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Role of external signals in regulating the pre-penetration phase of infection by the rice blast fungus, *Magnaporthe grisea*

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Abstract. The role of external signals (particularly the substratum surface and light) in regulating the pre-penetration phase of *Magnaporthe grisea* (Herbert) Barr [anamorph, *Pyricularia grisea* Sacc.] were analysed on rice (*Oryza sativa* L.) leaves, artificial substrata and in liquid suspension. Surface contact was found to be essential for appressorium induction but not conidium germination. Both a high surface hydrophobicity and light favoured the formation of short differentiated germ tubes and large numbers of appressoria, but neither factor was essential for their induction. Light intensity had a graded effect on the lengths of differentiated germ tubes but not on the number of appressoria formed. Higher numbers of appressoria differentiated on rice leaves than on artificial substrata suggesting that the host provides additional factors, and thus a more conducive environment, for promoting appressorium formation. Our study indicates that the pre-penetration phase of rice blast infection involves a programme of growth and differentiation triggered at conidium germination and regulated by multiple signals from the host and environment. No evidence was found for a single, external signal which initiates appressorium formation. Starvation is suggested as providing the necessary intracellular signal.

Key words: Appressorium – Contact sensing – Infection structure – *Magnaporthe* – *Oryza* – Rice blast

Introduction

Rice blast is considered the most important disease of rice in the world (Ou 1980, 1985). The causative agent, *Magnaporthe grisea* (Herbert) Barr [anamorph, *Pyricularia grisea* Sacc.], offers an excellent experimental system in which to address many of the most significant questions in plant pathology. In recent years, studies of the cell biology and molecular genetics of this fungus have permitted a number of critical elements of host-pathogen interactions to be analysed (Valent 1990; Valent and Chumley 1991; Dobinson and Hamer 1992). One important aspect of study has been the pre-penetration phase of infection.

The initial stages of infection by *M. grisea* usually require the deposition and attachment of three-celled conidia to aerial parts of the rice plant. Attachment typically involves the immediate and persistent adhesion of a conidium to the rice leaf by means of a glue (the *spore tip mucilage*) released from the spore apex. The spore tip mucilage will also stick strongly to various artificial surfaces, including 'non-stick' Teflon (Hamer et al. 1988). Germination of the conidium can occur from any of its three cells and results in one or more germ tubes which, after a period of growth, can differentiate terminal, pigmented appressoria (Peng and Shishiyama 1988; Bourett and Howard 1990; Howard et al. 1991b). The appressorium is even more tenaciously adherent to the host surface than are conidia, and generates a hydrostatic pressure (> 80 bar) of unprecedented magnitude in living eukaryotic cells. This pressure provides a mechanical force which seems to be the primary mechanism by which an emergent penetration peg pushes through the rice leaf cuticle (Howard and Ferrari 1989; Howard et al. 1991a).

The pre-penetration phase of infection by *M. grisea* involves differential gene expression. Three genes, differentially expressed during appressorium formation, have been cloned (Lee and Dean 1993b; Talbot et al. 1993) and one encodes a hydrophobin-like protein (Talbot et al. 1993). Other genes involved in appressorium morphogenesis and pigmentation have also been identified by mutant analysis (Woloshuk et al. 1983; Hamer et al. 1989; Howard and Ferrari 1989; Chumley and Valent 1990). However, little is known about how these different genes are regulated by signals from the host, environment or from within the fungus itself. Nevertheless, recent evidence suggests that cAMP, but not Ca^{2+}, plays an important role as an intracellular signal in mediating appressorium formation (Lee and Dean 1993a).

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A useful approach to the experimental analysis of signals which influence the initial stages of infection is to study the process in vitro. In *M. grisea*, the pre-penetration phase readily occurs on artificial substrata in drops of water (e.g. see Bourett and Howard 1990). In this way, one can avoid the complex influences of the host which, itself, may also be affected by the same environmental factors that directly affect the fungus.

