Inhibition of *Blumeria graminis* germination and germling development within colonies of oat mildew

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Germination by *Blumeria graminis* DC Speer ffl. spp. _avenae, hordei_ and _tritici_, was greatly suppressed when conidia fell within colonies of _f._ spp. _avenae_ or _hordei_ established on susceptible oat or barley, respectively. On healthy oat or barley, and when distant from powdery mildew colonies, all _f._ spp. formed normal appressoria. This was also true when conidia germinated within established barley mildew colonies. Within barley mildew colonies, appressoria of _f._ sp. _hordei_ penetrated epidermal cells (formed haustoria) more frequently than appressoria distant from colonies. Similarly, _f._ spp. _avenae_ and _tritici_, normally unable to infect barley, frequently penetrated epidermal cells subtending established barley mildew colonies. Thus, colony establishment induced barley epidermal cell accessibility, even to non-pathogenic _f._ spp. In contrast, when all three _f._ spp. germinated within established oat mildew colonies, most formed abnormal, hypha-like germ tubes. Since they did not form appressoria, and were thus unable to attempt penetration, it was impossible to determine whether oat mildew colonies induced accessibility of underlying oat epidermal cells. However, when superficial structures of established colonies were removed, germings of all _f._ spp. formed appressoria freely on cells containing oat mildew colony haustoria. Furthermore, these cells showed high level induced accessibility not only to _f._ sp. _avenae_ but also to the normally non-pathogenic _f._ spp. This indicated that factors disrupting germination and further development by conidia lying within oat mildew colonies were produced from the superficial colony structures and not by haustorium-containing plant cells. The factors appear to have limited mobility.

*Keywords:* *Blumeria graminis; Erysiphe graminis; oat mildew; barley mildew; germination; germ tubes; biocontrol.*

**INTRODUCTION**

*Blumeria graminis* DC Speer (syn. *Erysiphe graminis* DC) causes powdery mildew of cereals and other grasses. The disease is spread mainly by wind-blowing asexual conidia. Following deposition on a host, conidia germinate and germling morphogenesis proceeds through a series of distinct steps. Emergence of the primary germ tube (PGT) provides the first visible sign of germination (within 30–120 min). The PGT remains short (approx. 5–10 mm) and aseptate, and is thought to have at least three functions, namely, attachment to the host surface [5–7, 11, 26], gaining access to host water [2], and recognising host surface features [4, 6, 7, 38]. The features recognized probably include hydrophobicity and cutin and cellulose breakdown products released by fungal enzymes [5, 6, 10, 14, 32, 35, 36]. This recognition engages processes that lead to elongation of a second germ tube [4, 5, 38] that emerges shortly after the PGT [5, 6, 26, 38]. Elongation of the second germ tube is pre-requisite to it eventually differentiating a specialized infection structure, the appressorium. As it grows epiphytically, the elongating appressorial germ tube forms a single septum and itself recognizes host surface features [4–6, 17]. This recognition leads to swelling of the distal cell and eventually to differentiation of a hooked appressorial lobe at the tube apex (Fig. 1). At 20°C, appressoria are formed by 10–12 h after inoculation. Occasionally, first- or later-formed germ tubes fail to contact, or recognize contact with the leaf, in which case they remain as short, non-functional “subsidiary germ tubes” [7, 26, 38]. In this case, reserves permitting, a subsequent germ tube will take on the PGT functions and the next-formed germ tube will elongate. Similarly, elongated germ tubes sometimes fail to contact, or respond to, the host surface, in which case they remain as undifferentiated, hypha-like structures incapable of infection [4, 5, 38].

When a functional appressorium is formed, an infection peg emerges from beneath the appressorial lobe and attempts to penetrate the underlying epidermal cell (12–16 h after inoculation) [1]. The attacked cell responds and papilla deposition is initiated. However, in a compatible relationship, the infection peg often penetrates the
response site, enters the cell and an immature primary haustorium is formed (15–24 h). The haustorium then develops digitate processes from each end of the ellipsoidal haustorial body and absorbs nutrients from the infected cell [1, 3, 18]. These are exported to the appressorium, hyphae emerge from the appressorial germ tube or from the mother conidium, and an epiphytic hyphal weft grows at accelerating rate over the leaf surface [3, 18]. After 2–3 days, nutrients supplied by the growing primary haustorium become insufficient to support the young colony, and a second generation of hyphal appressoria is initiated in response to darkness during the daily cycle [3, 18]. These hyphal appressoria are usually sited either on the cell containing the primary haustorium or within one or two cells distance of it. Penetrations from these appressoria form secondary haustoria (usually between two and six) and hyphal growth accelerates further. Successive generations of haustoria (with increasing numbers in each generation) are formed nightly. By 4–5 days the first conidiophores develop and sporulation commences shortly afterwards.

Even in compatible host–B. graminis interactions where some primary penetration attempts succeed, many fail to penetrate the papilla defence. It is now well established that the ability of B. graminis to penetrate cereal epidermal cells is dramatically affected by the outcome of prior attacks by the fungus [8, 20, 21, 23, 24, 27–29, 33, 34]. This has implications for the field situation where individual cells may be confronted by sequential attacks as conidia are deposited continually from the aerial pool. Thus, if an initial attack is successful, the cell shows induced accessibility (sensu Ouchi et al. [34]) so that later attacks on that cell will almost invariably succeed. By contrast, failed initial attacks induce “inaccessibility” (sensu Kunoh et al. [21]) within the cell so that subsequent attacks almost invariably fail. While these induced changes are essentially localized phenomena, being expressed most strongly in the single cell directly subjected to earlier attack, there is some transmission of effects to immediately adjacent epidermal cells although the effects are often undetectable at two cells distance [8, 21, 23, 27–29, 33].

Lyngkjær and Carver’s previous studies [8, 27–29] involved double inoculation procedures whereby the first, “inducer” inoculum was allowed to develop and then its superficial fungal structures (conidia, germ tubes, hyphae) were removed before the second, “challenge” inoculum was applied. The inducer inoculum was given a maximum of 48 h incubation before removal of superficial structures i.e., where primary penetration had succeeded, a primary haustorium was formed but no hyphal appressoria or second generation haustoria were initiated. The success (or failure) of challenge attack was then determined in relation to the presence of inducer primary haustoria or papillae remaining as intracellular evidence of successful or failed inducer attack, respectively.

We undertook the present work to extend Lyngkjær and Carver’s earlier studies [8, 27–29]. Here, however, we aimed to allow colonies from inducer inoculum to develop second generation haustoria before applying challenge inoculum. We intended that germling appressoria of the challenge inoculum would attack host cells at the time that inducer colonies were initiating their third generation haustoria. Thus, our intention was to determine whether the presence of multiple haustoria (second and third generation) produced by a developing colony enhanced the expression of induced accessibility in nearby, unattacked cells.

We executed our first experiments using oat (Avena sativa L.) inoculated with a compatible isolate of B. graminis f. sp. avenae Marchal. However, we found it impossible to gather sufficient data to fulfil our objective. Where challenge conidia were deposited within the boundaries of an established f. sp. avenae colony derived
from inducer inoculation, very few of these conidia seemed to germinate. Furthermore, very few of those that did germinate formed an appressorium. The majority of germings produced highly abnormal hypha-like germ tubes and did not appear to attempt penetration of the host cells.

