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# Leaf wax layer may prevent appressorium differentiation but does not influence orientation of the leaf rust fungus *Puccinia hordei* on *Hordeum chilense* leaves

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## Abstract

The infection process of most rust fungi start with spore germination, directional growth of the germ tube towards a stoma, differentiation of an appressorium over the stoma, and penetration into the substomatal cavity. In the South American wild barley *Hordeum chilense* Roem. & Schult., wide variation occurs in the degree to which several rust fungal species are able to form appressoria over the stomata. Apparently, features of the plant may hamper early stages of the infection process. Such an early defence is called avoidance. In order to find out how germ tube growth is directed towards stomata, and whether the cuticular wax layer plays a role in this orientated growth and in appressorium differentiation, several orientation and differentiation parameters of *Puccinia hordei* germ tubes were measured on *H. chilense* leaves with and without the wax layer. Orientated growth of the germ tubes started upon contact with the epidermal cell junctions. The growth of lateral branches of the germ tube over the first epidermal cell junction that it meets, may help the germ tube to grow along the transverse axis of the leaf. No evidence was found of attraction of the germ tube to stomata. Removal of the cuticular wax layer did not result in loss of germ tube orientation. This suggests that the leaf wax layer has no role in the guidance of germ tubes. On high avoidance accessions, removal of the wax layer allowed appressoria to develop over stomata that would otherwise be overgrown. No effect of the cell widths in stomatal complexes was found on the chance that stomata were overgrown. This suggests that the overgrowth of stomata on *H. chilense* leaves by *P. hordei* germ tubes is mainly caused by the wax covering of the stomatal apparatus.

## Introduction

Rust fungi (Basidiomycotina, Uredinales, Pucciniaceae) are important plant pathogenic obligate biotrophs. The infection process of cereal rust fungi, such as the barley leaf rust fungus *Puccinia hordei* Otth, starts with hydration and germination of uredospores on the plant surface (Hoch and Staples, 1987). The germ tube grows across the longitudinally orientated epidermal cells until it contacts a stoma where it ceases growth and develops an appressorium directly over the stomatal opening (Littlefield and Heath, 1979; Hoch and Staples, 1987; Hoch et al., 1987). It has been proposed that this orientation of the germ tube growth along the transverse axis of the leaf increases the probability of encountering stomata, which are arranged in rows on the cereal leaves (Lewis and Day, 1972; Littlefield and Heath, 1979).

Orientated growth of the germ tube and formation of the appressorium are considered to be the responses to the stimuli from the host (Staples et al., 1983; Hoch and Staples, 1987; Hoch et al., 1987; Allen et al., 1991b; Collins and Read, 1997). These stimuli may be physical, e.g., the close spacing of epidermal cell junctions (Read et al., 1997) or the epicuticular wax crystals pattern (Lewis and Day, 1972), or may be chemical,

e.g. pH gradients around the stomata (Edwards and Bowling, 1986).

The effect of topographical features on the orientation of rust germ tube growth and on differentiation of appressoria can be studied in different ways. The rust may be applied to an inert artificial substratum with precise microtopography of defined dimensions (Staples et al., 1983; Hoch et al., 1987; Allen et al., 1991a,b; Collins and Read, 1997; Read et al., 1997) or to leaf surface replicas made of inert material (Collins and Read, 1997). No chemical signals from the host will be present in these cases.

Hoch et al. (1987) observed that the best signals for growth orientation of the bean rust fungus (*Uromyces appendiculatus*), germlings were ridges or grooves in the substrate spaced 0.5–15.0 μm apart. The regularity of orientation diminished as the spacing increased, and spacings greater than 30.0 μm were not effective. Orientation perpendicular to ridges and/or grooves was also observed for *P. hordei* and *P. graminis tritici* germ tubes after growing them over more than a few ridges or grooves on artificial membranes (Read et al., 1997). Hoch et al. (1987) suggested that the surface depressions located at the anticlinal walls of the epidermal cells could serve as the topographical features that orient germling growth. These depressions were spaced 15–30 μm apart on bean leaves, and this could explain why the germling orientation *in vivo* is not as strong as that observed with the closest spacing on the artificial membranes. These authors concluded that the depth of the ridges is not as critical for growth orientation as it is for appressorium differentiation since orientation occurred on ridges ranging in height from 0.1 to 5.0 μm.