Two important factors which can influence pathogenesis are light and the physical nature of the host surface (Emmett and Parbery 1975; Hoch and Staples 1991; Read et al. 1992). However, the role of neither of these factors has been the subject of rigorous analysis during the pre-penetration phase of *M. grisea*. Nevertheless, it is well established that successful infection of rice plants can take place in either the light or dark (Ou 1985). Furthermore, appressoria have been shown to differentiate on a wide range of artificial substrata with marked differences in their hydrophobicity, hardness and other surface properties (Araki and Miyagi 1977; Uchiyama et al. 1979; Yaegashi et al. 1987; Bourett and Howard 1990; Uchiyama and Okuyama 1990; Howard et al. 1991a; Lee and Dean 1993a,b).

The aim of the present study was to determine the roles of external signals (particularly the substratum surface and light) in regulating the pre-penetration phase of infection by *M. grisea*. For this purpose, we have quantified conidium germination, germination patterns, germ-tube lengths, and appressorium formation in the fungus under light and dark conditions on rice leaves, cellophane, Teflon and glass, and in liquid suspension. Our study indicates that appressorium induction occurs within a conducive environment providing multiple regulatory signals rather than being exclusively stimulated by a single external signal.

Materials and methods

Organism and growth conditions. Magnaporthe grisea, strain 0–42 (kindly supplied by Dr. Barbara Valent, DuPont Co., Wilmington, Del., USA) was bulked up and stored as described by Valent et al. (1991). For experimentation, oatmeal-agar plates (1.5% agar containing 50 g per litre of Scottish porridge oat flakes) were inoculated with small pieces of filter paper bearing the fungus (Valent et al. 1991) and incubated at 25° C for 18–24 d under continuous light from fluorescent lamps at 45 μmol photons·m^{-2}·s^{-1} in a cooled incubator. Conidia were isolated as described by Bourett and Howard (1990). The concentration of conidia in suspension was adjusted to $1 \cdot 10^4$–$2 \cdot 10^4$·ml^{-1} in sterile, double-distilled water. At conidial concentrations higher than this, it was not possible to determine accurately the percentage conidium germination and appressorium formation. Illumination was minimised during conidial isolation by wrapping the centrifuge tubes containing conidia in aluminium foil. All conidia produced by this isolation procedure lacked spore tip mucilage.

In-vitro system on solid substrata. For all in vitro experiments, unused glass slides (Blue Star; Chance Propper, Warley, UK) were washed in Fairy Liquid detergent (Procter and Gamble, Newcastle upon Tyne, UK) and rinsed thoroughly with double-distilled water. Three silicon gaskets (Swinnex-13; Millipore, Bedford, Mass., USA), covered in melted dental wax (Anutex; Associated Dental Products, Swindon, UK), were applied to the slides to which they adhered once the wax cooled. The gasket rings prevented run-off of the inoculum drop during subsequent manipulations. To minimise microbial contamination during experimentation, the slides with gaskets were treated with UV-A irradiation for at least 4 h prior to inoculation. For experiments on cellophane (gauge 525, uncoated 'rayophane' from A.A. Packaging, Walmer Bridge, Lancs., UK) or Teflon (polytetrafluoroethylene from DuPont Co., Wilmington, Del., USA), 10-mm-diameter circles of the substrata were cut using a cork borer. After autoclaving, the circles were placed within the silicon gaskets. Experiments on glass were performed directly on the slides. A 20-μl conidium suspension was applied to the substratum and the slides placed in sterile, 12 cm·12 cm square Petri dishes. To maintain high humidity, sterile tissue paper, saturated with sterile water, was placed in each dish which was then sealed with Parafilm. Except where stated, the fungal samples were grown for 24 h and incubated at 25° C under continuous light from fluorescent tubes at 45 μmol photons·m^{-2}·s^{-1} in a cooled incubator. Dark-grown samples were covered in light-tight bags normally used to store photographic paper. To determine the effects of different light intensities, Petri dishes were wrapped in polyester neutral density filters (Lee Lighting, Glasgow, UK) of different grades (50%, 25% and 15% transmission). Temperature measurements with a digital thermometer showed that the temperatures within sealed Petri dishes grown in the light and dark, and within the incubator, were the same (± 0.5° C).

