INFECTION OF KIKUYU GRASS

(*PENNISETUM CLANDESTINUM*)

BY THE RUST FUNGUS

PHAKOPSORA APODA

by

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ABSTRACT

A rust fungus on kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chiov.) was identified as *Phakopsora apoda* (Har. & Pat.) Mains., and was reported for the first time in South Africa.

An investigation was conducted into the direct penetration mechanisms employed by the urediospore germlings, using light, scanning and transmission electron microscopy. The infection processes of *P. apoda* were found to parallel closely those of *Phakopsora pachyrhizi*.

Infection structures of *P. apoda* produced on artificial membranes are similar to those observed in the host leaf. The binucleate urediospore germinates to form a typically short germ tube, which differentiates an appressorium, delimited by a septum. The two nuclei migrate into the appressorium, where mitosis occurs, resulting in four nuclei in the mature appressorium.

Appressoria appear to form preferentially at epidermal cell junctions. When germinated on artificial membranes, germlings differentiate appressoria against microfabricated ridges and on smooth surfaces, possibly with a marginal preference for ridge-associated differentiation (significant at the 5% but not the 1% level of significance).

A penetration pore develops in the basal wall of the mature appressorium, over the infection site. A cone-like structure develops around the pore. The basal wall of the appressorium is thinned and electron dense in the vicinity of the cone, and the cone appears to be attached to the appressorial wall by means of a collar of similar, electron-dense material. The membrane-bound cone is elaborately branched, and numerous, electron-dense glycogen-like particles are associated with these cone elaborations.

A penetration peg, its walls continuous with an inner wall layer of the cone, penetrates the epidermal cell wall and expands into an intracellular penetration hypha within the host cell.

The penetration hole formed by the peg has smooth edges, and there is little deformation of the epidermal cell wall fibrils, indicating a predominantly enzymatic mode of penetration.

The penetration hypha traverses the epidermal cell, and emerges into an intercellular space of the mesophyll, narrowing to form a penetration neck at the exit site. Both the penetration peg and the neck, contain multivesicular bodies associated with parallel arrays of microtubules. The intercellular penetration hypha contains an elaborate endomembrane system, and is virtually devoid of glycogen-like particles. Lomasomes often occur in the vicinity of the penetration neck.

Signs of host disruption and the formation of papilla-like structures are apparent in mesophyll cells adjacent to the infection site 12 hours after inoculation.

A septum delimits the penetration hypha from the primary hypha, which extends further into the mesophyll and forms secondary branches.

PREFACE

The research described during the course of this study, was undertaken in the Department of Microbiology and Plant Pathology, at the University of Natal, Pietermaritzburg, under the supervision of Prof. F.H.J. Rijkenberg.

To facilitate easy conversion of chapters 2, 3 and 4 into papers for publication, each of these chapters has been written as a separate entity, with its own introduction and discussion. As a result, a certain amount of repetition could not be avoided. A general introduction, including a literature review on direct penetration in the rust fungi, and a general discussion, have been included to provide some continuity and broad observations of the penetration processes of *Phakopsora apoda*. The general discussion has been divided into two parts, to accommodate a fairly detailed examination of some of the techniques used and technical challenges encountered during the course of the study, without cluttering and detracting from the essence of the research itself.

Chapters 2 and 3 represent work that was presented at the 33rd and 34th annual conferences of the South African Society for Plant Pathology, in 1995 and 1996 respectively. Abstracts were published in the South African Journal of Science, vols 91 and 92.

I hereby declare that the information presented in this thesis is the result of original work by the author, and has not been submitted, in any form, to another university. Where reference has been made to the work of others, it has been acknowledged accordingly within the text.

R. Adendorff

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CHAPTER 1 GENERAL INTRODUCTION

1.1 A RUST ON KIKUYU GRASS IN NATAL

Kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chiov.) is one of the major pasture grasses in Natal, especially in the mist-belt areas of the KwaZulu-Natal Midlands. According to Hall (1991), kikuyu pastures constitute 24% of the pastures planted for summer grazing in the KwaZulu-Natal region. During the 1986/87 season, Hall (1991) discovered an outbreak of rust in kikuyu grass pastures in Richmond, KwaZulu-Natal, South Africa. Hall and Rijkenberg (1989) appear to have made the first report of rust on kikuyu grass in South Africa, and tentatively identified the causal organism as *Puccinia stenotaphri* Cumm.

During the course of Hall's (1991) studies, it became apparent that this was an unusual rust fungus. Uredial morphology was strikingly different from most other rust fungi, displaying a dense array of finger-like paraphyses along the periphery of the uredium. Hall (1991) also discovered that host penetration from urediospores is effected directly through the cuticle and epidermis of the host leaf, as opposed to the 'normal' or more commonly studied form of penetration, via the stomata.

The aim of this study was to investigate the disease symptoms and the uredial morphology of the fungus, to accurately identify the causal organism, and to elucidate the processes of host infection by the pathogen from its urediospores.

1.1.1 Identification of the rust fungus on kikuyu grass in KwaZulu-Natal

Since Hall and Rijkenberg's (1989) report of rust on kikuyu grass in Richmond, the disease has been found at Hilton, Greytown (Lowe, pers. comm.[1]) and Dargle in the KwaZulu-Natal midlands, as well as in the more coastal Kloof area. According to Hall

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1 K. Lowe, plant pathology Honours student at the University of Natal, Pietermaritzburg.

(1991), since its initial discovery in Richmond in 1987, the rust 'has spread rapidly to numerous districts of the region'. Unfortunately he did not specify which districts. It is possible that the rust was already widespread in KwaZulu-Natal, but had not been detected or reported earlier due to the low levels of disease that are often present, and also perhaps owing to the unspectacular nature of the symptoms.

As the original identification of the fungus causing rust on kikuyu grass was of a tentative nature, the present author decided to examine the symptomatology and uredial morphology more closely to make a definitive identification possible.

1.1.1.1 Disease symptoms

The disease is characterised by the presence of small, chestnut brown, amphigenous uredia, which are scattered or in close clusters of 2-4 on the leaf, tending to be more abundant along the edges and towards the distal third of the lamina (Fig. 1). Occasionally, in very severely infected leaves, uredia occur on the leaf sheath near the ligule. Uredia have not been observed on the stem.

When very numerous, uredia are often associated with chlorosis and necrosis of the leaf tissue, especially towards the distal portions of the affected leaves (Fig. 1). The 'green island' effect is usually evident in the early stages of chlorosis.