Appressorium differentiation occurs as a response to different ridge heights, and varies between and even within a rust species (Allen et al., 1991a; Collins and Read, 1997). In *U. appendiculatus*, it has been suggested that topographical features of the stomatal complex provide the signals for appressorium differentiation over stomata (Hoch et al., 1987; Wynn, 1976). However, the role of topographical signals in the induction of cereal rust appressoria is much less clear (Read et al., 1997). In the bean rust, the optimal inductive artificial topographical signal closely resembles the ridge or 'ledge' at the guard cell lip of the host plant, *Phaseolus vulgaris* (Hoch et al., 1987). The stomatal complexes of cereal leaves do not possess such a prominent guard cell ridge (Read et al., 1997).

An important distinction between the topographical signals involved in appressorium differentiation *in vivo* for the bean rust and for the cereal rusts has already been stressed by Read et al. (1997). In the case of the bean rust, a ridge (guard cell lip) appears to be the important factor, whilst furrows (cell junctions and stomatal pore) seem to be significant for the cereal rusts. Read et al. (1997), using microfabricated substrata, showed that not only the ridge height but also the spacing between ridges was influencing appressorium differentiation. Germ tubes of *P. hordei* differentiated over closely spaced, multiple ridges and grooves, but not to a significant extent over single ridges or flat surfaces. Of the 1.5, 2.5 and 50 μm ridge spacings tested, the most inductive spacing was 1.5 μm for all the heights analysed (up to 2.5 μm) and the greatest level of appressorium differentiation was recorded over ridges which were 2 μm high (Read et al., 1997). The same authors suggested that cereal rust, in contrast to bean rust, respond to the close spacing of cell junctions of the dumbbell shaped cereal guard cells. Nevertheless, these authors acknowledge that they had not identified the precise topographical features that are involved in appressorium differentiation *in vivo*.

These previous studies with artificial membranes, suggested that physical features of the host alone are sufficient to orientate germ tube growth and to induce appressorium formation, but are insufficient to explain the high level of appressorium formation observed *in vivo*. A reason for the discrepancy with *in vivo* observations may be that the host provides a combination of chemical and topographical signals which allows stomata to be accurately recognised by the cereal rust (Collins and Read, 1997). Also an imperfect reproduction of the host physical features by the replication method, notably the cuticular wax crystals, may explain these discrepancies.

The wax crystal lattice of the cuticle has been implicated in directing germ tube growth (Lewis and Day, 1972). Nevertheless, Jenks and Ashworth (1999) hypothesised that the wax structure, covering the leaf surface, may disorient fungal hyphal growth across plant surfaces.

On some *Hordeum chilense* accessions, a large percentage of germ tubes grew over one or more stomata, without differentiating an appressorium. Appressorium differentiation on these accessions may occur at 10–30% of the frequency of that on other *H. chilense* accessions or on cultivated cereals (Rubiales and Niks, 1996). As a consequence, no penetration of the stoma and no further infection can take place (Rubiales and Niks, 1996). This feature was coined 'avoidance'

(Rubiales and Niks, 1992). Growth of germ tubes over stomata on high avoidance accessions might be due to the absence of an effective appressorium inducer structure or to the failure of the germ tube to contact the inducer structure. Scanning electron microscopy indicated the presence of an extensive wax covering over the stomata of these accessions. Extensive wax covering over the guard cells, obscuring features that normally induce appressorium differentiation, was therefore suggested to be the cause of overgrowth of stomata in *H. chilense* accessions with high avoidance levels (Rubiales and Niks, 1996).

The first aim of the present study was to distinguish whether germ tube growth of *P. hordei* on *H. chilense* leaf surfaces is directed towards stomata or whether it is merely perpendicular to the long axis of the leaf. The second aim was to clarify the role of the cuticular wax layer in orientation of the germ tube to the stomata. The third aim was to determine the role of the cuticular wax layer and of the dimensions of the stomatal cells on the appressorium differentiation by *P. hordei* in the wild barley *H. chilense*.