In-vitro system in liquid suspension. In order to analyse spore germination in liquid suspension, hanging-drop cultures were prepared. This involved adding 20-μl drops of spore suspension to glass slides, immediately inverting the slides, and then incubating them on a support over damp tissue paper in Petri dishes (as described in the previous section). Spore germination and appressorium formation in the hanging drops were assessed after 24 h using an Nikon Diaphot (Tokyo, Japan) inverted microscope.

Fixation and quantitative analysis. After 24 h (48 h for the temperature experiments), the silicon gaskets were removed and each sample immobilised and fixed by applying a molten drop of 20 μl 2% agarose (Type VIII; Sigma, Dorset, UK) containing 5% glutaraldehyde (Sigma) at 50° C. For the time-course experiments, samples were immobilised and fixed at the times indicated. Percentage conidium germination, germination patterns and appressorium formation were assessed using a Reichert-Jung Polyvar Photomicroscope with a ×10 plan apo objective and a total magnification of ×125. Germ-tube lengths were measured using a Bio-Rad (Watford, Herts., UK) MRC-600 laser scanning confocal system mounted on an Nikon Diaphot inverted microscope, and the images analysed with CoMOS (Bio-Rad) software. Unless stated otherwise, six replicates were performed for each experiment which was done at least twice. All conidia (100–300) were counted in each replicate. Percentage appressorium differentiation was taken as the percentage of germinated conidia (sporelings) that were differentiated. An appressorium was regarded as a rounded, pigmented, terminal swelling of a germ tube (Fig. 1). Statistical analyses were performed using either Fig P for Windows v. 1.0 (Biosoft, Cambridge, UK) or Microsoft Excel v. 4.0. Statistical tests (*t*-test and analysis of variance) were done after arcsine transformation.

Plant growth conditions and infection. Rice (*Oryza sativa* L.) plants (M-201; California Cooperative Rice Research Foundation Inc.) were grown in standard soil (Fisons, Type M3) and watered with Hoagland's nutrient solution (Hoagland and Arnon 1950). Plants were grown under a 14-h photoperiod at 27° C in the light (300 μmol photons·m^{-2}·s^{-1}) and 21° C in the dark. Plants were transferred to a cooled incubator 14–18 days after seed germination. The third or fourth leaf of each plant was placed in a Petri dish (50 mm diameter) by guiding it through small openings cut in the dishes. Care was taken not to damage the leaf or touch the leaf surface to be studied. Damp filter paper was placed in the base of the Petri dish to maintain a high humidity. Prior to inoculation, the leaves were left for at least 8 h in open Petri dishes, after which 8-μl drops of conidial suspension were pipetted onto the upper surfaces of

leaves. The Petri dish lids were then replaced. For dark samples, the Petri dishes were wrapped in black photographic bags in such a way that they were light-tight. Incubation conditions were as described for the in-vitro system.

Staining of infected leaves. Leaves were removed and sprayed with 0.05% Calcofluor M2R (Sigma, Poole, Dorset, UK) 24 h after inoculation. Quantitation of germination patterns and appressorium formation was performed immediately afterwards by fluorescence microscopy, using the U1 filter block (containing a 330- to 380-nm excitation filter, 420-nm dichroic mirror, and 418-nm-long path barrier filter) of the Reichert-Jung (Slough, UK) Polyvar microscope. Germ-tube lengths were measured using CoMOS image analysis software.

Results

Conidium germination. The mean percentage of conidium germination, on the different substrata and in hanging drops, ranged from 92.8 ± 3.5 (SE) to 98.3 ± 1.3 (SE) in the light and dark. In the hanging drops, it was notable that after 24 h none of the germ tubes differentiated appressoria unless they made contact with the glass surfaces from which the drops were suspended.

