded.

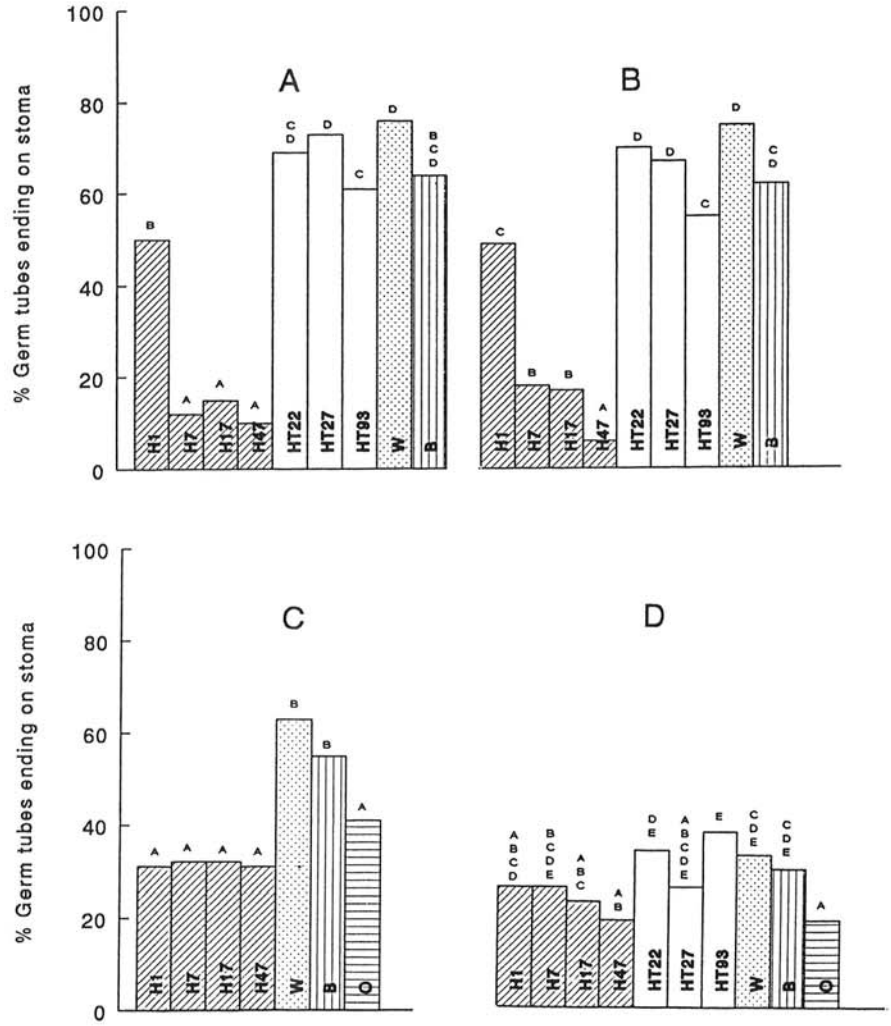

Fig. 1. Percentage of germlings of **A,** wheat leaf rust (*Puccinia recondita*); **B,** barley leaf rust (*P. hordei*); **C,** oat crown rust (*P. coronata*); and **D,** wheat yellow rust (*P. striiformis*) ending on the stomata of seedlings of four *Hordeum chilense* lines (H numbers), three tritordeums (HT numbers), wheat (average of the checks Little Club and Morocco) (W), barley (average of the checks Topper and line L94) (B) and oat Cebeco (O). The data for *P. recondita* and *P. hordei* are the averages of two experimental runs; the data for *P. coronata* are from one run; and the data for *P. striiformis* are the average of three runs. Per rust species, the same letters over columns indicate that differences are not statistically significant ($P \leq 0.01$).