1.1.1.2 The causal organism

- **Uredia**

Uredia of this fungus are small, approximately **0.15-0.4 x 0.1-0.2 mm**, and are circular, or slightly elongated parallel to the leaf venation. If left undisturbed, they produce short columns of bright orange urediospores (Fig. 2). On the opposite side of the leaf, the uredium is characterised by a chestnut lesion of the same size which, in some cases, may also be accompanied by rupture of the epidermis and the production of spores.

Figure 1 (i) and (ii): Symptoms of kikuyu grass rust: note the greater number of chestnut-brown uredia towards the distal ends of the leaves, and the associated chlorosis and necrosis of the leaf tissue.

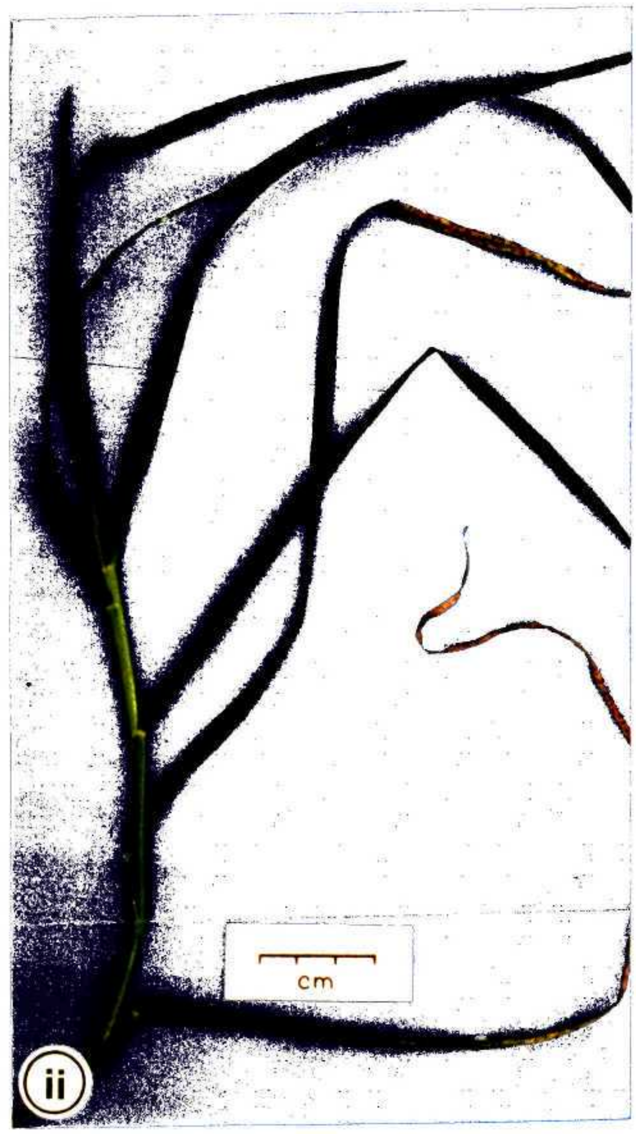
Figure 2: Uredia (U) of *P. apoda* as seen under the dissecting light microscope. The persistent epidermis can be seen in Fig. 2(i) (small arrows), whereas in Fig. 2(ii) the uredium is fully exposed. In Fig. 2(ii) the paraphyses (P) are just visible as a pale, raised ring along the periphery of the uredium. The bright orange urediospores (Ur) are visible in clumps within the uredia.

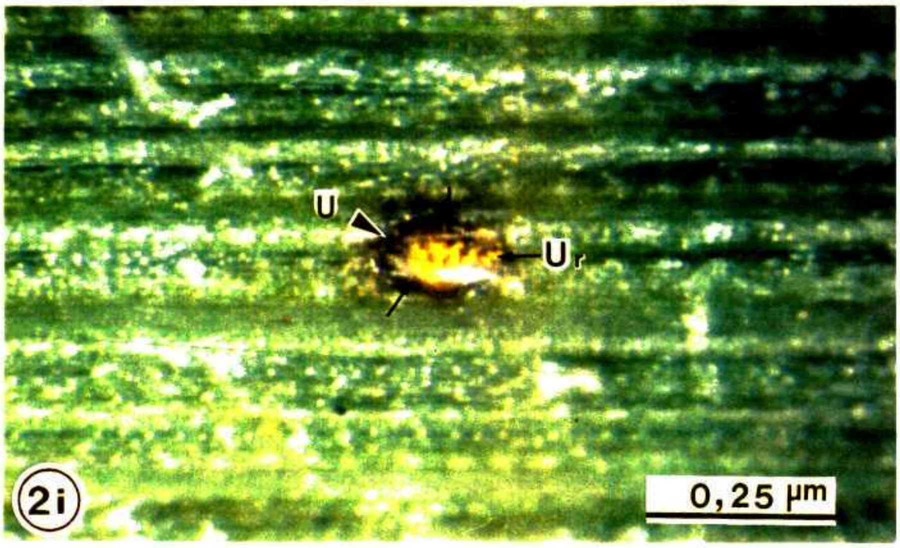
Figure 3: Scanning electron micrograph of uredia of *P. apoda*.

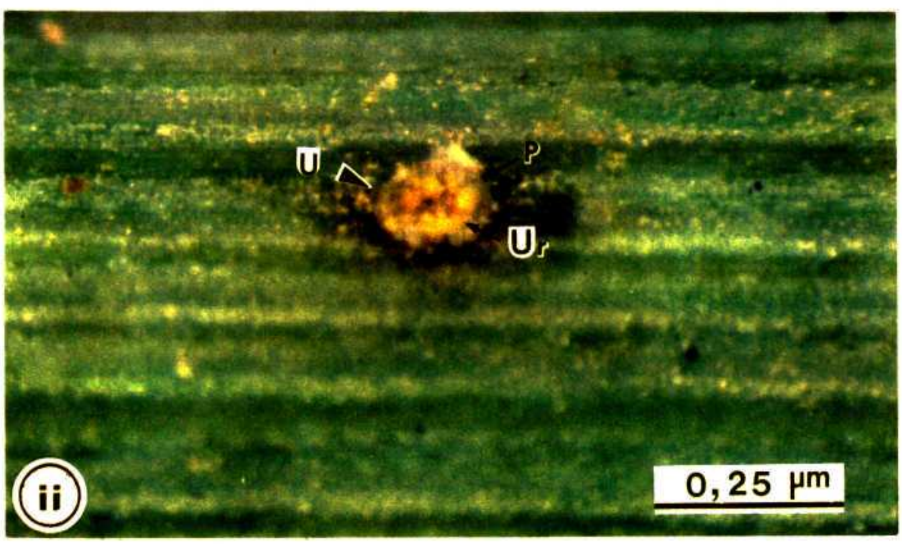
(i) This is a fairly small uredium (U). Note the incurved paraphyses (P), and the persistent epidermis (E).