Germination and differentiation patterns. With the exception of the lengths of germ tubes (see below), the general appearance of germinated conidia bearing appressoria was very similar on rice leaves and artificial substrata. Mature appressoria were pigmented and typically circular in profile (Figs. 1, 2). Sometimes, however, the appressoria on rice leaves were irregular in outline (not shown) because they tend to mould themselves around papillae on the leaf surface (see Hirooka et al. 1982).

Four patterns of germination and differentiation were representative of > 90% of all patterns exhibited by sporelings (Fig. 3). The relative proportion of sporelings within each of these four classes was broadly the same on all substrata. Conidia germinated from one or more of their three cells although < 2% germinated from their middle cells. Greater than 70% of sporelings possessed

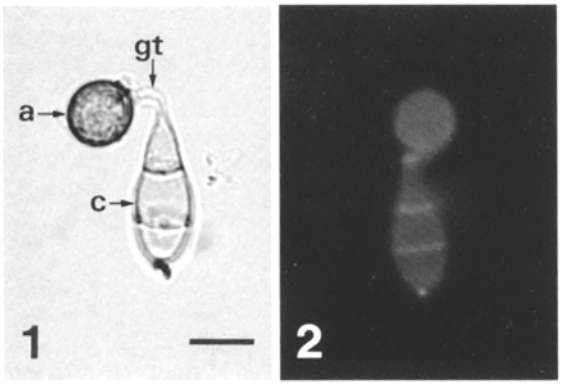

Fig. 1. Brightfield light micrograph of a germinated conidium of *Magnaporthe grisea* (*c*) from which a germ tube (*gt*) has differentiated an appressorium (*a*) on Teflon. Bar = 10 μm

Fig. 2. Fluorescence light micrograph of a germinated conidium from which a germ tube has differentiated an appressorium on a rice leaf. Magnification same as Fig. 1

Fig. 3. Differentiation patterns of germinated *M. grisea* conidia on rice leaves, cellophane, Teflon and glass in the light (□) and dark (■) after 24 h. Bars represent SEs

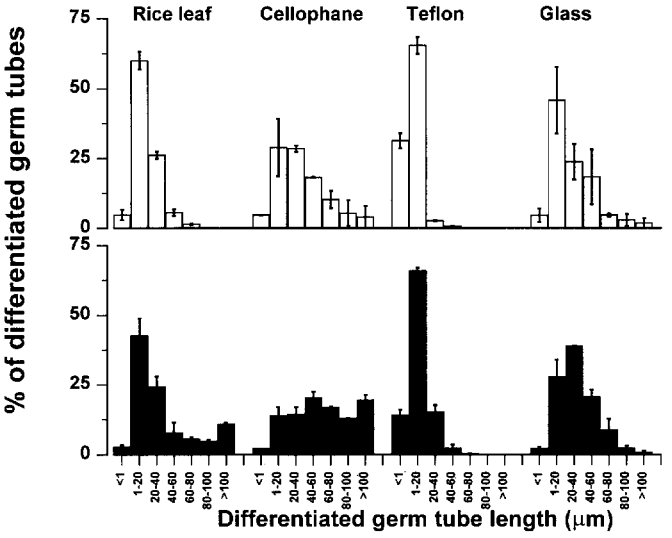

Fig. 4. Percentage of differentiated *M. grisea* germ tubes in defined length classes, on rice leaves, cellophane, Teflon and glass in the light (□) and dark (■) after 24 h. Bars represent SEs

one germ tube only; the most common type had one differentiated germ tube which had emerged from the apical cell of the conidium. The differentiation patterns did not differ significantly ($P < 0.05$) if conidia were incubated in the light or dark.

Lengths of differentiated germ tubes. On the rice leaf, germ tubes with appressoria showed a marked tendency to be shorter in the light than the dark: 97% and 77% of the germ tubes were < 60 μm long in the light and dark, respectively (Fig. 4). Of the artificial substrata, only differentiated germ tubes on cellophane were significantly shorter in the light (80% < 60 μm long) than the dark (51% < 60 μm long). On Teflon, 100% and 97% of germ tubes were < 60 μm in the light and dark, respectively; on glass, 93% and 90% were < 60 μm in the light and dark, respectively.