## Material and methods

### *Fungus*

Uredospores of *P. hordei* Otth (isolate 1-2-1; Parlevliet, 1976) were produced in a green house on the susceptible barley (*H. vulgare* L.) line L98.

### *Plants*

Two separate experiments were conducted. Orientation studies included *H. chilense* Roem. & Schult. accessions H7, H46 and H47, selected on the basis of their relatively high stoma density on the abaxial leaf surface (Rubiales and Niks, 1996). Susceptible barley line L94 was included as a reference plant. Countings and measurements were done on the flat abaxial leaf surface as no significant differences on wax coverage between adaxial and abaxial leaf surface were detected using SEM (Vaz Patto, Wageningen University, the Netherlands, unpubl.). For the appressorium differentiation studies, eight accessions of *H. chilense* were selected on the basis of their appressorium inducing capacity. H250, H47, H46 and H7 have a low appressorium inducing capacity (high level of avoidance) and H304, H1, H211 and H75 a moderately high appressorium inducing capacity (low level of avoidance) (Vaz Patto et al., 2001). Countings and measurements were done on the upper (adaxial) leaf surface. All the *H. chilense* accessions were obtained from Prof. A. Martín (Instituto de Agricultura Sostenible, Córdoba, Spain). Plants were grown in a greenhouse compartment at 18–25 °C and ambient humidity, at the Laboratory of Plant Breeding, Wageningen University, the Netherlands.

### *Removal of the epicuticular wax*

About three $cm^2$ of the adaxial or abaxial (depending on the experiment) leaf surface of four sixth leaves of tillers of plants in the vegetative stage were treated with celloidin in order to remove the cuticular wax layer (Rubiales and Niks, 1996). This treated part and the adjacent untreated part were detached from the plants and placed, adaxial or abaxial surface up (depending on the experiment), on damp filter paper in Petri dishes.

### *Inoculation procedure*

Inoculations took place in an inoculation tower. *P. hordei* spores (4 mg, about 190 spores/$cm^2$), mixed with *Lycopodium* powder (1 : 10, v : v), were applied for the orientation studies. *P. hordei* spores (6 mg, about 400 spores/$cm^2$), mixed with *Lycopodium* powder (1 : 10, v : v) were applied for the appressorium differentiation studies. After inoculation, the Petri dishes were covered and incubated overnight in a green house compartment inside a black polyethylene bag. This procedure results in very small water droplets on the leaf surface. Large droplets of water might disturb germ tube growth. The leaf segments were stained after 24 h, taking care not to disturb the position of the germ tubes. The leaf segments were flooded in lactophenol–ethanol for 2–3 days, gently rinsed in water, and flooded in a solution of 0.1% Uvitex in 0.1 M Tris/HCl buffer (pH 8.5) for 3 min. Finally the segments were immersed in 25% glycerol solution for 5 min. Counting and measurements of the germlings were made by using an epifluorescence microscope (Zeiss Axiophot, exciter filter BP 395–440, dichroic beam splitter FT 460 nm, and barrier filter LP 420).

The efficiency of wax removal was checked by examining one leaf segment per plant accession and per wax treatment using cryoscanning electron microscopy as described by Rubiales and Niks (1996).

*Orientation of the germ tubes*

In one trial, four leaves per accession H7, H46 and H47 were inoculated in two consecutive replications in which the following were recorded:

- direction of emergence of germ tube from the uredospore to determine if orientation at germination is already towards the stomatal rows, i.e. transverse direction (Figure 1).
- length of the first five lateral branches of the germ tubes (Figure 1). These lateral branches develop above the lateral junctions of the epidermal cells. Where two of such lateral branches emerged opposite to each other, the longest was measured.
- the minimal distance at which the germ tube grew past a stoma (Figure 1), to determine if there is an active attraction by the stoma.

In a second trial, four leaves per accession H7, H46 and H47 were inoculated with and without the wax removal treatment. The following observations were recorded:

- length of germ tube from uredospore to the first stoma reached.