(ii) This is a particularly large uredium (U). Note how the incurved paraphyses (P) which densely line its periphery (and also impinge on the centre of the uredium) are not long enough to traverse the entire uredium, giving the effect of an 'open' uredium. In fact, the incurvature of the paraphyses is the same as in 3(i).


cm
1i


cm
ii


U
Ur
2i
0,25 µm


U
P
Ur
ii
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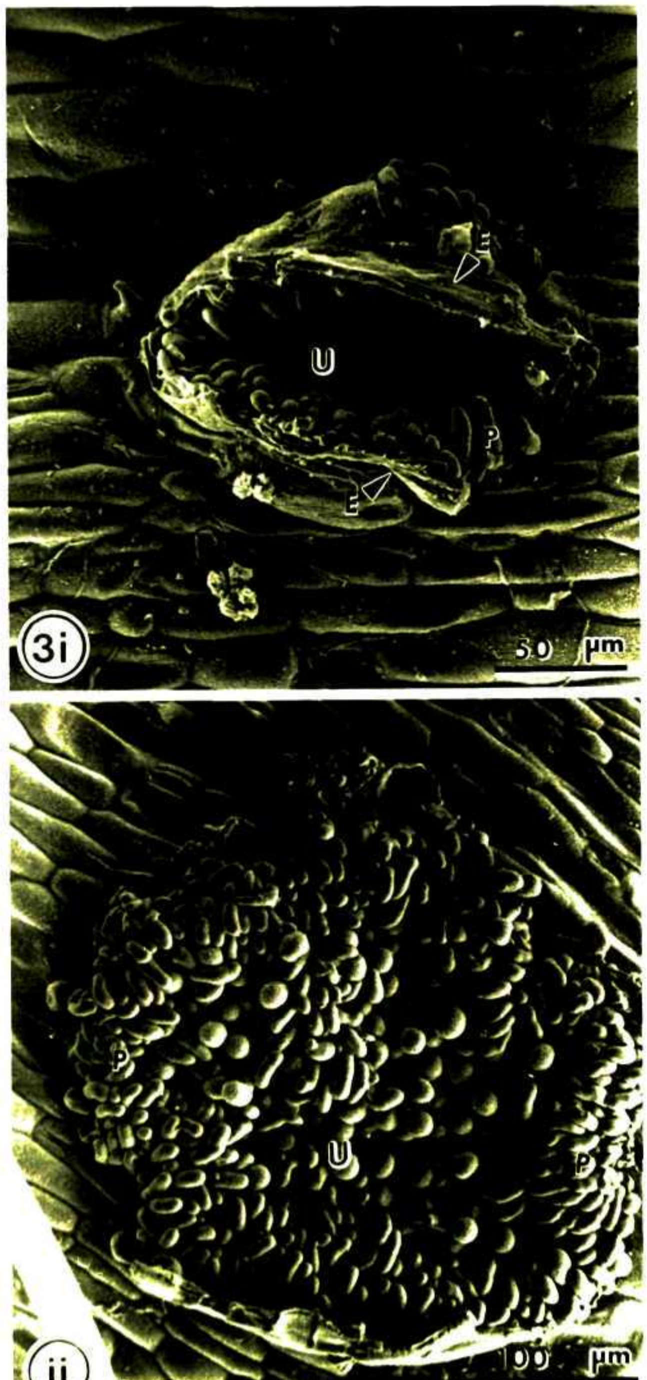
E
E
U
P
E
3i
50 µm
P
U
P
ii
100 µm

Abundant paraphyses line the periphery of the uredium. These hyaline structures are clavate to cylindrical and are incurved towards the centre of the pustule (Fig. 3). Hand sections of uredia, viewed under the light microscope, revealed that the walls of the paraphyses are dramatically thickened apically and dorsally. It would appear that the dorsal/apical walls are sometimes 4 to 5 times as thick as the inner walls.

- **Urediospores**

The urediospores are ellipsoid to obovoid, echinulate and have pale/hyaline walls with orange-yellow cytoplasm. A range of sizes was observed in a sample of 100 spores: (15-)**19-24**(-26) **x** (17-)**23-28**(-32) **μm**. Because of the small size of the uredia, the number of spores produced per uredium is small.

1.1.1.3 The literature

Identification of the rust fungus could only be done on the basis of uredial characteristics, as no telial stage of the fungus was found.

To the present author's knowledge, only 4 rust fungi have been reported on *Pennisetum clandestinum*:

1. *Puccinia subcentripora* Arth. & Cumm. (Cummins, 1971);
2. *Puccinia stenotaphri* (Syd.) Cumm. (Hall and Rijkenberg, 1989); 3. *Puccinia substriata* Ell. & Barth. var. *penicillariae* (Speg.) (Laundon and Waterson, 1964; Ramachar and Cummins, 1964; Walker, 1988);
4. *Phakopsora apoda* (syn. *Puccinia apoda* (Har. & Pat) Mains) (Mains, 1938; Dingley, 1977; Gjaerum and Dennis, 1982; Gardner, 1984; Walker, 1988).

From the literature, descriptions of *Phakopsora apoda* most closely correspond to observations made by the present author.

The following is a summary of concurrent points:

(i) Gardner (1984) made very similar observations about disease development and symptoms, e.g. uredia occur mainly on the lower leaves; infection is enhanced by cool, wet, shady conditions; the fungus is very sensitive to the microhabitat (disease development can be severe in a shaded area, with no apparent infection in sunny patches just meters away); severe infection causes chlorosis and necrosis, especially at distal leaf portions.