The work reported here was therefore redirected towards exploring the effects that established colonies might have on germination and germing development by *B. graminis* conidia deposited within established *B. graminis* colonies. We investigated these effects with respect to f. sp. *avenae* colonies on susceptible oats and f. sp. *hordei* colonies on susceptible barley.

**MATERIALS AND METHODS**

*Pathogens and plants*

An isolate of *B. graminis* f. sp. *avenae* race 5, was multiplied on plants of oat cv. Selma grown in a spore-proof glasshouse under natural lighting conditions and minimum temperature of 12°C. Similarly, isolate CC1 of *B. graminis* f. sp. *hordei* and an isolate of *B. graminis* f. sp. *tritici* (originally supplied by A. Daniels, Aventis, U.K.), were multiplied on barley cv. Pallas and wheat cv. Riband, respectively. One day before conidia were required for experimentation, infected leaves were shaken to remove older conidia and ensure a supply of vigorous young conidia for inoculum.

For all experiments, fully expanded second-formed leaves of cereal plants were used. Studies of oat (*A. sativa* L.) used cvs Selma and Maldwyn. Neither has known major gene resistance to *B. graminis* f. sp. *avenae* race 5. Studies of barley (*Hordeum vulgare* L.) used cv. Pallas, which is susceptible to *B. graminis* f. sp. *hordei* isolate CC1. Since the second leaves of these cvs are susceptible to virulent fungal isolates, penetration attempts from many appressoria are successful and relatively few attacks fail in association with papilla deposition by living epidermal cells, and few result in death of attacked cells ([8], Lyngkjaer and Carver, unpublished).

Neither oat cv. nor Pallas barley is susceptible to *B. graminis* f. sp. *tritici*, Pallas is not susceptible to *B. graminis* f. sp. *avenae*, and the oat cvs are not susceptible to *B. graminis* f. sp. *hordei*. As expected from previous studies [13], preliminary observations suggested that failure of infection by inappropriate ff. spp. was explained by a combination of two factors: either papilla responses prevented penetration or attacked cells died rapidly in response to attack. Importantly, even on inappropriate cereal spp., conidia of all fungal ff. spp. appeared to germinate and develop normally up to the stage of appressorial differentiation.

To produce disease-free experimental plants, seeds were sown singly in paper tubes (15 x 120 mm) containing soil : peat : sand (60 : 30 : 10; v : v : v). Plants were grown to full expansion of the second-formed leaf (approx. 18 days) in a spore-proof glasshouse under natural lighting conditions and a minimum temperature of 12°C.

**Experimental designs and procedures**

*Double inoculation experiments: established colonies in place.* All of these experiments involved using first a low density inoculation to establish young colonies on test leaves and then, 72 h later, applying a second, high density inoculation. In this way, conidia of the second inoculation were deposited both within established colonies where they lay touching or between the superficial hyphae, or distant from colonies when they lay on cells that had no contact with hyphae of established colonies. For the first inoculation, in all cases nine leaves of Selma or Maldwyn oat, or of Pallas barley were inoculated with conidia of the appropriate f. sp. (*avenae* or *hordei*, respectively). Paper tubes containing the plants were laid on a bench, and their second-formed leaves were arranged adaxial surface up (and held flat using small weights at leaf apex and base), before placing a spore settling tower over them. Conidia were blown from infected leaves into the tower and a slide placed among the leaves was used to trap conidia and monitor spore density which was adjusted to approx. 2–5 conidia mm⁻². The inoculated plants and four additional healthy plants were then transferred to a growth cabinet at 20°C and under a 12 h light/12 h dark cycle, with 200 µmol m⁻² s⁻¹ photon flux density during the light period. The first light period commenced immediately after inoculation.

Under these conditions, previous studies [3, 9, 12] allowed us to anticipate the pattern of colony development. Following establishment of a functional primary haustorium at around 20 h after inoculation, secondary hyphal elongation and branching was expected to accelerate up to the dark period beginning 60 h after inoculation, when, in the 60–72 h dark period, hyphal appressoria and then second generation haustoria would be initiated. One of the nine inoculated leaves was cut, fixed, cleared and microscopically examined 71 h after inoculation to confirm that colonies were developing as expected. When this was confirmed, four of the inoculated leaves were fixed 72 h after inoculation for reference.

At 72 h, a second inoculation was applied to the remaining four inoculated leaves, and the second-formed leaves of the four healthy plants were inoculated simultaneously to provide a control. The second inoculation was adjusted to approx. 50 conidia mm⁻². According to requirement, conidia for the second inoculation were
either of the f. sp, appropriate to the host (i.e. the same isolate as used for establishing colonies via the first inoculation) or of an inappropriate f. sp, normally unable to infect the test leaves. Following the second inoculation, leaves were returned to the growth cabinet and incubated for a further 24 h before all were fixed.

**Double inoculation experiments: established colonies removed.**

Thirteen second-formed leaves of Selma oat were inoculated with approx. 2 conidia mm\(^{-2}\) of *B. graminis* f. sp. *avenae* race 5. These, and 12 healthy (uninoculated) leaves, were inoculated as described above, but for 96 h, although one inoculated leaf was fixed, cleared and microscopically examined 95 h after inoculation. Microscopy of this leaf confirmed the anticipation [3] that the third generation of haustoria had been initiated by this time. At 96 h after the first inoculation, the adaxial surface of all leaves (including the healthy ones) was painted with cellulose acetate/acetone solution which was allowed to dry (approx. 2–5 min) before the dried acetate film was peeled away. This removed all superficial fungal structures due to the first inoculation (colony hyphae, failed germlings and conidia) and the epicuticular leaf waxes from inoculated and healthy leaves [10, 27]. As expected [27], epidermal cells containing haustoria formed by colonies on leaves treated with the first inoculum remained alive (as judged by lack of cytoplasmic granulation and whole-cell autofluorescence). Four leaves that had received the first inoculum were then inoculated for a second time using 50 conidia mm\(^{-2}\). These, and 12 healthy leaves, were incubated as described above, but for 96 h, although one inoculated leaf was fixed, cleared and microscopically examined 95 h after inoculation. Microscopy of this leaf confirmed the anticipation [3] that the third generation of haustoria had been initiated by this time. At 96 h after the first inoculation, the adaxial surface of all leaves (including the healthy ones) was painted with cellulose acetate/acetone solution which was allowed to dry (approx. 2–5 min) before the dried acetate film was peeled away. This removed all superficial fungal structures due to the first inoculation (colony hyphae, failed germlings and conidia) and the epicuticular leaf waxes from inoculated and healthy leaves [10, 27]. As expected [27], epidermal cells containing haustoria formed by colonies on leaves treated with the first inoculum remained alive (as judged by lack of cytoplasmic granulation and whole-cell autofluorescence). Four leaves that had received the first inoculum were then inoculated for a second time using 50 conidia mm\(^{-2}\) of either f. sp. *avenae*, f. sp. *hordei* or f. sp. *tritici*, and different sets of four healthy leaves were also inoculated simultaneously with the different f. spp. All were incubated for a further 36 h before fixation.