Appressorium differentiation. Table 1 shows that on rice leaves 98% of germinated conidia differentiated appressoria in the light whilst fewer (92%) did in the dark. On

Table 1. Percentage differentiation of *M. grisea* appressoria on rice leaves, cellophane, Teflon and glass in the light and dark after 24 h

	Rice Leaf	Cellophane	Teflon	Glass	Means
Light	98.0	79.9	91.2	69.0	84.5
Dark	91.5	54.5	68.1	39.0	63.3
Means	94.7	67.2	79.6	54.0	1% LSD 1.9

Table 2. Influence of photon flux density on percentage differentiation of *M. grisea* appressoria after 24 h incubation on cellophane

	Photon flux density ($\mu mol \cdot m^{-2} \cdot s^{-1}$)					1% LSD
	110	60	30	15	0	
% Appressorium Differentiation	87.0	85.7	86.4	80.9	58.9	3.6

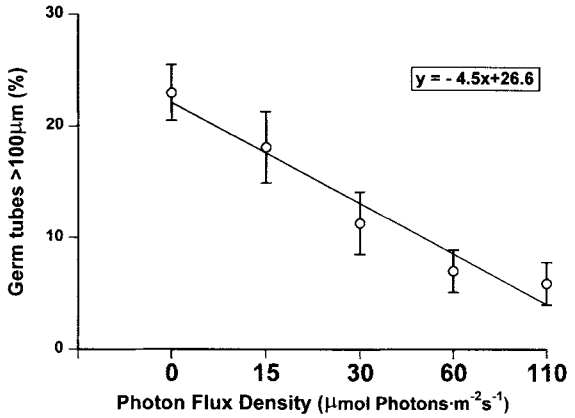

Fig. 5. Influence of photon flux density on the percentage differentiated of *M. grisea* germ tubes > 100 µm long after 24 h incubation on cellophane. Bars represent SEs of nine replicates per treatment. The line was fitted by regression analysis

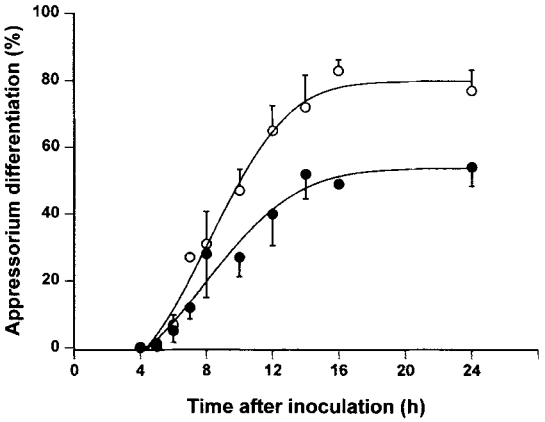

Fig. 6. Time course of differentiation of *M. grisea* appressoria in the light (○) and dark (●). Bars represent SEs

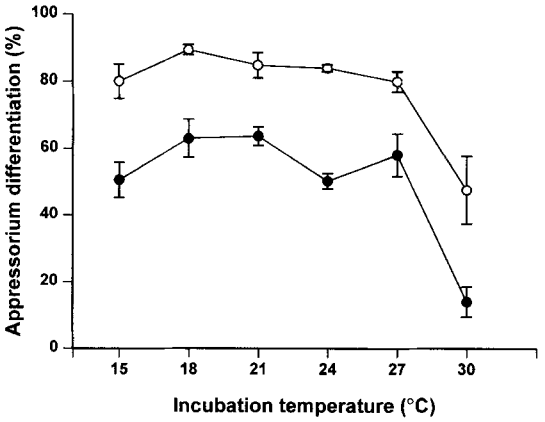

Fig. 7. Differentiation of *M. grisea* appressoria on cellophane at different temperatures after 24 h in the light (○) or dark (●). Bars represent SEs

the artificial substrata, differentiation was ⩾ 69% in the light and 20–30% lower in the dark. On each substratum, the percentage differentiation in the light was significantly greater than in the dark ($P < 0.01$). Comparisons of all the light-grown treatments, or all of the dark-grown treatments, showed that percentage differentiation on rice leaves was significantly higher ($P < 0.02$) than on any of the artificial substrata. The results were subjected to a two-way analysis of variance to detect significance of difference between the effects of the light/dark treatments and the type of substratum used. The analysis showed a significant influence ($P < 0.01$) of light and the substratum type, but no significant interaction between these factors, on the percentage of appressoria formed.