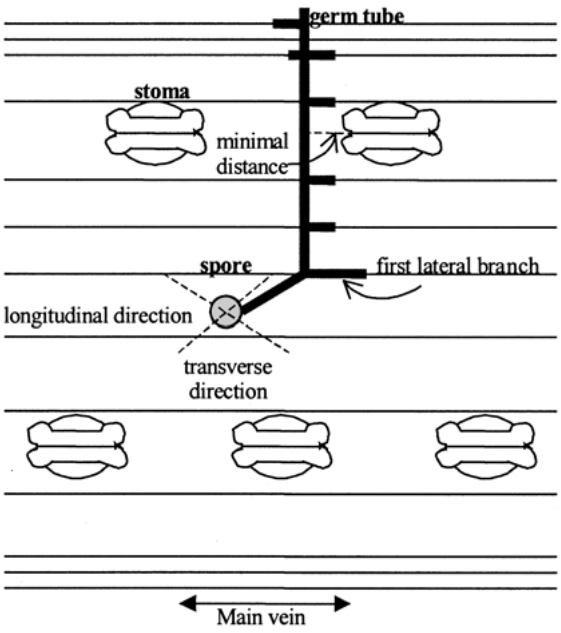


*Figure 1.* Diagramatic representation of a *H. chilense* leaf surface on which are indicated possible directions of *P. hordei* spore germination, germ tube lateral branches over epidermal cell junctions and the minimal distance from the germ tube to the stoma.

- length and width of the projection of the germ tube on the long axis of the leaf.

Measurements were performed on 20 germlings per leaf segment. Averages over the 20 germlings were used for statistical analysis. Part of the data on orientation of germ tubes has been presented before (Vaz Patto and Niks, 2000).

*Appressorium differentiation by the germ tubes*

Four leaves of all the *H. chilense* accessions were inoculated with and without the wax removal treatment, in two consecutive replications. The following observations were recorded:

- the percentage of germ tubes not reaching any stoma.
- the percentage of germ tube/stoma encounters resulting in appressorium differentiation.
- the percentage of germ tubes that form an appressorium.

Statistical analysis was performed on the percentages (100 counts) per leaf.

*Cell dimensions on stomatal complex*

The widths of guard and subsidiary cells of 20 stomata (10 overgrown, 10 with differentiated appressorium) per leaf segment were measured on four H75 leaf segments where no wax had been removed (Figure 2C). Measurements were made using an eyepiece micrometer at 1000× magnification.

## Results

*Orientated-growth studies on the abaxial leaf surface*

The adaxial leaf surface of *H. chilense* is much more ridged than the abaxial leaf surface. Since the ridged structure of the adaxial epidermis could compromise orientation measurements, the flat abaxial leaf surface was used to study the orientation of germ tubes. On the abaxial surface, germ tubes showed very pronounced directional growth, perpendicular to the epidermal cell junctions (Figure 3). Of all the 630 spores observed in the two repetitions, 328 spores started the germination in a transversal direction. This was not significantly more than 50% of the spores and implies that the direction of germination of the spores occurs in a random way, and is not influenced by features of the leaf.