**Preparation for light microscopy**

As described previously (e.g. [13]) leaves were fixed and cleared by a procedure that avoids displacing ungerminated conidia and loosely attached germlings. Briefly, central 30 mm segments of leaves were excised, and laid inoculated surface up on tissue paper pads moistened with 3:1 ethanol:glacial acetic acid (v:v) to fix and bleach the leaves. They were then transferred to pads moistened with water and subsequently to pads moistened with lactoglycerol (lactic acid:glycerol:water, 1:1:1, v:v) to clear. For studies of fungal development where leaves are fixed before large colonies are allowed to develop, it is possible to attain good resolution of pathogen and host using unstained leaves mounted without coverslips, and viewed with no-coverslip objectives. However, because of diffraction around hyphae of colonies established by the first inoculum, it was impossible to use this approach to resolve details of second inoculum development within established colonies. We needed, therefore, to stain specimens.

The first experiment involved double inoculating Selma oat with *B. graminis* f. sp. *avenae*, leaving established colonies from the first inoculation in place. Here, staining was achieved by placing a smear of Trypan blue in lactoglycerol (0.1 g per 100 ml) on a coverslip, lowering the adaxial (inoculated) surface of the leaf segment onto the coverslip, and then inverting the mount onto a microscope slide smeared with lactoglycerol. This procedure was devised to allow staining without displacing loosely attached germlings. We used this to gather data on the developmental characteristics of germlings formed by conidia of the second inoculum in relation to their proximity to established colonies. However, we found that even with care some ungerminated conidia were displaced; they could be seen moving over relatively long distances if specimens were viewed immediately after mounting when the liquid media were flowing over segments. Therefore, for this experiment we had limited confidence in data for germination frequency by conidia of the second inoculum in relation to their proximity to established colonies.

To allow assessment of germination frequency as well as the morphological characteristics of germlings formed by the second inoculum, we devised a staining technique that caused no detectable displacement of ungerminated conidia. This was used for all further experiments. As before, leaves were fixed, bleached, and cleared, but dishes containing the segments supported on tissue paper were held on a slant to avoid accumulation of free liquids around segments and the possibility that free liquid would move onto the segment surface. To stain, segments supported on slanted dishes were sprayed very lightly using a hand-pumped atomizer (Schott, Germany) from a distance of approx. 40 cm with alcoholic methyl blue stain (0.2 g methyl blue in 100 ml 95% ethanol). A small amount of spray (six pumps) was applied and then spraying was suspended for 1 min to allow the ethanol to evaporate. In this way, no visible droplets of liquid accumulated. A further spray was then applied. This process was repeated until microscope inspection showed colonies, germlings and ungerminated conidia to be stained sufficiently (usually three cycles). Repeated observation (before and after spray staining, without coverslip) of spores located distantly from established colonies, showed that the procedure did not displace ungerminated conidia. Following staining, specimens were mounted without a coverslip and viewed by transmitted light and differential interference contrast microscopy using 20 x or 40 x objectives. Observations were made immediately because although staining remained good for approx. 24 h, it faded thereafter.
Data collection

Double inoculation experiments: established colonies in place.

Our aim in these experiments was to determine the effect colonies established from the first inoculation might have on conidial germination and the development of germlings from the second inoculation.

For the first experiment where coverslips were fitted, on each control leaf (inoculated once, 24 h before fixation) 50 randomly selected, germinated conidia were examined. Conidia were classified as germinated if at least one short germ tube had been produced. For each of these germlings, note was taken of whether they had developed abnormally or had developed normally to differentiate an appressorial germ tube (illustrated in Fig. 1). Appressorial germ tubes were recognized by the presence of a single septum within the tube, swelling towards the tube apex, and the presence of a hooked apical lobe. Note was also made of whether appressoria were associated with successful penetration as evidenced by the presence of a haustorium. Abnormal germlings were those which had either failed to form an elongated germ tube (at least 15 μm), or where the elongated germ tube had not differentiated into an appressorial germ tube. On double-inoculated leaf segments fixed 24 h after application of the second inoculum, observations were made for 50 germinated conidia that lay on leaf epidermal cells located “distant” from the nearest colony, and germlings were classified in the same way as on controls. Distant cells were defined as being at least four epidermal cells away from the nearest cell in contact with hyphae belonging to colonies formed by the first inoculum. Similar classifications were made for 50 germlings arising from conidia located within the outer “boundaries” of colonies established by the first inoculum. The colony boundary was defined by an imaginary line joining the tips of a colony’s leader hyphae (Fig. 2).

Of course, on double inoculated leaves, some germlings deriving from the first inoculation failed to penetrate and establish colonies, and we could not distinguish between these and germlings from the second inoculation. Some of these failed germlings would inevitably be included in our samples. However, since the density of the second inoculation (approx. 50 conidia mm⁻²) was 10–25 times greater than the first (approx. 2–5 conidia mm⁻²), and since many germlings from the first inoculation formed colonies, the great majority of data would necessarily relate to germlings derived from the second inoculation. Therefore, we introduced only small errors due to inclusion of observations of germlings from the first inoculation.

We wished to gauge the growth made by colonies in the period 72–96 h after inoculation i.e. from the time of applying second inoculum to the time of fixation for double inoculated leaves. Drawings of two “typical” B. graminis f. sp. avenae colonies are shown in Fig. 2(a) and (b) (for 72 h and 96 h colonies, respectively). As measures of colony size, the maximum longitudinal and lateral distances from tip to tip of the longest hyphae [Fig. 2(a) and (b)] were measured for 10 colonies on each leaf segment fixed 72 h after inoculation, and for 10 colonies fixed 96 h after inoculation. For the 96 h segments, measurements were also made of the maximum longitudinal and lateral distances between the last branches on leader hyphae [Fig. 2(b)]. From these measurements, it was deduced that the lengths and breadths of 72 h colonies were approximately equivalent to the last-branch length and breadth distances seen in 96 h colonies. Thus, when observing 96 h colonies, the position of the last branch on leader hyphae offered an approximate, but reasonable, indication of the location of leader hyphal tips at 72 h when the second inoculum was
applied. This information allowed us to modify data collection criteria for subsequent experiments.

In remaining experiments where the fungus was spray-stained, additional data were collected. As described above, on controls (inoculated once, 24 h before fixation) 50 germlings were classified for normality of development, but in addition, 100 randomly selected conidia were examined to determine whether they had germinated. Similarly, on double-inoculated leaves fixed 24 h after the second inoculation, 50 germlings lying on epidermal cells distant from colonies were classified for normality of development, and 100 conidia on such cells were examined for germination. In relation to established colonies, data were collected from two different zones within colonies. The inner zone was defined by an imaginary boundary line linking the points at which the last hyphal branch occurred on the colony’s major runner hyphae [Fig. 2(c)]. The outer zone lay between the inner boundary and the outer boundary which was, as before, defined by an imaginary line joining the tips of a colony’s major hyphal branches [Fig. 2(c)]. On each leaf segment, many randomly selected colonies were examined and data were collected from within each zone until we had accumulated observations from 100 conidia to determine whether they had germinated, and from 50 germinated conidia for normality of germling development.

As considered above, on double inoculated leaves, only small errors would have been introduced due to inclusion of data deriving from the first inoculum in the data sets.

For all leaf segments fixed 24 h after the second inoculation and in all experiments, mean percentages were calculated, as appropriate, for spore germination, abnormal germling development, and haustorium formation. Data sets were gathered from each location on double inoculated leaves (distant from colonies, and, as appropriate, within colony outer and inner zones) and for controls inoculated once 24 h before fixation. Percentages were transformed to arcsine square roots (transformed value = 180/π × arcsine [√/100]) to normalize data and stabilize the variance throughout the data range, before being subjected to analysis of variance (using Genstat [15]).