Influence of irradiance. Of the three artificial substrata tested, only cellophane resembled rice leaves by inducing the fungus to produce shorter differentiated germ tubes in the light than the dark (Fig. 4). It was, therefore, chosen as a substratum to investigate the effects of irradiance on germ tube lengths and appressorium formation.

Irradiance had a marked effect on the lengths of differentiated germ tubes and this was a graded response: the higher the photon flux density, the lower the number of long (> 100 µm) differentiated germ tubes (Fig. 5).

Although light per se, enhanced the number of appressoria formed (Table 1), the photon flux density (15–110 µmol photons $\cdot m^{-2} \cdot s^{-1}$) did not seem to be important in this response (Table 2). Under all irradiances used, the percentage of conidia which produced appressoria was > 20% higher than in the dark. This difference was highly significant for all treatments ($P < 0.01$) and no significant difference between irradiances was found.

Time course of appressorium differentiation. On cellophane, conidium germination in the light and dark and was completed within 8 h (data not shown). On the same substratum, germ-tube differentiation began 5 h post inoculation in both the light and dark (Fig. 6). However, the first morphological indicator of appressorium formation (hook formation, Bourett and Howard 1990) was observed 1 h earlier in the light than the dark (data not shown). Under both conditions, differentiation of appressoria ceased 14 h post-inoculation. The number of ap-

pressoria which differentiate per unit of time over the 6- to 12-h period was approximately 50% higher in the light than in the dark (Fig. 6).

Influence of temperature on appressorium differentiation. On cellophane, the percentage differentiation of appressoria in the light was significantly greater than in the dark over the temperature range 15–30° C ($P < 0.05$). Thus the promotive effect of light on appressorium differentiation was independent of temperature. At 30° C, far fewer appressoria were formed in both treatments (Fig. 7).

Discussion

Our data indicate that the pre-penetration phase of infection by *M. grisea* involves a programme of growth and differentiation triggered at spore germination and regulated by multiple signals from the host and surrounding environment.

Role of contact sensing. Surface contact was found to be essential for appressorium induction but not conidium germination. It is well established that contact of a germ tube with a solid surface is an essential prerequisite for appressorium formation in many plant pathogens (Emmett and Parbery 1975; Hoch and Staples 1991; Read et al. 1992). Our study does not support the conclusions of Lee and Dean (1993a) who recently reported for *M. grisea* that contact with a surface induces conidium germination whilst a high surface hydrophobicity, as found on a rice leaf, induces appressorium formation. Unfortunately, we cannot comment on their findings regarding conidium germination because of the lack of experimental details in their paper. These authors did show that appressoria differentiated readily on substrata with a high hydrophobicity (wax paper, polystyrene and polyester) but not on glass and agarose which were more hydrophilic. However, *M. grisea* has been shown to form appressoria on cellophane (Hashioka 1972; Araki and Miyagi 1977; Uchiyama et al. 1979; Hirooka et al. 1982; Bourett and Howard 1990) and glass (Uchiyama et al. 1979; Yaegashi et al. 1987; Uchiyama and Okuyama 1990), and our results confirm this.