(ii) Uredia:

- amphigenous (Mains, 1938; Cummins, 1971; Dingley, 1977);
- 0.2 - 0.3 mm in diameter (Dingley, 1977);
- numerous, incurved, hyaline to light brown/golden, peripheral paraphyses, with apical and dorsal thickening (Mains, 1938; Cummins, 1971; Dingley, 1977; Gjaerum and Dennis, 1982);

(iii) Spores:

- spore sizes observed during the course of this study are exactly within the ranges proposed for *P. apoda* (Mains, 1938; Cummins, 1971; Dingley, 1977; Gjaerum and Dennis, 1982);
- echinulate, yellowish (Cummins, 1971), walls hyaline to yellowish (Mains, 1938; Gjaerum and Dennis, 1982).

It seems reasonable to conclude, on the basis of the information available, that the rust fungus infecting kikuyu grass in KwaZulu-Natal is *Phakopsora apoda* (Har.& Pat.) Mains. This identification has been confirmed by Professor J.F. Hennen[2], and a Disease Note has been published in *Plant Disease* (Adendorff and Rijkenberg, 1995a).

Phakopsora apoda has apparently been found in several northern, central and southern African countries (Dingley, 1977), and Gjaerum and Dennis (1982) mentioned that *P. apoda* had been reported on *Pennisetum* in South Africa, although they did not

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2 The Arthur Herbarium, Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907, U.S.A.

mention which species of *Pennisetum* this report pertained to, and they did not cite the source of this information. Attempts to contact either of the authors proved to be unsuccessful. Since Dennis was working at the Royal Botanic Gardens at Kew when the paper in question was submitted for publication, investigations were conducted as to whether the report of *P. apoda* in South Africa may have originated from the mycological herbarium at Kew. Unfortunately, although a number of African specimens of *P. apoda* have been lodged at the mycological herbarium, none originated in South Africa (Russ[3], pers. com.). The present author has subsequently lodged a specimen of kikuyu grass infected with *P. apoda* (PREM number: 54236), at the National Collection of Fungi, in the National Herbarium in Pretoria.

According to Dingley (1977), pycnial and aecial stages of the rust are unknown. The telial stage has been described (Cummins, 1971), but appears to be uncommon. To the present author's knowledge the telial stage has not been found naturally in Hawaii (Gardner, 1984) or New Zealand (Dingley, 1984), and Hall's (1991) attempts to induce the formation of teliospores, were unsuccessful.

1.1.2 Epidemiology of *Phakopsora apoda*

1.1.2.1 Disease development

The development of the kikuyu grass rust was observed in the sample collection area at Hilton over three years (1994-1996). The following observations were made:

- In the early stages of the disease cycle (early summer), infection was usually observed in shady or very moist areas, and was especially severe in areas of permanent or nearly permanent shade. The fungus seems to be particularly sensitive in this respect, as in some places, severely infected plants in the shade would be only a few meters away from

3 Ian Russ, Mycological Herbarium, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AE, England.

seemingly unaffected plants which were exposed to more sunlight or a drier environment;

- Disease levels increased gradually over the summer months and reached their peak in late autumn/early winter. Corresponding to this increase in uredial abundance, was an increase in the range of habitats where infected grass was found;

- The hyperparasite *Sphaerellopsis filum* Fr. O. Eriks. became increasingly abundant in the uredia towards the end of summer each year;

- During winter, the kikuyu grass died back (especially after frosting), with very little green growth persisting. From around July to January in 1994 and 1995, the rust appeared to be very scarce. In many of the patches that were observed to have been heavily infected towards the end of summer, there was no sign of the disease by the following spring. No telial stage of the rust was found;

- The winter months of 1996 had unusually high rainfall figures, and as a result the kikuyu grass did not die back as it had in previous years. In spite of this however, disease levels did decline dramatically, and by late August the present author was hard-pressed to find infected material. On the few infected plants that were found, the uredia were, almost without exception, severely infested with vigorously sporulating pycnidia of the hyperparasite *S. filum*. Sporulation of the rust fungus was minimal in all uredia observed.

It appears that *P. apoda* can survive from season to season in the uredial stage, without the formation of other spore types, and hence without a sexual stage. This is especially significant in terms of the genetic diversity of the fungus, which must be restricted to some extent by its purely asexual perpetuation. Another member of the genus *Phakopsora*, *Phakopsora ziziphi-vulgaris* on jujube (*Ziziphus sativa*), has been shown to overwinter in the form of summer sori in diseased leaves, although the average germination rate of overwintered urediospores was much lower than that of fresh spores (3.15 % as opposed to 43.93 %) (Zheng and Lu, 1992).

The yearly fluctuation in kikuyu rust levels may result from a typical exponential growth cycle terminated by the death of the host leaves each winter, or seasonally controlled environmental factors may have a direct effect on the ability of the fungus to infect and proliferate in the host. In 1996 the kikuyu grass did not die back as in previous years and yet disease levels still declined dramatically: it seems logical to assume that temperature and moisture effects play a large role in the disease cycle of this rust.

Another factor that cannot be ignored, is the effect of the hyperparasite *S. filum*. The high levels of the hyperparasite in late winter may have been the culmination of exponential population growth during summer, both on *P. apoda* and perhaps other rust fungi as well, or perhaps was the result of a fungal host more predisposed to infection after exposure to unfavourable climatic conditions. It is possible that *S. filum* contributed to the decline in rust levels during the cool season, both as a result of its hyperparasitic nature, and perhaps also as a result of increased leaf tissue necrosis caused by the release of toxins into the host tissue (Bruton[4], pers. comm.). According to Kranz (1981), the presence of a single pycnidium in the sorus of *Puccinia recondita* Roberge ex Desmaz. reduced the mean number of urediospores produced by between 10 and 70 % in comparison to uredia that were free of *S. filum*. This may be especially important in *P. apoda* which produces so few spores as it is.

It would seem that seasonal fluctuations result from both seasonal temperature variations and from a cycle driven by the hyperparasitic interaction between *P. apoda* and *S. filum*. Actual levels of disease, even at the peak of the 'epidemic', were relatively

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4 A.G. Bruton, Head of the Centre for Electron Microscopy, Natal University, Pietermaritzburg, South Africa.

low, and the disease could perhaps be described as endemic, co-existing with the host from season to season.

1.1.2.2 Significance and control of kikuyu grass rust

Even though rust on kikuyu grass does not seem to culminate in death of the host, the photosynthetic area of the grass laminae is substantially reduced when infection is heavy. The exact effects of the disease on the nutritional value of kikuyu pastures would have to be determined to establish the economic significance of this rust fungus.