Double inoculation experiments: established colonies removed.

Our aim here was to determine whether any effects of the first inoculation on subsequent performance of conidia and germlings arising from the second inoculation, depended: (1) upon the presence of colony hyphae derived from the first inoculation; or (2) whether the presence of haustoria within epidermal cells was sufficient to mediate these effects.

On each control leaf segment, data to assess germination and normality of germling development were gathered as described above. On leaves inoculated twice, germination was determined for 50 conidia located on epidermal cells at least four cells away from the nearest cell containing a haustorium formed by colonies arising from the first inoculum (distant cells), and 25 germlings on distant cells were scored for their developmental characteristics. Similarly where conidia of the second inoculation lay on epidermal cells containing haustoria formed by colonies of the first inoculation, germination was determined for 50 conidia and germling development was assessed for 25 germinated conidia.

As described above, mean data from each leaf segment were calculated as percentages that were transformed prior to analysis of variance.

Low temperature scanning electron microscopy (LTSEM)

To complement light microscopy and aid image interpretation, sub-samples of double inoculated leaves were examined by LTSEM. Thus, when the central 30 mm leaf segments were excised and fixed for light microscopy, the adjacent basipetal 10 mm leaf blade segment from some samples was taken for LTSEM. Following established procedure [11], these segments were attached to a copper SEM stub with colloidal graphite and frozen rapidly by introducing the stub to a pre-cooled stub holder (approx. −190°C) under an argon flush, and transferring the assembly to the pre-cooled (−190°C) stage of an Emscope SP2000A sputter-cryo system. Cryo-fixation in this way avoided ice crystal formation on specimens. The specimen was then sputter coated with gold before detailed observation on the cold stage (approx. −160°C) of a JEOL JSM 840A SEM, with 3.0 or 5.0 kV electron accelerating voltage. SEM images were recorded using a digitizer board and software (SemAfore®, JEOL) running under a Windows® operating system. The figures presented here are from digitized images enhanced using Adobe Photoshop® to maximize details of areas of interest.

RESULTS

Selma oat double-inoculated with f. sp. avenae, coverslips applied.

Data for spore germination will not be described because applying coverslips to specimens was seen to displace many ungerminated spores. Fig. 3 shows that deposition within the boundary of an established colony had a dramatic effect on germling development. On control leaves (inoculated only once, 24 h before fixation) and when germlings were distant from established colonies on double-inoculated leaves, the great majority of germlings developed normal appressoria. By contrast, when conidia were located within the boundary of an established colony, more than 70% of germlings were abnormal.
apex. When germlings were within the boundary of established colonies, they frequently made direct contact with hyphae of the established colony [Fig. 4(a), (b), (d) and (e)]. Contact could be via the conidium, the germ tubes, or both. However, abnormal germlings were formed even where no direct contact was made [Fig. 4(c) and (f)].

On controls and when germlings were distant from established colonies on double inoculated leaves, approx. 15–30% of germlings successfully penetrated leaf epidermal cells to form a haustorium [Fig. 3(b)]. By contrast, when germlings were located within the boundary of an established colony, less than 5% of germlings formed haustoria.

Experiments where specimens were viewed without coverslips, and hyphae of established colonies remained in place

Oats inoculated first with f. sp. avenae and secondly with f. sp. hordei or tritici

Germination. When spray stained specimens were mounted without coverslips to avoid displacing ungerminated conidia we felt confident that data gathered to describe spore germination were valid. Here, therefore, we considered percentage germination of conidia located on control leaves, and, where leaves bore colonies established from the first inoculation, we considered the germination of conidia deposited distant from colonies and lying within the outer and inner zones of colonies [Fig. 2(c)].

Fig. 5(a) and (b) show that when f. sp. avenae conidia were inoculated onto Maldwyn or Selma oat leaves bearing established f. sp. avenae colonies, germination rates were significantly depressed if conidia lay within the inner zone of an established colony. Here, germination percentages were approximately halved compared with locations distant from colonies or on controls inoculated only once. This was true for experiments involving both oat cvs. There was no difference between controls and locations distant from colonies on double-inoculated leaves. In both experiments, there was a tendency for conidia within the outer zone of colonies to show slightly lower germination than at distant locations or on controls, but this effect was small and significant only for the experiment with cv. Maldwyn.

When f. sp. tritici conidia were inoculated onto Maldwyn oat leaves bearing f. sp. avenae colonies, effects of location on germination by f. sp. tritici conidia were very similar [Fig. 5(c)] to those seen in Fig. 5(a) and (b). Germination frequency by conidia located within the inner zone of f. sp. avenae colonies was about half that seen for conidia distant from colonies or on controls, but germination was not depressed if conidia were within the outer zone of colonies.
For f. sp. hordei, the suppression of germination associated with deposition within f. sp. avenae colonies appeared even greater than for conidia of other spp. [Fig. 5(d)]. When f. sp. hordei conidia were deposited within the inner zone of colonies, germination was reduced by nearly 70% (to approx. 17%) compared with locations distant from the colonies (approx. 53% germination). Again, suppression of germination was less marked for conidia deposited within the outer zone of f. sp. avenae colonies, although even here a significantly lower percentage germinated than if located distant from colonies. Interestingly, there was also a small but significant reduction in germination for f. sp. hordei conidia located distant from f. sp. avenae colonies on double inoculated leaves compared with controls. This effect was not seen in any other combination.
Germling development. Fig. 6(a) and (b) show that when f. sp. *avenae* conidia germinated on Maldwyn or Selma oat leaves bearing established f. sp. *avenae* colonies, a very high percentage (>74%) developed abnormally if conidia lay within the inner zone of an established colony. As described for the first experiment (Fig. 4), abnormalities were infrequent (approx. 4–12%) for germlings on controls or located distant from colonies. They were also relatively infrequent when germinated conidia lay within the outer zone of established colonies (<18%).

When f. sp. *tritici* germlings developed on Maldwyn oat leaves bearing f. sp. *avenae* colonies, effects of location on germling development by f. sp. *tritici* [Fig. 6(c)] were very similar to those seen in Fig. 6(a) and (b). On controls and in locations distant from colonies, very few germlings developed abnormally (<7%). Within the outer zone of f. sp. *avenae* colonies, the abnormality frequency was slightly but significantly higher (approx. 16%). However, when f. sp. *tritici* conidia were within the inner colony zone, the majority of germlings developed abnormally (77%). The abnormalities were similar to those shown in Fig. 4.

As with effects on germination, the effects of germling location on the frequency of abnormal germling development appeared greater for f. sp. *hordei* than other f. sp. spp. As in other cases, when f. sp. *hordei* germlings were on control leaves or distant from f. sp. *avenae* colonies, a very low frequency of abnormality was seen (<9%, Fig. 6(d)), and when located within the inner zone of colonies abnormalities were extremely frequent (82%, Fig. 6(d)]. However, in contrast to other combinations, a relatively high abnormality frequency [approx. 49%, Fig. 6(d)] was seen when germinated f. sp. *hordei* conidia were located in the outer zone of f. sp. *avenae* colonies. These data indicated that f. sp. *hordei* was more sensitive.
to the influences of f. sp. \textit{avenae} colonies than either f. sp. \textit{avenae} itself or f. sp. \textit{tritici}.