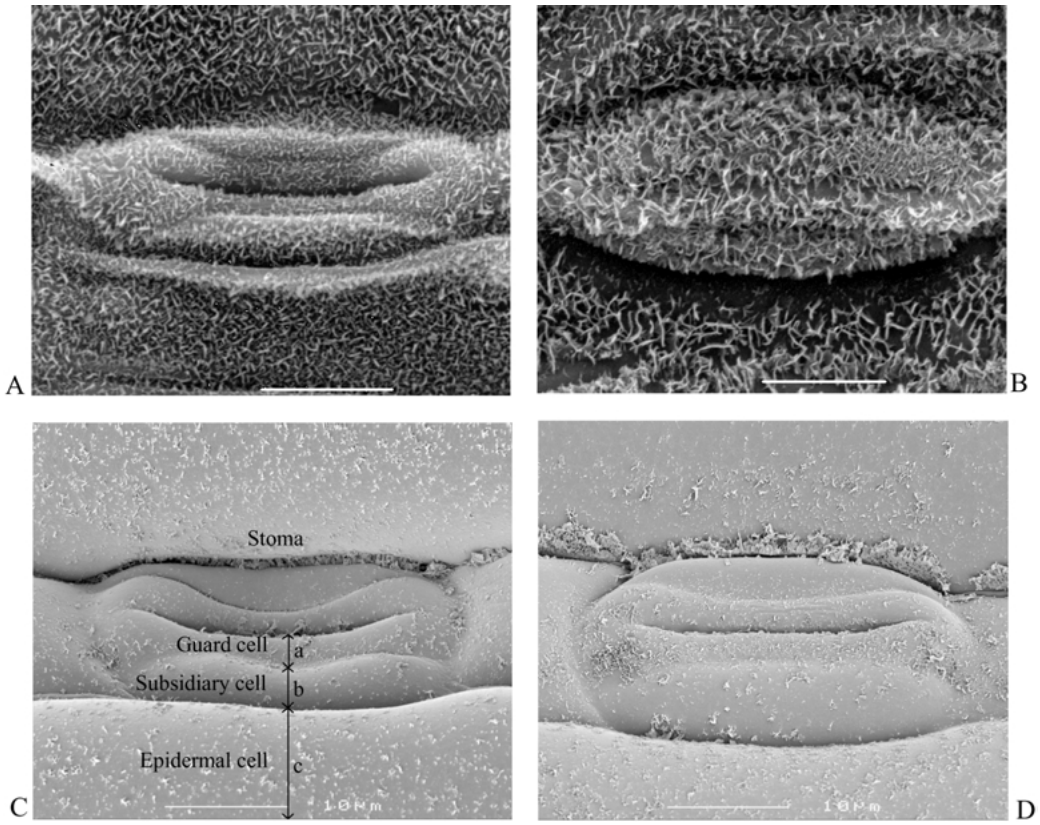


*Figure 2*. Ultrastructure of stomata of *H. chilense* line H1 (low avoidance) (control (A) and wax removed (C)), and line H7 (high avoidance) (control (B) and wax removed (D)). In Figure 2C, indication of measurements done: a = guard cell width, b = subsidiary cell width and c = adjacent epidermal cell width. Note the absence of a regular lattice on the wax crystal microstructure covering a stoma on *H. chilense* leaf surface (Scale bars, 10 μm).

The first encounter of the germ tube to an epidermal cell junction induced a significantly longer lateral branch than the subsequent encounters (Table 1). It appeared that the first encounter was crucial in the transition from the random germ tube growth to orientated growth perpendicular to the long axis of the leaf (Figure 3). Germ tubes that failed to form lateral branches appeared to be poorly attached to the leaf surface. Once germ tubes grew perpendicularly to the long axis of the leaf, they would either pass one or more stomatal rows, or encounter a stoma on which it might form an appressorium.

Germ tubes did not seem to change direction in order to reach a nearby stoma. Stomata that were encountered, were typically in the way of the straight growing germ tubes. Germ tubes could pass a stoma even at a very short distance (<1 μm) without changing direction towards that stoma.

*Effect of wax amount*

Celloidin treatment effectively removed the wax layer from the leaf surface, as is evident from the SEM studies (Figure 2C,D). In none of the accessions did the wax removal change significantly the percentage of germ tubes that failed to reach a stoma (Table 2). The length of the germ tube till it reached the first stoma was not different on the leaves from which the wax had been removed (Table 3). This implies that wax removal did not result in a more erratic growth direction in the germ tubes. This conclusion can also be drawn from the lack of effect of wax removal on the length/width projection of the germ tube (Table 3). Germ tubes tend to grow perpendicular to the long axis of the leaf and the projection of the germ tube on the long axis of the leaf is referred to as width. The ratio length/width of the projection of the germ tube would be expected to be high