There is an interesting discrepancy in the literature regarding appressorium formation on glass. Although, as indicated, a number of papers have shown that appressoria of *M. grisea* can develop on this substratum, other workers have reported that they do not (Hamer et al. 1988, 1989; Howard et al. 1991a; Lee and Dean 1993a). The reason for this inconsistency is not clear but may be attributable to differences in the type of glass used, strain variation or instability of certain genes involved in appressorium formation. In relation to the latter point, of possible relevance is that *M. grisea* is highly mutable at certain genetic loci (Valent and Chumley 1991). One such locus is *SMO* and Hamer et al. (1989) found that smo$^-$ mutants, in contrast to the wild type from which they were isolated, formed significant numbers of appressoria on glass.

The ability of germ tubes to differentiate into appressoria may be related to how they adhere to their substratum – an important aspect of plant-pathogen interactions about which little is known (Nicholson and Epstein 1991). It has been suggested that a hydrophobin-like protein produced by the *MPG1* gene may be important for appressorial adhesion through hydrophobic interactions with the rice leaf (Talbot et al. 1993). However, it seems unlikely that such a mechanism is involved in the adhesion of appressoria to cellophane considering the latter's hydrophilic nature.

Evidence was found for germ tubes, but not conidia, exhibiting contact-sensing. Firstly, a solid surface was required for germ tubes to differentiate. Secondly, a high substratum hydrophobicity (of Teflon or rice leaves) resulted in shorter differentiated germ tubes than on more hydrophilic substrata (glass and cellophane). This may be a result of earlier differentiation, or alternatively slower growth, on the hydrophobic substrata. However, it should be noted that the conidia used in our analysis lacked spore tip mucilage (Hamer et al. 1988) and it is conceivable that this may play a role in conidial contact sensing. This aspect needs to be addressed in a future study.

Role of light. There is a dearth of knowledge on the significance of light in regulating the pre-penetration phase of plant pathogens (Emmett and Parbery 1975). We found on rice leaves and cellophane that the lengths of differentiated germ tubes were, on average, shorter in the light than the dark. A possible explanation of this phenomenon is that light stimulates germ tubes to differentiate earlier after germination than in the dark, but this needs to be confirmed by further analysis. On all substrata, germinated conidia incubated in the light produced consistently more appressoria than those kept in the dark.

The mechanistic basis of how light is perceived by germ tubes is unknown. However, it was interesting to note that the lengths of differentiated germ tubes, but not the number of appressoria formed, were significantly influenced by the photon flux density of the light. Thus the effect on the length of differentiated germ tubes was a graded response to the irradiance, whilst increased appressorium numbers were not. This distinction suggests that these two responses involve different signal transduction pathways.

Increasing light levels usually are associated with higher temperatures in those parts of the world where rice blast is endemic. Our data demonstrated that temperatures above 30° C resulted in reduced appressorium formation. Conidia of *M. grisea* are dispersed mainly at night (Ou 1985). Since it takes several hours after spore deposition for appressoria to be initiated, then this process will sometimes occur in early daylight as temperatures start to rise. The advanced formation of appressoria at this time before temperatures rise too high may be a distinct advantage for *M. grisea*, particularly since appressoria can endure more adverse conditions than germ tubes (Emmett and Parbery 1975).

A 'conducive environment' is required for appressorium initiation. Signalling during the pre-penetration phase of infection by *M. grisea* inevitably involves an extremely complex network of interactions rather than independent, linear pathways of signalling events. Visualizing appressorium initiation in *M. grisea* as being controlled by just one or even a few external factors is probably too simplistic. Instead, a whole network of external and internal signals may exert control but each component to varying extents (e.g. see: Kacser and Porteous 1987; Trewavas 1987). Appressorium initation might be achieved in a variety of ways depending on the precise mixture and balance of signals and signal transduction elements present. Emmett and Parbery (1975) concluded that few plant pathogens require specific external stimuli for appressorium induction but instead, need a *conducive environment* for the process to take place. We suggest that what comprises a conducive environment may vary depending on the signalling capabilities of the germ tube and the environmental factors prevalent.