In addition to being more sensitive to the influence of f. sp. \textit{avenae} colonies, f. sp. \textit{hordei} germlings appeared to show a greater range of morphological abnormalities (Fig. 7) than germlings of the other \textit{C\text{\textsubscript{128}}} spp. Haustorium formation. Germlings of f. sp. \textit{hordei} never formed haustoria in oat cv. Maldwyn irrespective of whether they attacked control leaves or leaves previously infected with f. sp. \textit{avenae}. Haustorium formation by f. sp. \textit{tritici} was also extremely rare; no germlings formed haustoria in control leaves or on cells distant from established f. sp. \textit{avenae} colonies. Within the outer zone of f. sp. \textit{avenae} colonies, only 1\% of f. sp. \textit{tritici} germlings formed a haustorium and within the inner zone, only 1.5\% did so.

There were no differences in the percentages of f. sp. \textit{avenae} germlings forming haustoria in epidermal cells of control leaves and cells distant from established colonies of f. sp. \textit{avenae} (Fig. 8). On Maldwyn [Fig. 8(a)], there was no difference between germlings distant from colonies and those within the outer zone of colonies, although within the inner zone of colonies significantly fewer germlings formed haustoria than in any other location. In the experiment using Selma [Fig. 8(b)], similar percentages of germlings formed haustoria in controls, in cells distant from colonies and in the inner zone of colonies. However, when germlings were located within the outer zone of colonies, a significantly higher percentage formed haustoria than in any other location.

\textit{Barley cv. Pallas} inoculated first with f. sp. \textit{hordei} and secondly with ff. spp. \textit{hordei}, \textit{avenae} or \textit{tritici}. 

\textbf{FIG. 6.} Percentages of \textit{B. graminis} ff. spp. \textit{avenae}, \textit{tritici} and \textit{hordei} conidia that germinated but formed abnormal germ tube structures (failed to form appressoria) 24 h after inoculation onto second-formed oat leaves of healthy controls, or onto leaves bearing \textit{B. graminis} f. sp. \textit{avenae} colonies derived from a first inoculation 72 h earlier. All material was fixed 24 h after second inoculation. Data are for f. sp. \textit{avenae} conidia on oat cvs Maldwyn (a) and Selma (b), and for ff. spp. \textit{tritici} (c) and \textit{hordei} (d) on cv. Maldwyn. On controls ( ), data are based on counts of 50 randomly selected germlings, while on leaves bearing colonies, counts were made for 50 germlings located distant from colonies ( ), 50 within the outer zone of colonies ( ), and 50 within the inner zone of colonies ( ), on each of four replicate leaves in all cases. Percentage data were transformed to angles for analysis of variance. Where columns are headed by different letters, LSD tests applied to angle-transformed data indicated a significant difference ($P < 0.05$) between means within a data set.
Germination. Data from barley showed trends very similar to those from oat (Fig. 9). Conidia of all f. sp. germinated well on Pallas barley control leaves, and equally well on cells distant from established colonies of f. sp. hordei. There was a slight tendency for germination to be reduced when conidia were located within the outer zone of f. sp. hordei colonies, but the reduction was small and significant only in the case of f. sp. avenae. In all cases, germination was significantly reduced where conidia were located within the inner zone of colonies. However, the degree of reduction in germination was relatively smaller than that seen within the inner zone of f. sp. avenae colonies on oat (where germination rates were at least halved; Fig. 5). The maximum reduction was seen with f. sp. avenae where on distant cells approx. 69% germinated compared to approx. 39% within the inner zone of f. sp. hordei colonies (i.e. a reduction of 43%) while there were only 32 and 31% reductions in germination of f. sp. hordei and tritici, respectively.

Germling development. Extremely low frequencies of germling abnormality were seen when conidia of any f. sp. germinated within f. sp. hordei colonies growing on barley (Fig. 10). This was in complete contrast to the situation seen when conidia germinated within colonies of f. sp. avenae growing on oat. Even when the germlings

![Fig. 7. Light micrographs showing examples of abnormal germling structures formed by B. graminis f. sp. hordei when conidia germinated within the boundary of f. sp. avenae colonies growing on Maldwyn oat leaves. Colonies were established from a first inoculation applied 72 h earlier than the second inoculation from which the germlings derive. All material was fixed 24 h after the second inoculation. (a) The elongated germ tube is a simple, hypha-like structure showing little sign of swelling or apical hooking, and two septa are evident. The first is close to the spore, at the position where the single septum forms in morphologically normal appressorial germ tubes, and the second is approximately 35 μm from the conidium i.e. approximately where the apical appressorial hook is formed by a normal appressorial germ tube. Note: three ungerminated conidia are located close to hyphae (top right of micrograph). (b) As in (a), two septa are present in the elongated germ tube. The central cell of this tube is swollen, but a further hypha-like cell has been formed. Small protruberances are evident on the central cell, the most distal of which (directed downwards) is reminiscent of a poorly developed appressorial lobe. (c) Again two septa are present in the elongated germ tube. The central cell is swollen and shows distinct protruberances, but these are clearly too small to be mistaken for appressorial lobes. The distal cell of the germ tube is slightly swollen with a club-like apex. (d) When first seen, this germling was mistakenly thought to have formed a normal appressorium with a hooked apical lobe (double arrowhead). The branch cell, separated from the hooked cell by a septum and growing left from the central portion of the hooked cell, was thought to be secondary hyphal growth. However, careful examination showed that no haustorium was present beneath the hooked structure. Therefore the cell growing left represented abnormal branching of the elongated germ tube and the hooked cell cannot be interpreted as a normal appressorial structure. Several examples of similar germlings were seen. Cu = ungerminated conidium; Hy = hypha of established colony; PGT = primary germ tube; arrowheads indicate septa in elongated germ tube; double arrowheads indicate appressorium-like structure.}
Colonies derived from a first inoculation 72 h earlier. All material was fixed 24 h after the second inoculation. On controls (inner zone of f. sp. hordei), there was a significant increase in the frequency of abnormality within the inner zone of colonies compared with controls, in abnormality frequency between germlings located distant from colonies or those located within the colony outer zone. For f. sp. avenae and tritici, there was a significant increase in the frequency of abnormality within the inner zone of f. sp. hordei colonies. However, even here only approx. 10% of germlings appeared abnormal (as compared with 70–80% abnormality for any/C128 spp. germinating within the inner zone of f. sp. tritici, respectively, Figs 12 and 13), and even if they were located within the outer zone of colonies, a few germlings formed haustoria (4 and 2%, respectively). From casual observation, it was apparent that when germlings of the inappropriate f. spp. located within a f. sp. hordei colony formed a haustorium, this haustorium was most frequently formed in an epidermal cell that also contained a haustorium of the established colony (illustrated in Fig. 13). However, data to confirm this observation were not collected.

**Haustorium formation.** When f. sp. hordei germlings attacked control barley leaves, epidermal cells distant from established colonies, or were located within the outer zone of colonies, 13–14% of appressoria were associated with successful penetration attempts as evidenced by the presence of a haustorium beneath their appressoria (Fig. 12). However, when germlings were located within the inner zone of colonies, the percentage of appressoria with haustoria was significantly increased and almost doubled in frequency (22.5%).