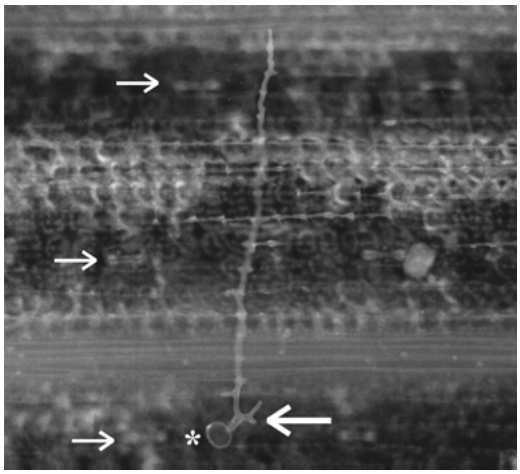

*Figure 3*. Marked directional growth of the germ tubes of *P. hordei* perpendicular to the epidermal cell junctions on *H. chilense* (H47) leaf surface. Germ tube grows from spore (*) showing a first lateral branch (←) longer than the subsequent lateral branches. Note that the first lateral branch marks the beginning of the transverse orientation of the germ tube. Narrow arrow indicates a stoma row.

*Table 1*. Length (μm) of the first five germ tube lateral branches (LB) of *P. hordei* on three *H. chilense* and on cultivated barley (L94) leaf surface

| LB | H7 | H46 | H47 | L94 |
|---|---|---|---|---|
| 1 | $23^{*,b}$ | $21^{b}$ | $23^{b}$ | $24^{b}$ |
| 2 | $8^{a}$ | $12^{a}$ | $10^{a}$ | $17^{a}$ |
| 3 | $6^{a}$ | $10^{a}$ | $10^{a}$ | $17^{a}$ |
| 4 | $8^{a}$ | $10^{a}$ | $12^{a}$ | $18^{a}$ |
| 5 | $7^{a}$ | $10^{a}$ | $12^{a}$ | $16^{a}$ |

*Each value is the average of measurements on 20 germlings on four leaves per accession in two repetitions. [a–b]Values with letter in common in each column are not statistically significant ($p < 0.05$), Duncan test.

in the case of normal perpendicular orientation. In the case of disoriented germ tube growth, the average ratio would be close to one. We conclude that, in general, the cuticular wax does not provide essential clues for the germ tube to find the stomata.

On the control leaf segments, high avoidance accessions H250, H47, H46 and H7 showed a low percentage of germ tube/stoma encounters with appressorium differentiation (12% average). On these accessions, removal of the wax resulted in a six-fold (and significant) increase in appressorium differentiation (Table 2). Apparently, the removal of the wax layer allowed appressoria to develop over stomata that would otherwise be overgrown. On the low avoidance accessions H211, H1 and H304, a much higher percentage of encounters resulted in appressorium differentiation (68–85%) than on high avoidance accessions. Removal of the wax in these accessions did not increase the appressorium differentiation. After wax removal, all accessions had a similar percentage of

*Table 2*. Germ tube growth and appressorium formation of *P. hordei* on leaves of different *H. chilense* accessions

| | Germ tube/stoma encounters with appressorium differentiation (%)# | | Germlings not reaching any stoma (%)# | |
|---|---|---|---|---|
| | Control | Wax removal | Control | Wax removal |
| *High avoidance accessions* | | | | |
| H250 | $3.3^{a}$ | $66.7^{**,a}$ | $15.0^{b,c}$ | $11.6^{a,b}$ |
| H47 | $9.3^{a,b}$ | $65.2^{**,a}$ | $12.7^{b}$ | $13.0^{a,b}$ |
| H46 | $15.1^{b,c}$ | $65.6^{**,a}$ | $7.1^{a}$ | $10.4^{a}$ |
| H7 | $20.3^{c}$ | $72.1^{**,a,b}$ | $22.1^{d}$ | $16.6^{b,c}$ |
| *Low avoidance accessions* | | | | |
| H75 | $55.9^{d}$ | $74.7^{*,a,b}$ | $17.8^{c,d}$ | $15.6^{a,b,c}$ |
| H211 | $68.1^{e}$ | $67.0^{a}$ | $15.0^{b,c}$ | $16.6^{b,c}$ |
| H1 | $75.4^{f}$ | $72.0^{a,b}$ | $22.1^{d}$ | $16.0^{a,b,c}$ |
| H304 | $85.4^{g}$ | $79.4^{b}$ | $21.9^{d}$ | $21.2^{c}$ |

#Each value is the average of counts on at least 100 germ tubes or 100 stoma/germ tube encounters each on four leaves per accession in two repetitions. Half segment of each leaf was treated to remove the wax layer, while the other half was left untreated as a control. Differences between control and wax removal are statistically significant at 0.05 (*) or at 0.01 (**) level (ANOVA). [a–g]Values with the same letter per column are not significantly different (Duncan test, $p < 0.05$).