Hall (1991) observed that cattle are more reluctant to graze kikuyu grass that is heavily infected with rust fungus. This apparently leads to large areas of infected pastures not being utilized. However, this theory would be difficult to verify as disease levels seem to be higher on grass that is allowed to grow very tall, and cattle have a preference for grass of a more intermediate length.

Hall (1991) found that control of the fungus could be obtained by cutting and removing infected material from the field, as well as by improving grazing management so that the sward did not grow too long before being grazed.

The fact that taller grass plants appear to be more susceptible than those that have been cut back, may imply that this disease is unlikely to become a problem on lawn grass. The present author has found that infection in lawn grass does tend to be restricted to the taller grass plants that have escaped mowing under hedges, around trees etc., although the role of the microclimate in these sheltered areas would probably also have an important effect on disease development. Of course, rust on mown grass is considerably more difficult to see than on longer grass, and this may give a false impression of increased resistance.

Kikuyu grass rust is also unlikely to become a serious problem in areas where the host grass dies back in winter. Perhaps in more tropical areas where the host is not exposed to frosting or low temperatures, kikuyu grass rust is a greater problem. This could depend heavily on whether high levels of disease at the onset of winter result from a stressed host, a stressed pathogen, or from the natural, exponential increase of disease over the 'growing' season.

Since disease development would appear to be closely linked to highly localised and very specific climatic and seasonal conditions, there is always the possibility that kikuyu grass rust could become economically important in the event of unusual weather conditions.

1.1.3 The ultrastructure of *Phakopsora apoda*

1.1.3.1 The paraphyses

One of the most remarkable aspects of the uredial morphology of *Phakopsora apoda* is the ring of finger-like paraphyses lining the periphery of the uredium. The dissecting and light microscopes do not do justice to these structures. It is only under the electron microscope that they can be observed in any detail (Fig. 3).
Hall (1991) suggested that the paraphyses serve the purpose of controlling the release of spores depending on the climatic conditions they are exposed to, i.e. they interlock and hold the spores back when conditions are unfavourable for dispersal, and open out when conditions are favourable. However, examination of the scanning electron micrographs he referred to, indicated that in the smaller uredia (not necessarily younger uredia), the paraphyses were able to span the uredium and give the appearance of interlocking and holding back the spores. However, in larger uredia, the paraphyses, whilst exhibiting the same degree of incurvature, did not span the uredium and hence gave the appearance of being more 'open' (compare Figs 3(i) and (ii)). During the course of this study, the present author could find no evidence to suggest that an active opening and closing mechanism is associated with the paraphyses, although the heavy apical and dorsal thickening of the paraphysis walls did inspire thoughts of turgor effects and plant movements.

An alternative function of these paraphyses is not immediately apparent. The substantial thickening of the walls may aid in the reduction of water loss. The hairs may serve to trap a layer of air above the uredium and thereby reduce the moisture gradient between the fungus and the atmosphere. The small size of the uredia may also be related to moisture conservation. A reduction in the area of compromised host leaf cuticle would reduce the impact of the rust on the host, making it a more successful parasite.

A sensitivity on the part of the fungus itself to desiccation, may be reflected in the occurrence of the rust mainly in very shady, moist areas. Perhaps the availability of water plays a role both in the post-infective stages as well as during the actual infection process. It is also possible that the grass host is more predisposed to infection in the shade. Gardeners know well enough how patchy sun-loving grasses such as kikuyu can be in heavy shade. This lack of vigour may very well have an impact on the resistance of the grass to disease.

It would be interesting to investigate the behaviour and sensitivity of other rust fungi which produce peripheral paraphyses in their uredia, and to see if there are any similarities to *P. apoda*. One could also compare rust fungi that have dorsal and apical thickening of the paraphysis wall, with those that have regular wall thickening.

1.1.3.2 The infection process

Mycologically, the most fascinating aspect of *Phakopsora apoda*, is its mode of host infection from urediospores. Hall (1991) demonstrated how this rust fungus penetrates the host epidermis directly, and not via stomata, as has most commonly been observed in other rust fungi.

Hall's (1991) research was preliminary, and only dealt superficially with the extrafoliar infection structures of the fungus. During the present study, various techniques were utilized to examine the penetration and early colonization of the host from urediospores of *P. apoda*. In Chapter 2, plastic membranes were used to examine the differentiation and nuclear behaviour of the urediospore germlings. In Chapter 3 both the extra- and intrafoliar infection structures produced from urediospores were examined by means of scanning electron microscopy. This entailed scanning the surface of host leaves, and also scanning leaves in cross-section, providing a 3-dimensional view of the infection process. Chapter 4 describes how transmission electron microscopy was used to provide ultrastructural information about the infection structures, especially the unusual appressoria. The techniques that were used and developed during the course of this study, have been dealt with in the General Discussion, which also serves to provide a more composite picture of the infection process.

1.2 LITERATURE REVIEW OF DIRECT PENETRATION IN THE RUST FUNGI

1.2.1 Introduction

The following review of direct foliar penetration by the rust fungi has been compiled to allow comparisons to be drawn with the subject of this thesis, viz. *Phakopsora apoda*, a fungus that penetrates the host leaf directly through the cuticle from urediospore-derived infection structures. The paucity of information available on direct penetration of the host during infection from urediospores, has necessitated the inclusion of some information regarding other spores types, particularly basidiospores, in the hope of these investigations shedding light on the processes observed in urediospore germlings of *P. apoda*.

The spore germling probably represents the most vulnerable stage in the rust fungus life-cycle. This delicate, ephemeral, pre-infection stage is prone to desiccation, UV radiation, antagonistic organisms, harmful chemicals, heat, and numerous other factors that could compromise its genetic and physiological integrity. It is imperative that the germlings penetrate the host epidermis and establish a parasitic relationship with the host as rapidly as possible. Rust fungi have developed sophisticated and very specialised mechanisms of foliar penetration during their co-evolution with host plant species to maximise the speed and efficiency of this process. De Bary (Littlefield and Heath, 1979) would appear to have been one of the first to study the penetration processes employed by the different spore stages of rust fungi. He apparently reached the conclusion that there are two methods of host penetration in the rust fungi:

1) indirectly, through a stoma, from an appressorium directly over the stoma: typical of penetration during host infection from urediospores e.g. *Puccinia graminis* Pers.:Pers. f.sp. *tritici* Eriks and E. Henn. (Lennox and Rijkenberg, 1989), and aeciospores (Littlefield and Heath, 1979);
2) directly, through the cuticle, from an appressorium: typical of penetration by basidiospore germlings e.g. *Uromyces appendiculatus* (Pers.) Unger var. *appendiculatus* (Gold and Mendgen, 1984).