On controls and on cells distant from f. sp. hordei colonies, germlings of f. spp. avenae and tritici never formed haustoria (Fig. 12). By contrast, when germlings of these inappropriate f. spp. were located within the inner zone of f. sp. hordei colonies, they frequently formed a haustorium (17.5% and 14.5% for avenae and tritici, respectively, Figs 12 and 13), and even if they were located within the outer zone of colonies, they almost always formed normal appressoria (Fig. 12). However, when germlings were located within the outer zone of colonies, a few germlings formed haustoria (4 and 2%, respectively).

Removing the superficial hyphae of B. graminis f. sp. avenae colonies that had been previously allowed to develop for 96 h before re-inoculation, allowed us to determine whether effects on germination and germling development depended upon the presence of hyphae or whether the presence of haustoria within epidermal cells was sufficient to mediate these effects. Where colony hyphae were removed, haustoria formed by the colony were clearly visible, and epidermal cells containing those haustoria appeared to remain alive as evidenced by lack of whole-cell autofluorescence and absence of cytoplasmic granulation or disorganization. This was as expected from earlier studies [8, 27–29] where haustorium-containing cells also survived removal of superficial structures. Cells containing colony haustoria obviously laid within the outer boundary of colonies as defined earlier by an imaginary line linking tips of leader hyphae. Furthermore, from our observations and Hirata’s [18]
Inhibition of *B. graminis* development

**Fig. 9.** Percentages of *B. graminis* ff. spp. *hordei* (a), *avenae* (b) and *tritici* (c) conidia that had germinated 24 h after inoculation onto second-formed cv. Pallas barley leaves of healthy controls, or onto leaves bearing *B. graminis* f. sp. *hordei* colonies derived from a first inoculation 72 h earlier. All material was fixed 24 h after the second inoculation. On controls (■), data are based on counts of 100 randomly selected conidia, while on leaves bearing colonies, counts were made for 100 conidia located distant from colonies (■), 100 within the outer zone of colonies (■), and 100 within the inner zone of colonies (■), on each of four replicate leaves in all cases. Percentage data were transformed to angles for analysis of variance. Where columns are headed by different letters, LSD tests applied to angle-transformed data indicated a significant difference (*P* < 0.05) between means within a data set.

**Fig. 10.** Percentages of *B. graminis* ff. spp. *hordei* (a), *avenae* (b) and *tritici* (c) conidia that germinated but formed abnormal germ tube structures (failed to form appressoria) 24 h after inoculation onto second-formed cv. Pallas barley leaves of healthy controls, or onto leaves bearing *B. graminis* f. sp. *hordei* colonies derived from a first inoculation 72 h earlier. All material was fixed 24 h after the second inoculation. On controls (■), data are based on counts of 50 randomly selected germlings, while on leaves bearing colonies, counts were made for 50 germlings located distant from colonies (■), 50 within the outer zone of colonies (■), and 50 within the inner zone of colonies (■), on each of four replicate leaves in all cases. Percentage data were transformed to angles for analysis of variance. Where columns are headed by different letters, LSD tests applied to angle-transformed data indicated a significant difference (*P* < 0.05) between means within a data set.
detailed studies, it is most likely that haustorium-containing cells lay within the inner zone of colonies whose hyphae had been removed. On the other hand, epidermal cells four cells distant from haustorium-containing cells probably lay outside (for acropetal and basipetal cells), or only just within (for lateral cells), the boundaries of colonies that had been removed. Thus, these epidermal cells were regarded as comparable to cells previously defined as distant from colonies.

Germination and germling development. Overall, slightly but significantly \( (P < 0.05) \) fewer conidia germinated on control leaves (approx. 63 %) than on double inoculated leaves. However, unlike experiments where colony hyphae were left in place, there was no difference in germination between conidia distant from cells containing colony haustoria (germination = approx. 68 %), and conidia overlying epidermal cells containing colony haustoria (germination = approx. 69 %). There was a significant \( (P < 0.001) \) difference in germination between the three fungal \( \text{f.spp.} \), with \( \text{f.spp. hordei} \) conidia germinating slightly less well (approx. 62 %) than either \( \text{f.spp. avenae} \) (approx. 70 %) or \( \text{tritici} \) (approx. 68 %). These small differences presumably reflect uncontrolled variation in inoculum quality between \( \text{f.spp.} \). However, there was no interaction between conidium location and \( \text{f.spp.} \) indicating consistency of these small effects within the experiment.

For germinated conidia, there was only a very low frequency of germling abnormality in all locations and most germlings formed apparently normal appressoria. For all \( \text{f.spp.} \) and all locations, abnormality was never seen for more than 6 % of germlings even if the germlings were in contact with epidermal cells containing haustoria formed by \( \text{f.spp. avenae} \) colonies that had been removed.

Thus, in complete contrast to experiments where colonies were undisturbed, the location of conidia on epidermal cells that had been within the boundaries of colonies established by the first inoculum had no effect on either their germination or on subsequent germling development.

Haustorium formation. Although location had no effect on appressorium formation, it had a dramatic effect on the percentage of germlings that penetrated epidermal cells successfully to produce haustoria (Fig. 14). When \( \text{f.spp. avenae} \) germlings attacked control leaves or epidermal cells distant from cells containing a haustorium formed earlier by an \( \text{f.spp. avenae} \) colony, approx. 42 % formed haustoria. However, when attack was on a cell containing a haustorium formed earlier, 92 % did so. Thus, cells that contained colony haustoria were conditioned to a high level of “accessibility” to subsequent attack by \( \text{f.spp. avenae} \).

The presence of \( \text{f.spp. avenae} \) colony haustoria in epidermal cells of oat also conditioned them to a high level of accessibility to subsequent attack by germlings of \( \text{f.spp. tritici} \) and \( \text{hordei} \). Thus, 90 % of \( \text{f.spp. tritici} \) and 58 % of \( \text{f.spp. hordei} \) germlings penetrated successfully and formed haustoria when attacked cells contained \( \text{f.spp. avenae} \) colony haustoria. By contrast, neither of these inappropriate \( \text{f.spp.} \) formed haustoria in control oat leaves or in cells distant from cells containing \( \text{f.spp. avenae} \) colony haustoria.

DISCUSSION

We were very surprised by our initial finding from Selma oat that germination and germling development were markedly and adversely affected when \( B. graminis \) conidia...
Inhibition of *B. graminis* development

![Diagram](image)

**Fig. 13.** Light micrograph of two *B. graminis* f. sp. *tritici* germlings located within the inner zone of a *B. graminis* f. sp. *hordei* colony. The colony was established from a f. sp. *hordei* conidium deposited 72 h earlier than the f. sp. *tritici* conidia. The specimen was fixed 24 h after deposition of the f. sp. *tritici* conidium. Both f. sp. *tritici* germlings produced normal appressoria from which haustoria were formed. Apph = primary appressorium of f. sp. *hordei* colony; Appt = appressorium of f. sp. *tritici* germling; Ch = conidium of f. sp. *hordei*, Ct = conidium of f. sp. *tritici*. Hh = primary haustorium of f. sp. *hordei* colony; Hat = primary haustorium of f. sp. *tritici* germling.

were deposited within established oat mildew colonies. This was because a considerable body of evidence indicates that deposition of *B. graminis* conidia, and conidia of other powdery mildew fungi, close to an established *B. graminis* colony can greatly enhance the chances of these conidia producing successful infections.