*Table 3*. Germ tube growth of *P. hordei* on three *H. chilense* and on cultivated barley (L94) leaf surface without (control) and with treatment to remove the wax layer

| Accessions | Germ tube length from spore to first stoma (μm) | | Ratio length/width of germ tube projection | |
|---|---|---|---|---|
| | Control | Wax removal | Control | Wax removal |
| H47 | $50^{a}$ | $53^{ns}$ | 11 | $11^{ns}$ |
| H46 | 50 | $51^{ns}$ | 11 | $9^{ns}$ |
| H7 | 52 | $57^{ns}$ | 10 | $11^{ns}$ |
| L94 | 57 | $57^{ns}$ | 9 | $7^{ns}$ |

[a]Each value is the average of measurements on 20 germlings on four leaves per accession. Half segment of each leaf was treated to remove the wax layer, while the other half was left untreated as a control.
Differences between control and treatment are statistically significant at 0.05 level (ANOVA). ns = no significant difference.

*Table 4*. Cell widths (μm) of stomata of *H. chilense* accession H75, that were overgrown or on which an appressorium was formed by *P. hordei*

| Dimensions | Overgrown | Appressoria formed |
|---|---|---|
| Width of guard cells | 4.0[a] | 3.9[ns] |
| Width of subsidiary cells | 5.8 | 5.7[ns] |
| Width of adjacent epidermal cell | 11.8 | 10.8[ns] |

[a]Each value is the average of measurements on 10 stomata on four leaves. Differences in dimensions between overgrown stomata and stomata on which an appressorium was formed are statistically not significant at 0.05 level (*t*-test). ns = no significant difference.

germ tube/stoma encounters leading to appressorium formation (Table 2).

*Effect of cell dimensions on stomatal complex*

On control leaf segments of *H. chilense* accession H75, overgrowth of stomata was about as common as appressorium differentiation: 44% versus 56% of germ tube/stoma encounters (Table 3). Wax removal increased this appressorium differentiation to 75%. In this accession, no significant differences were observed in stomatal cell dimensions between stomata that were overgrown and stomata on which an appressorium was differentiated (Table 4).

## Discussion

Germ tubes of several rust fungi have been shown to grow perpendicular to the epidermal cells junctions of the leaf surface (Wynn, 1976; Read et al., 1997) or at right angles to the structural lines of artificial membranes (Wynn, 1976; Staples et al., 1983, Hoch et al., 1987; Read et al., 1997). This growth is directed by physical contact of the growing tip with the surface. Close adherence is needed (Allen et al., 1991a). It is proposed that, because of the arrangement of the stomata in the leaf surface, this orientation almost ensures that the germ tube reaches a stoma, if it grows far enough (Wynn, 1976). In addition, it is advantageous for the rust fungus to reach the stoma as efficiently as possible. The more energy it spends in germ tube growth, the smaller the chance that the infection unit will establish a vigorous colony (Niks, 1990).

The present study was undertaken to understand if the germ tube growth of *P. hordei* on a *H. chilense* leaf surface is directed towards stomata or if it is merely perpendicular to the long axis of the leaf, and how the wax layer can affect the orientation and the appressorium formation. For this purpose we used leaves rather than artificial membranes to avoid imperfect reproduction of host physical structures, notably the cuticular wax crystals (Collins and Read, 1997). Growth of the germ tubes started in a random direction at spore germination, until they contacted an epidermal cell junction. Since the outer periclinal walls of the leaf epidermis are convex, a sharp downward longitudinal fold occurs over the junction of anticlinal cell walls. This longitudinal fold seems to induce a lateral branching of the germ tubes, that follows the fold. This event appears to be associated with the germ tubes taking a growth direction perpendicular to this first cell junction.