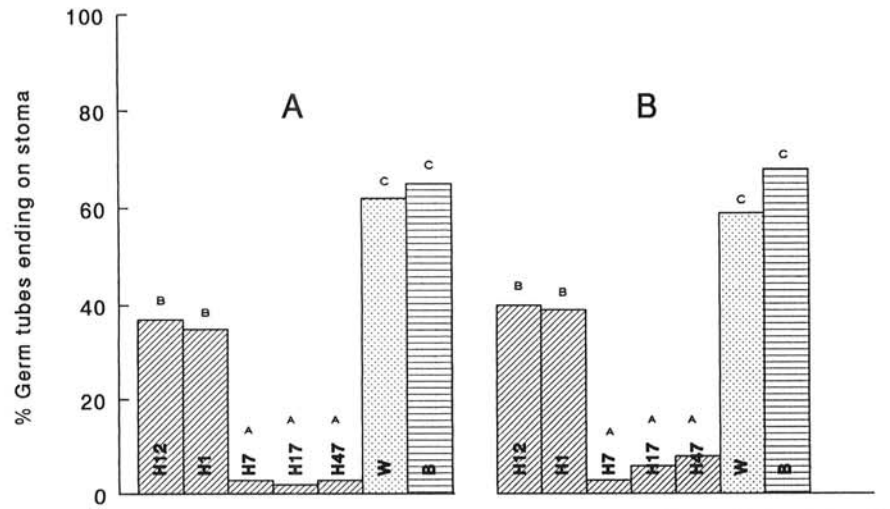

Fig. 2. Percentage of germlings of **A,** wheat leaf rust (*Puccinia recondita*) and **B,** barley leaf rust (*P. hordei*) ending on the stomata of 14-wk-old plants of five *Hordeum chilense* lines (H numbers), wheat check Little Club (W), and barley check line L94 (B). The data are the average of two experimental runs. Per rust species, the same letters over columns indicate that differences are not statistically significant ($P \leq 0.01$).

in all accessions. Despite significant genotype effects, there was no obvious grouping between or within plant species.

Mechanisms of appressorium failure. The low appressorium formation by *P. recondita* and *P. hordei* on H7, H17, and H47 was not due to reproducible and substantial differences in spore germination. Many germ tubes on these accessions grew erratically without orientation towards the stomata (Fig. 3A). Frequently, germ tubes that grew over a stoma on these accessions did not differentiate an appressorium but continued to grow away from the stoma. On lines with a high rate of appressorium formation, disorientation of germ tubes also occurred (Fig. 3B,C), but overgrowth of stomata without appressorium differentiation was rare. A quantification of these events, however, was not attempted.

Expression of low appressorium formation in tritordeum. The tritordeums had either H1 (high appressorium formation) or H7 or H47 (low appressorium formation) as parents (Table 1). The amount of appressorium formation on these tritordeums did not differ substantially from that on wheat and barley (Fig. 1). *P. recondita* and *P. hordei* tended to form fewer appressoria on HT93 than on the other two tritordeums. This may be the effect of the H47 parent of HT93. The magnitude of this pre-appressorium resistance of HT93 was small.

DISCUSSION

The low appressorium formation by leaf rust fungi on some *H. chilense* lines in a previous study (29) was corroborated in the present experiments. The phenomenon occurs both in seedlings and mature plants. Two mechanisms may have contributed to the low appressorium formation: disorientation of the germ tubes (Fig. 3A–C) or germ tubes that cross several stomata but do not differentiate an appressorium (Fig. 3A). In this study, we did not try to quantify the role of the two mechanisms. Low appressorium formation due to characteristics of the plant leaf surface occurs before intimate contact between the fungus and the plant and could, therefore, be regarded as a case of avoidance.