However, not all rust fungi conform to these trends. Some species of rust fungi penetrate indirectly during infection from basidiospores, through stomata without the formation of appressoria, i.e. the germ tube grows through the stomatal pore, as occurs in some coniferous tree rusts, e.g. *Cronartium ribicola* J.C. Fisch. ex Rabenh. (Patton and Johnson, 1970), and at least one fungus penetrates directly through the cuticle during host infection from its aeciospores, viz. *Arthuriomyces peckianus* (Howe in Peck) Cumm. Hirat. (Swann and Mims, 1991). Five species of rust fungus are known to penetrate directly through the cuticle from urediospores (Hunt, 1968; Hall, 1991). In two early light microscopy studies, basidiospore germlings of *Puccinia graminis* Pers.:Pers. and *Puccinia triticina* Eriks were observed to penetrate directly through the cuticle, without the formation of appressoria (Waterhouse, 1921; Allen, 1930, 1932a).

According to Gold and Mendgen (1984), there have even been reports of direct penetration by infection hyphae arising from the metabasidium. Allen (1934, 1935) has also reported direct penetration from pycniospore germlings of *Melampsora lini* (Ehrenb.) Desmaz. and *Puccinia malvacearum* Bert.. No appressoria were observed, with penetration being effected by the germ tubes.

Most of these unusual reports, however, have never been investigated further with electron microscopy, notable exceptions being *A. peckianus* (Swann and Mims, 1991) and the direct penetrator from urediospore-derived infection structures, *Phakopsora pachyrhizi* Syd. (Koch, Ebrahim-Nesbat and Hoppe, 1983).

The early work on rust fungi generally centred on basidiospore-derived infections of stem rust of cereals (e.g. Allen, 1930, 1932a, 1932b, 1934, 1935). Studies on penetration therefore focused on direct/cuticular penetration. Since then, the emphasis of rust research has gradually shifted its focus to the more economically and epidemiologically important urediospore stage of the rust fungus life cycle. As a result, extensive research into the mechanisms of indirect, urediospore germling penetration of leaf surfaces has been conducted in the past decade or so, with particular attention to the induction of penetration structure formation (Littlefield and Heath, 1979; Wynn and Staples, 1981; Hoch and Staples, 1987; Hoch and Staples, 1991).

Comparatively little research has been published on the direct-penetrating rust fungi. This has largely been due to the fact that very few of the economically important rust fungi are direct penetrators in their uredial stage.

In the following account, the infection processes employed by the rust fungi will be followed from the point of fungal adhesion to the host surface, to the growth of mycelium within the leaf tissue. The germination process itself has not been included, and neither has the development and structure of haustoria been considered in any detail. Rather, the emphasis has been placed on the actual penetration of the leaf epidermis and early infection structure formation.

1.2.2 Adhesion of infection structures to the host surface

In the past, a commonly held view was that fungal spores become entrapped on the plant, and that adhesion does not take place before germination and differentiation. It has subsequently been demonstrated that fungi take a more active role in adhesion, and the process may begin upon host contact, before appressorium development (eg. Kunoh, Nicholson and Kobayashi, 1991).

Attachment of fungal spores and germ tubes to the surface would appear to be an essential pre-infection event, determining the success of infection (Kunoh *et al.*, 1991; Nicholson and Epstein, 1991). The release of enzymes or adhesives from spores or germlings may be crucial for differentiation and growth of fungal germlings, and may be associated with host recognition (Kunoh *et al.*, 1991).

Secretion of mucilaginous adhesive materials from germinating spores has been reported for numerous fungi, including the direct-penetrating basidiospores of numerous rusts, and many direct-penetrating non-rust fungi (e.g. Waterhouse, 1921; Gold and Littlefield, 1979; Landes and Hoffmann, 1979; Littlefield and Heath, 1979; Rijkenberg, de Leeuw and Verhoeff 1980; Metzler, 1982; Gray, Amerson and van Dyke, 1983). The gel-like material is usually deposited in a thin layer on top of the appressorium, accumulating laterally, and tapering off below the appressorium (Mendgen and Deising, 1993).

Basidiospores produce an extracellular matrix that accumulates along all the areas of contact between the host and fungus, especially at the periphery of the appressorium (Gold and Mendgen, 1984). This probably represents a non-specific contact response, as it has also been shown to occur at contact points between basidiospores (Jacobi, Amerson and Mott, 1982). Mims and Richardson (1989), found that basidiospore germlings of *Gymnosporangium juniperi-virginianae* Schw. produced greater amounts of extracellular matrix on dialysis membrane than on the host leaf, indicating that the germlings can modify the amount of material required for adequate adhesion to the substrate (Mims and Richardson, 1989).

Swann and Mims (1991) observed the presence of an electron dense, extracellular material surrounding the base of the direct-penetrating aeciospore germling appressoria of *A. peckianus*, which they deduced was responsible for adhesion to the substrate upon which the spores were germinated. The material was associated with the germ tubes as well, being most prominent at the sites of contact with the substrate.

Koch and Hoppe (1988) felt that these extracellular exudates may be a feature associated with direct penetration in particular, but there have been many reports of these substances being produced by the urediospores and germlings of indirect-penetrating rust fungi, e.g. *Uromyces viciae-fabae* (Pers.) Schroet. (Deising, Nicholson, Haug, Howard and Mendgen, 1992; Clement, Martin, Porter, Butt, and Beckett, 1993) and *Puccinia hordei* G. Otth. (Read, Kellock, Knight and Trewavas, 1992).

The mechanisms by which adhesion is mediated are poorly understood (Mendgen and Deising, 1993). Very often, the properties of the surface upon which the spores are germinated, has an effect on adhesion, e.g. the general response of some rusts of graminaceous hosts is that they adhere more tightly to hydrophobic, rather than hydrophillic surfaces (Wynn and Staples, 1981).