The germ tube seems to act as a specialised 'sense organ' which grows out from the spore. So what environmental signals, influencing its differentiation into an appressorium, does it sense? We have shown that surface contact, surface hydrophobicity, light, light intensity and temperature are all important. However, other factors are also influential, including water, pH, and signals derived from the host. Free water is required for conidium germination and a high relative humidity is essential for infection (Ou 1985). Germ tubes form most appressoria in the pH range 5.0–6.0 (data not shown). Host-derived signals must also be important because we found that consistently higher numbers of appressoria were formed on rice leaves than on any of the artificial substrata used in our study. Rice leaves consistently provided the most conducive environment for appressorium formation under the different illumination conditions employed. Whether these host-derived signals are chemicals or physical attributes (e.g. microstructure) of the leaf surface, is not clear.

Of signals identified as playing a role in regulating the pre-penetration phase, we can distinguish between those that are essential and those which are not. The essential factors (e.g. surface contact and a high relative humidity) seemed to be passive in effect rather than providing active stimulation of appressorium formation. The passive role of surface contact is indicated by the observation that germ tubes did not differentiate immediately on making contact with a surface but grew to different lengths before forming appressoria. Other factors (e.g. light and the precise physical and chemical make up of the contact surface) may not be indispensible but can play important roles as *modulatory signals* providing the conducive environment for appressorium initiation. It is possible that all of these external signals act in concert to make the germ tube competent to respond to an, as yet unidentified, internal signal (see next section).

Although most fungal leaf pathogens, like *M. grisea*, do not seem to rely on specific external signals for appressorium formation (Emmett and Parbery 1975), some clearly do. In this respect, rusts deserve special mention. A number of rusts respond to well-defined topographical features as primary signals of appressorium induction (Allen et al. 1991a; Hoch and Staples 1991; Read et al. 1992). For example, > 90% of germ tubes of the bean rust (*Uromyces appendiculatus*), grown on artificial substrata, are optimally induced to differentiate appressoria over steps with a height of 0.5 μm (Hoch et al. 1987; Allen et al. 1991a). This topographical signal was closely correlated with the guard-cell lip (or ledge) of the host plant *Phaseolus vulgaris* (Hoch et al. 1987; Allen et al. 1991b; Terhune et al. 1991). Rusts have evolved this type of sensing process in order to precisely locate appressoria over stomata through which they penetrate. *Magnaporthe grisea* and most other leaf pathogens, however, have no requirement for such a mechanism because they directly penetrate through the leaf cuticle and thus do not need to form appressoria at specific locations on the leaf surface.

Induction of conidium germination and appressorium differentiation. Our work shows that the pre-penetration phase of *M. grisea* involves a defined programme of cell lineage to which the fungus becomes committed at conidium germination. A similar conclusion was reached by Howard et al. (1991b) who described this succession of growth and developmental events as representing a *morphogenetic unit*. What triggers germination is not definitely known. However, since percentage germination was > 92% in the light and dark on all substrata and in liquid suspension, this indicates that water is probably important because it was a common, external factor in all these experiments. However, whether spore hydration provides a primary stimulus for germination, or whether water allows the release from spores of a germination self-inhibitor (Macko 1981), remains to be determined.

As indicated earlier, we found no evidence for an external inductive signal for appressorium differentiation. This suggests that appressorium initation is dependent on intracellular signalling. Since the whole of the pre-penetration phase can occur in water devoid of external nutrients, starvation may provide this stimulus. In support of this is the finding that RNA transcripts of the *MPG1* gene, which is differentially expressed at a high level during appressorium formation, are also elevated in cultures starved of a carbon or nitrogen source (Talbot et al. 1993). Furthermore, cAMP, which is elevated in response to starvation in some eukaryotes (e.g. in *Dictyostelium*, Gerisch 1987), also seems to be involved in appressorium induction in *M. grisea* (Lee and Dean 1993a).

It is clear from our study that the different artificial substrata used did not completely mimic results obtained on the more conducive environment of rice leaves. This indicates a shortcoming of soley using in-vitro systems to experimentally analyse the pre-infection phase in *M. grisea*, and probably other pathogens as well. It emphasizes the need to perform experiments in parallel on the natural host substratum.

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