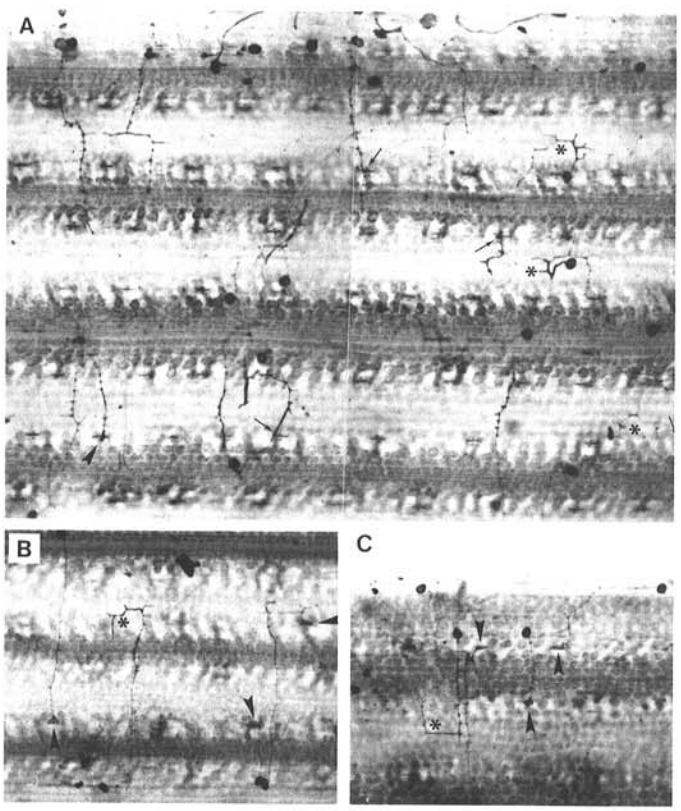

Fig. 3. Micrographs of germlings of *Puccinia hordei* on primary leaves of **A,** *Hordeum chilense* line H47; **B,** *H. chilense* line H1; and **C,** barley line L94. Germlings could form an appressorium over a stoma (▶), go astray (*), or overgrow a stoma without producing an appressorium (→). A, ×124; B and C, ×105.

Germ tube orientation is facilitated by morphological and ultrastructural aspects of the leaf surface. The wax crystal lattice of the cuticle (6,19) has been implicated in directing germ tube growth. Gradients in pH at the leaf surface may also play a role in the ability of the germ tubes to find stomata (7).

The differentiation of an appressorium may be triggered by morphological features of the stoma (e.g., a cuticle lip on the guard cells) (34). For the bean rust fungus, Hoch et al (14) have shown that a single 0.5 μm deep scratch on a hard surface can replace the signal for appressorium formation and that the depth of the scratch corresponds to the height of the stomatal lip. Lewis and Day (19) suggested that disjunction of the germ tube and the crystal lattice over the stomatal pore may trigger the formation of the appressorium.

The poor germ tube orientation on the *H. chilense* lines H7, H17, and H47 may be caused by ultrastructural features. On the lines with high appressorium formation, germ tubes could go astray as well (Fig. 3B,C) but perhaps at a lower frequency. One would expect that low appressorium formation could also be the consequence of low stoma frequency or high hair density. However, *H. chilense* had lower appressorium formation than the cereal checks but did not have fewer stomata or consistently more hairs (Table 1). Within *H. chilense*, line H1 had the highest appressorium formation as well as the fewest stomata and the most hairs.

The overgrowth of stomata in H7, H17, and H47 by germ tubes of leaf rust fungi suggests that in these lines the appressorium-triggering features may be poorly developed. The *H. chilense* accessions have smaller stomata than the cereal checks (Table 1). The morphology and dimensions of the stomata should be investigated at the ultra-structural level. Such studies could elucidate the cause of poor stoma recognition on the *H. chilense* accessions.

The poor appressorium formation on the *H. chilense* lines occurred with *P. r. tritici*, *P. hordei* (the present study), and with the leaf rust of *H. jubatum* (probably *P. r. agropyri*) and the leaf rust of rye (*P. r. recondita*) (29). Apparently these rust species use the same features to find and recognize stomata. Such features occur rather universally in the Gramineae, because the leaf rusts form appressoria on several gramineous nonhost species at an equally high rate (12,16,25,33,36). The mechanisms that hamper the rusts from finding stomata on *H. chilense* are not very effective on *P. coronata* and *P. striiformis*. This suggests that *P. coronata* and *P. striiformis* may use other features to find and recognize stomata. The poor germination and germ tube growth of *P. striiformis* made it difficult, however, to draw firm conclusions.

Because this type of avoidance of H7 and H47 hardly shows up or does not show up at all in the tritordeums, it is unlikely that the feature could be transferred to wheat. Tritordeum is an amphiploid that combines the HH genomes from *H. chilense* and the AABB(DD) genomes from *Triticum* spp. In this amphiploid, features such as resistance to rusts (28) and morphology (2,22,23) are very similar to those of the wheat parent. A similar dominance of *Triticum* features has been found in all other previously reported *Hordeum* × *Triticum* hybrids (10).

Although the transfer of this avoidance to wheat may not be feasible, the *H. chilense* lines in this study may be useful in the study of mechanisms that play a role in directional germ tube growth and stoma differentiation.

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