1.2.2.1 The role of attachment

The extracellular, adhesive matrix may have a number of important functions, such as:

- attachment of the fungal structures to the epidermis, preventing displacement by wind and water (Gold and Mendgen, 1984; Nicholson and Epstein, 1991);

- establishing close contact with the host, enabling the sensing of topography and thigmodifferentiation to take place (Nicholson and Epstein, 1991);

- 'sealing off' of the penetration site, and preventing the movement of toxins and enzymes away from the penetration site (Gold and Mendgen, 1984; Nicholson and Epstein, 1991);

• protection of the germ tube and appressorium against desiccation and other limiting environmental factors (Gold and Mendgen, 1984);

• a reservoir for 'penetration enzymes' (Gold and Mendgen, 1984).

Plant waxes may play an important role in the attachment of spores to the leaf surface. Wynn (1981) showed that *Puccinia sorghi* Schwein. germlings grew away from the leaf surface and failed to recognise stomata on waxless corn leaves, and *Puccinia coronata* Corda appeared to be bound to the leaf surface by a combination of fungal secretions and dissolved host wax. When the appressoria of many fungi are removed from the host leaf surface, a definite wax-less area or imprint in the cuticle can be seen, presumably as a result of the adhesion and subsequent stripping of the underlying wax layer beneath the infection structures (eg. Lewis and Day, 1972; Staub, Dahmen and Schwinn, 1974; Garcia-Arenal and Sagasta, 1980; Hau and Rush, 1982).

Lewis and Day (1972) suggested that the waxless areas under the germ tubes of *Puccinia graminis* could be attributed to adherence of the wax crystals to the hyphae. Nicholson and Epstein (1991) suggested that such imprints are the result of cuticular adhesion to the infection structures, implying that the fungal-cuticle bond is stronger than the bond between the cuticle and the leaf. An alternative theory is that the imprints are the result of enzymatic dissolution of the cuticle (Nicholson, 1984; Kunoh, Yamaoka, Yoshioka and Nicholson, 1988; Nicholson, Yoshioka, Yamaoka and Kunoh, 1988). Nicholson and Epstein (1991) postulated that this cuticular erosion could improve attachment of the infection structures to the leaf surface in two ways - firstly it could help to 'embed' the infection structures into the leaf surface, and secondly, it could alter the leaf surface in such a way as to create new sites for chemical bonding.

It is possible that if the wax crystals and other cuticular components at the site of spore germination and prospective penetration cannot be dissolved, then the close contact required for penetration and differentiation may not be achieved (Kunoh *et al.*, 1991). Cuticular erosion may be important in the initial preparation of the infection court to provide a surface that is recognized by the pathogen as amenable to penetration (Nicholson and Epstein, 1991).

However, in some fungi, e.g. in basidiospores of *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*, there is no apparent disruption of the wax surface beneath the appressoria, except at the point of actual penetration by the penetration peg (Gray *et al.*, 1983).

1.2.2.2 Composition of the extracellular matrix

Adhesive substances analyzed to date appear to be composed largely of polysaccharides, proteins or glycoproteins (Mendgen and Deising, 1993).

Cutinase, and possibly esterase activity, could alter the leaf surface and produce a surface with different adhesive properties (Nicholson and Epstein, 1991). The involvement of enzymes present in the extracellular matrix is difficult to prove (Mendgen and Deising, 1993), but erosion of the cuticle at the site of appressorium attachment has been attributed to the activity of hydrolytic enzymes such as cutinases and esterases (Deising *et al.*, 1992).

1.2.3 Growth and differentiation of pre-infection structures

1.2.3.1 Directional germ tube growth and penetration site selection

Johnson (1934) was probably the first to observe that the growth of germ tubes of indirect-penetrating fungi, very often occurs at right angles to the depressions on the epidermis created at the anticlinal junctures. He surmised that this growth habit, which he observed in *Puccinia graminis* f. sp. *tritici* on wheat leaves, increased the germling's chances of locating a stomatal opening in the leaf, since the stomata on graminaceous hosts are staggered in longitudinal rows, parallel to the leaf venation and the epidermal cell orientation. This phenomenon has been observed in a number of indirect penetrators (Dickinson, 1949, 1971; Lewis and Day, 1972; Wynn, 1976; Wynn, 1981; Staples, Hoch, Epstein, Laccetti and Hassouna, 1985). Edwards and Bowling (1986) found evidence to suggest that pH gradients may also be involved in growth orientation in *Uromyces viciae-fabae*.

It would make sense for germlings infecting dicotyledonous hosts to utilise a host stimulus other than the epidermal cell junctions when locating a stoma, as the epidermal cell arrangement in dicotyledonous plants is not as regular as in monocotyledonous plants. In the case of the bean (*Phaseolus vulgaris* L.) however, even though the epidermal cell junctions are not parallel, the germ tubes of *Uromyces appendiculatus* (Pers.) Ungervar. *typica* have been shown to grow at right angles to the larger ridges formed by the curvature of the epidermal cells (Wynn, 1976).

The basidiospore germ tubes of some rust species would appear to grow randomly over the host surface (Hansen and Patton, 1977; Gray *et al.*, 1983; Morin, Brown and Auld, 1992). Aside from the tendency to grow along an epidermal junction once it has been reached, there is no convincing evidence that directional germ tube growth occurs in direct-penetrating rusts. This is understandable since direct penetrators have only to grow across a single epidermal cell to find a suitable penetration site.

It would seem that many direct-penetrating fungi respond to the indentations between epidermal cells, and direct-penetrating germlings often form appressoria preferentially at or near these epidermal cell junctions (Hunt, 1968; Bonde, Melching and Bromfield, 1976; Wynn and Staples, 1981; Bonde, Bromfield and Melching, 1982; Gold and Mendgen, 1984; Staples and Macko, 1984; Morin *et al.*, 1992). According to Wynn and Staples (1981), at least 22 species in 14 genera have been documented to form appressoria frequently at cell junctions, and they regarded this penetration site selection to be a generalised phenomenon amongst the direct-penetrating fungi.

However, as Rijkenberg *et al.* (1980) illustrated in their studies of fungal infection from conidia of *Botrytis cinerea*, even if appressorium formation does occur at a particular site on the host, it does not necessarily mean that the infection will be successful and lead to the proliferation of the fungus. In their study, Rijkenberg *et al.* (1980) found that although approximately 40 % of the germlings of *B. cinerea* attempted penetration at the epidermal cell junctions of tomato fruit, none of these infections proved to be successful. With the aid of transmission electron microscopy, they demonstrated that only germlings that penetrated the centre of the epidermal cell surfaces, successfully infected the host tissue.