This evidence comes from investigations of wheat and barley infected with their appropriate f. sp. and subsequently inoculated with an inappropriate f. sp. of *B. graminis* or with less closely related *Erysiphaceae*. Thus Moseman and his co-workers [30, 31] found that wheat could be attacked by *B. graminis* f. sp. *hordei* if leaves were already infected with a virulent isolate of *B. graminis* f. sp. *tritici* or if the two fungi were inoculated simultaneously. Tsuchiya and Hirata [37] inoculated *B. graminis* f. sp. *hordei*-infected barley with f. sp. *tritici*, a *B. graminis* isolate from *Agropyron*, or with powdery mildew fungi from 49 different dicotyledonous species; the fungi included species from the genera *Erysiphe*, *Sphaerotheca*, *Podosphaera*, *Microsphaera* and *Uncinula*. Of the 51 fungi tested, 45 were able to infect the barley and 30 formed sporulating colonies. Tsuchiya and Hirata [37] noted that these infections almost invariably occurred close to established f. sp. *hordei* colonies and detailed histological studies of the *B. graminis* isolate from *Agropyron* and of *Sphaerotheca fuliginea* showed that successful infections by these fungi were almost invariably established in barley epidermal cells containing *B. graminis* f. sp. *hordei* haustoria or in neighbouring cells. Similarly, Ouchi et al. [33] concluded that induced ‘accessibility’ of barley to attack by wheat...
mildew and S. fuliginea was a phenomenon expressed locally in the immediate vicinity of established barley mildew colonies. More recent and detailed studies by Kunoh and his co-workers [22, 24, 25] again showed that induced accessibility of barley coleoptile cells to attack by the non pathogen Erysiphe pisi, is an extremely localized effect dependent on prior or near simultaneous infection by B. graminis f. sp. hordei. Thus, many studies have shown that barley and wheat can be rendered susceptible to powdery mildew fungi that are normally unable to infect them, if they are previously infected by a pathogenic powdery mildew fungus. However, this depends upon the normally non-pathogenic fungus being in close proximity to established infections of the pathogenic B. graminis. This in turn clearly suggests that proximity to an established B. graminis colony does not greatly impede germination or development of later-applied propagules and in fact favours their ability to infect. Hence, we were surprised to find that this was not the case when B. graminis conidia were deposited within oat mildew colonies.

Nevertheless, our observations of the behaviour of three different B. graminis f. f. spp. deposited within the inner zone of established barley mildew colonies were broadly in line with expectation. Although germination by conidia of all three f. f. spp. was somewhat depressed within these colonies, and the frequency of abnormal long germ tubes was slightly increased within the inner colony zone, the majority of germinated conidia differentiated morphologically normal appressoria. The functionality of these appressoria was demonstrated by the fact that a higher percentage were associated with successful penetration and haustorium formation than where conidia were deposited on healthy leaves, or on leaves that had supported colonies. On controls (■) data are based on counts of 50 randomly selected germlings, while on leaves that had supported colonies data are based on counts of 25 germlings whose appressoria were on epidermal cells that were distant from cells containing haustoria formed earlier by colonies (■), and 25 germlings with appressoria overlying cells containing haustoria formed earlier by colonies (■), on each of four replicate leaves in each case. Data for f. f. spp. triïci and hordei on healthy oat control leaves and on epidermal cells distant from haustorium-containing epidermal cells were zero in all cases (not shown).

When conidia of all three f. f. spp. were deposited within established oat mildew colonies germination percentages were depressed. This was similar to, but even more marked than the situation seen within barley mildew colonies. We know from previous work [8] that the

**FIG. 14.** Percentages of B. graminis f. sp. avenae, triïci and hordei germlings that formed appressoria and penetrated Selma oat epidermal cells to form a haustorium 36 h after inoculation. Data are for germlings on healthy control leaves, or on leaves that had supported f. sp. avenae colonies derived from a first inoculation applied 96 h before test conidia were inoculated, but from which all superficial fungal structures (conidia, hyphae) were removed immediately before inoculation with test conidia. On controls (■) data are based on counts of 50 randomly selected germlings, while on leaves that had supported colonies data are based on counts of 25 germlings whose appressoria were on epidermal cells that were distant from cells containing haustoria formed earlier by colonies (■), and 25 germlings with appressoria overlying cells containing haustoria formed earlier by colonies (■), on each of four replicate leaves in each case. Data for f. f. spp. triïci and hordei on healthy oat control leaves and on epidermal cells distant from haustorium-containing epidermal cells were zero in all cases (not shown).
presence of a haustorium within an oat epidermal cell should have induced accessibility to subsequent attack by *B. graminis* f. sp. *avenae*. However, we could not be sure from our observations of conidia deposited within established oat mildew colonies whether the plant cells had been affected. This was because very few germinated conidia produced morphologically normal appressoria, and even where appressorial germ tubes formed an apparently normal, hooked apical lobe, we could not be sure if the structure was functional. Thus, very few germings were capable of attempting infection and this alone could explain the very low frequency of haustorium formation seen within oat mildew colonies.

Nevertheless, there was some evidence from Selma oat (Fig. 8) that accessibility to *B. graminis* f. sp. *avenae* may have been induced within the outer zone of established colonies, and some evidence of induced accessibility to f. sp. *tritici* within oat mildew colonies established on Maldwyn oat. However, clear evidence for induced accessibility in oat epidermal cells was provided only when we removed the superficial structures of the oat mildew colonies prior to applying the second inoculum. Thus, when germings of all three f. sp. attacked epidermal cells containing a haustorium formed earlier by a oat mildew colony, a substantial proportion were successful in penetrating the cells and establishing a functional haustorium. For f. sp. *avenae*, this was a quantitative effect where the frequency of successful penetration was increased relative to control leaves or epidermal cells distant from haustorium-containing cells. For f. sp. *tritici* and *hordei* the effect was qualitative in the sense that these fungi were virtually unable to infect oat cells that did not contain a f. sp. *avenae* haustorium.

The available evidence clearly shows that prior infection of cereal epidermal cells by *B. graminis* has dramatic, albeit localized, consequences for the ability of cells to resist subsequent attack by the fungus. These effects affect not only the outcome of attacks by compatible isolates of the appropriate f. sp., but extend to permit invasion of cells by inappropriate f. spp. and non pathogenic species. The basis of induced changes in accessibility remain unknown.

At present, we have no idea what causes suppression of germination by conidia deposited within established powdery mildew colonies. However, it seems that similar factors operate in colonies of both barley and oat powdery mildew, and the factors were effective against all three f. sp. that we tested. It is a truisim that powdery mildew conidia do not germinate while they are within the developing spore chain attached to the mother conidiophore, but that they germinate rapidly after release. It seems that the same factor(s) suppress germination by conidia deposited within an established colony. The factor(s) are not particularly mobile as the suppressive effect was usually greater when conidia lay within the colony inner zone. The inner zone recognized at the time of fixation probably represented (approximately) the limits of hyphal development extant at the time when second inoculations were made (Fig. 2) suggesting that conidia were close to, or in contact with, hyphae at the time of their deposition. It is not clear however, whether spores that failed to germinate were actively and rapidly killed by factors released by the colony or whether they survived for some time (perhaps until fixation) although processes leading to germ tube emergence were inhibited. Further studies should be able to distinguish these possibilities and in turn shed light on the likelihood that similar factor(s) suppress the germination of conidia attached to the spore chain which obviously can remain alive for some time before release.