The first lateral branch was generally longer than the subsequent lateral branches. The data suggest that the first, longer, lateral branch will initiate the orientation, while the subsequent, shorter, branches serve to maintain the direction of growth.

Contrary to the positive attraction reported by Edwards and Bowling (1986) on the broad bean rust fungus, in our study there was no evidence of active germ tube growth to stomata. Germ tubes growing in the proximity of a stoma did not converge to it as would be expected if they were responding to stimuli from that stoma. Germ tubes did not necessarily grow towards the nearest stomata and, even when a spore germinated adjacent to a stoma, the germ tube might grow in the opposite direction.

According to Jenks and Ashworth (1999) and Lewis and Day (1972), the wax crystal microstructure might influence the orientation of germ tube growth. If this is the case, wax removal should affect the orientation of the germ tube growth. However, no improvement or worsening of germ tube orientation was observed, after removal of the wax layer. Scanning electron microscopy showed that the wax crystals covered the entire leaf surface exclusive of the trichomes. The wax crystals were not arranged in a lattice pattern (Figure 2A,B), as suggested would be the case for wheat (Lewis and Day, 1972). Because of the apparently random distribution of wax crystals, it was not surprising that the wax layer did not improve the orientation of the germ tube growth.

Rubiales and Niks (1996) studied a subset of these *H. chilense* accessions using the percentage of germ tubes forming appressoria on leaves as an avoidance parameter. They observed that removal of wax resulted in a substantial increase in appressorium

differentiation on high avoidance accessions, but to the level of the untreated low avoidance accessions. They also reported a significant reduction of appressorium differentiation on the low avoidance accessions. They presumed that these results were due to a poor germ tube orientation and suggested that the wax layer plays a role in the orientation of germ tube growth across the leaf towards the stomata (Rubiales and Niks, 1996). None of the accessions used in the present study show a significant change in the percentage of germ tubes that failed to reach a stoma, following wax removal (Table 2). This is an indication, contrary to the suggestion of Rubiales and Niks (1996), that the leaf wax layer does not contribute to, nor does it hamper the orientation of the germ tube.

In the present study, using the same avoidance parameter as Rubiales and Niks (1996), wax removal resulted in a complete restoration of appressorium differentiation on high avoidance accessions, but there was no consistent and significant reduction in appressorium differentiation on the low avoidance accessions (data not shown). Only on the low avoidance accession H211, did removal of the wax significantly reduce the appressorium differentiation. Since no effect of the wax layer on the orientation of germ tubes was observed in our work, the differences between our results and the results of Rubiales and Niks (1996) on appressorium differentiation are possibly due to a technical artefact.

Read et al. (1997), using artificial membranes and the fungus *P. hordei*, found that the optimal differentiation signal was a multiple of 2.0 μm high ridges spaced 1.5 μm apart. This spacing does not correspond with the spacing of adjacent cell walls in the stomatal complex. The width of the guard cells and of the subsidiary cells reported in the present work was about 4.0 μm and 5.8 μm, respectively. Unfortunately, Read et al. (1997) did not test the appressorium inducing capacity of ridge spacings that correspond to the cell widths reported here (4.0–5.8 μm). Therefore it remains to be investigated whether the spacing of cell junctions of *H. chilense* stomatal complexes is the inductive signal that triggers appressorium differentiation.

From this study we conclude that epidermal cell junctions direct the germ tube growth along the transverse axis of the leaf, without any role of the cuticular wax layer, and without attraction by stomata. We also suggest that the growth of the rust germ tubes over stomata was mainly due to the more extensive wax covering of the stomatal apparatus on the high avoidance accessions rather than to differences in the stomatal cell dimensions (width). In the *H. chilense* high avoidance accessions, the epicuticular waxes were probably covering the appressorium inducer structure of the stoma, reducing the chance for effective appressorium induction. On the low avoidance accessions, where the wax covering was not so extensive, the inducer structures of the stoma were more exposed to the germ tube tip, increasing the probability of differentiation of an appressorium.

An intriguing question to be addressed in the future is what are the precise features of the stoma in *H. chilense* that induce the appressorium differentiation of rust fungi.

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