Abnormal germling development was extremely frequent when conidia of all *B. graminis* f. sp. germinated within oat mildew colonies. By comparison, abnormalities were rarely seen within barley mildew colonies, even where germings of the second inoculum were in very close contact with hyphae (Fig. 11). The latter finding appears to contradict Hirata’s [18] observations of the barley mildew system. Hirata [18] stated that . . . “when (barley) leaves bearing old (barley) mildew colonies are inoculated (with *B. graminis* f. sp. *hordei* conidia), conidia that fall on or near the peripheral part of the colonies germinate and develop long septate germ tubes. The germ tube does not generally form an appressorium and the primary haustorium”. Hirata’s drawings of these germ tubes resemble the abnormal germings we saw within the inner zone of oat mildew colonies. However, Hirata did not quantify the frequency of this occurrence, and he did not indicate exactly the age of the colonies involved in his observations. It is possible that the ability of powdery mildew colonies to generate/release chemicals that cause abnormal germling development is a function of their age. Thus, although our oat mildew colonies had obviously released the factor(s) within 4 days of inoculation, barley mildew colonies of this age had not done so. If we had incubated our barley mildew colonies for longer before we applied the second inoculum, we may have detected effects on germling development.

Hirata [18] also found that anastomosis occasionally occurred when abnormal long germ tubes encountered hyphae. We never saw this. He went on to show that such anastomosis could occur between conidial germ tubes of powdery mildew from *Agropyron* (a non-pathogen of barley) and hyphae of established barley mildew colonies. From Tsuchiya and Hirata’s [37] further discussion of these studies, it appears that following anastomosis with barley powdery mildew, the powdery mildew from *Agropyron* derived nutrients from the barley mildew colony. This allowed the *Agropyron* fungus to develop hyphae, conidiophores and conidia capable of re-infecting *Agropyron*. Clearly, Hirata and his co-workers’
studies have important implications with respect to the intimacy of genetic and physiologic association resulting from anastomoses formed between fungal individuals both within and between ff. spp. of *B. graminis* and possibly between genera of the *Erysiphales*. There is immense scope to pursue these studies.

Presumably the effective factor(s) produced by oat mildew colonies that caused abnormal germling development in our experiments must be different from those that suppress germination. This conclusion arises from our finding that germination frequency was consistently reduced within colonies of both f. sp. *hordei* and f. sp. *avenae*, whereas our barley powdery mildew colonies had little if any detectable effect on germling development. Again, however, we have no idea what factor(s) may cause abnormal germling development. We believe the factor(s) has limited mobility, because, as with suppression of germination, greater effect was evident within the inner than the outer colony zone. Nevertheless, direct contact with a hypha was not necessary for abnormal germling development. The factor's major effect was to inhibit the later stages of appressorial germ tube differentiation. Thus, elongating germ tubes remained hypha-like, failed to swell and differentiate a hooked apical lobe, and often developed peculiar branches and multiple septa. Such abnormalities could result from failure of the elongating tube itself to recognize plant surface features [4–7]. The factor's major effect was to inhibit the later stages of appressorial germ tube differentiation. Thus, elongating germ tubes remained hypha-like, failed to swell and differentiate a hooked apical lobe, and often developed peculiar branches and multiple septa. Such abnormalities could result from failure of the elongating tube itself to recognize plant surface features [4–7]. This seems unlikely, however, because the effective factor(s) did not affect the PGT's ability to recognize such features. Alternatively, it may be that the oat mildew colonies release factor(s) that block intracellular signaling or signal transduction pathways that regulate appressorial formation. Temporal regulation of such pathways is known to be associated with maturation and hooking of the appressorial germ tube; pharmacological inhibitors of these pathways can prevent appressorium formation and lead to the development of undifferentiated, hypha-like elongated germ tubes [16, 17, 19]. Perhaps, our oat powdery mildew colonies produced chemicals with similar effects. Whatever the explanation, it is intriguing that the effective factor(s) disrupt germling development but are presumably harmless to the established colony.

Our experiments suggest strongly that the factors suppressing *B. graminis* germination and causing abnormal germling development were directly associated with presence of oat mildew hyphae, and are not due to indirect effects mediated via modification of the host oat plant's physiological or biochemical status. Thus, when superficial hyphae were removed from oat leaves, spores deposited on haustorium-containing cells germinated and normal, functional appressoria developed with at least the same frequency as on healthy leaves. The induced accessibility of haustorium-containing cells showed that they remained radically altered, at least in terms of their accessibility, by the presence of pre-formed haustoria. If factors inhibitory to germination and germling development were generated within haustorium-containing cells, it seems highly likely that removal of the epicuticular wax layer (that inevitably occurs during cellulose acetate stripping [7, 10, 11, 27]) would have facilitated their movement to the cell surface and increased inhibitory effects. This was clearly not so. These findings again appear to contradict Hirata's [18] observations. Hirata found that abnormal germlings often developed when barley mildew conidia were inoculated onto a leaf from which older colonies had previously been “scraped” away. We assume that “scraping” involved a physical procedure that could have ruptured the cell walls of superficial fungal structures (hyphae, conidiophores, etc.) releasing their cell contents onto the leaf surface prior to application of test conidia. If the factor(s) that cause germling abnormality are generated within cells of the colony, these may well have been released to remain as residue on the leaf surface following “scraping” in Hirata's experiment. Our technique would have removed all such residues along with the leaf epicuticular wax. We suggest this explains the apparent difference between our observations and Hirata's.

Our current work indicates that when *B. graminis* conidia are deposited within an established colony their germination is suppressed. Our experiments and Hirata's seminal studies show that where conidia do germinate within a colony, their subsequent development can be disrupted so that they do not form appressoria or haustoria, and are therefore incapable of independent existence and reproduction. Why should established colonies produce factors that inhibit germination and disrupt germling development? In situations with genetically diverse host and pathogen populations, there could be obvious selective advantages for an established colony in preventing potential competitors from infecting nearby plant epidermal cells which have been rendered highly accessible to penetration by the presence of haustoria formed by the established colony. These competitors may be less-well adapted to parasitize a particular plant genotype, and may even be avirulent. If this were so, avirulent individuals could kill attacked cells and deprive the adapted colony of potential feeding sites. On the other hand, some conidia produced by a colony are likely to be deposited close to that colony; suppressing their development would prevent competition for nutrients in finite local supply within a niche already occupied by that...
pathogen genotype. Similar effects are likely to apply within agricultural crops although their selective advantage may be less marked. According to Hirata’s observations, conidia that germinate and form abnormal germ tubes at the periphery of an established colony, may be able to form anastomoses with hyphae of that colony and, in effect, parasitize the colony by using its resources. It remains to be determined how frequently this happens, but it seems unlikely that the frequency of anastomosis compensates in any significant way for the suppressive effects established colonies have on germination and appressorium formation by potential competitors.

Currently we are attempting to isolate the factors responsible for inhibiting spore germination and germling development within oat mildew colonies. It is conceivable that this may reveal novel means of disease control.

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