Bonde *et al.* (1976), found that approximately 85% of the *Phakopsora pachyrhizi* appressoria they observed, developed over the anticlinal walls separating adjacent epidermal cells. Seventy-five percent of appressoria of basidiospore germlings of *U. appendiculatus* were found to form within 3.5 µm of the anticlinal wall junctions, although some apparently exhibited random growth on the leaf surface (Gold and Mendgen, 1984).

Cutler, Alvin and Price (1982) suggested that this site preference may be related to the greater availability of nutrients and moisture due to a higher rate of exosmosis in these areas and/or the chemical composition and physical structure of the wax layer that affects both the pH and wettability of the leaf surface. The increased 'leakiness' of these areas to exudates from within the leaf, may result from the fact that cuticular waxes are often thinner towards the epidermal cell junctions (Schönherr and Bukovac, 1970), and that the anticlinal walls serve as a wick for the exudates (Hoch and Staples, 1991). Germ tubes of direct-penetrating germlings of a number of different fungi show a tendency to grow along these junctions prior to appressorium formation, and this behaviour may be induced by either contact or chemical responses by the germ tubes (Wynn and Staples, 1981). Morin *et al.* (1992) suggested that the marked tendency of *Puccinia xanthii* Schw. to end germ tube growth or to produce appressoria near epidermal cell junctions, may be related to the fact that basidiospores tend to be deposited mainly in the cleavage line between cells after their natural discharge from metabasidia.

The appressoria of many indirect-penetrating rust fungi apparently enhance their attachment to the leaf surface by wedging their bases between the outer edges of the stomatal opening (Mims, Taylor and Richardson, 1989). Perhaps direct- penetrating species have a preference for epidermal cell junctions because these sites afford greater purchase for attachment. The 'valley' would allow a greater surface area of appressorium to be in contact with the cuticle.

An interesting pattern that emerges when comparing the germ tube lengths of direct and indirect-penetrating rust species/spore stages, is that in direct penetrators, the germ tubes are on average much shorter or even absent. After comparing two stoma-penetrating rusts with two direct-penetrating rusts, Hunt (1968) found that the length of the germ tube may be related to the mode of penetration. He found that the average germ tube length produced by the stomatal penetrator *Sphaerophragmidium acaciae* (Cke.) Magn. was over ten spore diameters, whereas that of the direct penetrator *Puccinia psidii* Wint., was 1-4 spore diameters. *Ravenelia humphreyana* P. Henn., must rank as the least specific rust in terms of infection site selection. It does not appear to show any preference for particular areas on the host epidermis, as evidenced by its germ tubes being typically short or absent. Basidiospores of *U. appendiculatus* var. *appendiculatus* were observed, by Gold and Mendgen (1984), to produce short germ tubes before the differentiation of appressoria. About 60 % of the germ tubes were shorter than the diameter of a spore.

The reduced selectivity of direct penetrators in penetration site selection, allows them to reduce the amount of energy spent on germ tube growth.

1.2.3.2 Classification of appressoria

Typically, both in the direct and indirect penetration processes, penetration occurs from appressoria. The only well substantiated exceptions known to the present author occur in some basidiospore germlings which penetrate indirectly through stomatal openings, via the extension of their germ tubes without the formation of appressoria (e.g.

Cronartium ribicola; Patton and Johnson, 1970).

Generally, rust fungi (Uredinales) form two types of appressoria. Urediospore and aeciospore germlings produce a large, well-defined swelling which is delimited from the germ tube by a septum, whereas basidiospore germlings usually give rise to a 'protoappressorium' (Emmett and Parbery, 1975), which may be little more than a swollen germ tube tip, typically not delimited by a septum.

When no septum delimits the appressorium, then the distinction between the germ tube and appressorium becomes vague (Emmett and Parbery, 1975). Sometimes, virtually no swelling of the germ tube tip occurs, and it is only in a functional sense that appressorium formation occurs. The broadest definition of an appressorium given by Emmett and Parbery (1975) is based on the capacity of a structure to adhere to a host surface, and its ability to 'germinate' and penetrate the host.

1.2.3.3 Differentiation of appressoria

Considerable research has been conducted on the factors leading to the differentiation of infection structures from urediospore germ tubes (e.g. Wynn and Staples, 1981; Staples and Hoch, 1984; Staples and Macko, 1984; Staples *et al.*, 1985; Hoch, Staples, Whitehead, Comeau and Wolf, 1987b; Allen, Hazen, Hoch, Kwon, Leinhos, Staples, Stumpf and Terhune, 1991). Unfortunately, very few investigations have been made into the factors leading to the differentiation of appressoria in basidiospore, aeciospore and direct-penetrating urediospore germlings.

Various chemotropic, thigmotropic and environmental conditions signal the induction of appressoria in indirect-penetrating rust fungi, and the importance of each stimulus would appear to vary from species to species (Allen *et al.*, 1991). Studies on a variety of rust fungi indicate that the entire infection process, from development of the appressorium to the infection hypha, is triggered by a single stimulus (Wynn and Staples, 1981).

As mentioned previously, close contact with the substrate is very important for the induction of appressoria. Koch and Hoppe (1988) investigated the role of close contact between *Phakopsora pachyrhizi* germlings and nitrocellulose filters with different pore sizes, in the differentiation of infection structures. On filters with large pore sizes (0.20 - 0.65 µm), only a limited portion of the growing germ tube was touching the substrate, and close contact could not be established. Appressorium formation was negligible on these surfaces. On the other hand, when spores were germinated on filters with smaller pores (0.10 - 0.01 µm), there was closer contact between the germ tubes and the filter surface, and more than 90% of the germinated spores developed appressoria.

Close adhesion of the infection structures is especially important for the detection of topographical signals on the host surface.

1.2.3.3.1 *The role of topography in differentiation*

A great deal of research has been directed towards the mechanisms involved in the sensing of host topography, and the subsequent differentiation of infection structures. Staples and Macko (1984) introduced the term 'thigmodifferentiation' to describe the inductive properties of topography in appressorium formation, as opposed to 'chemodifferentiation', the response to chemical signals. According to Mendgen and Deising (1993), Büsgen, in 1893, was one of the first to study the role of the stoma in the induction of appressorium formation in rust fungi. Since then, many experiments have been conducted to characterise the stimuli and mechanisms involved in appressorium induction and formation.

Many of these studies have made use of artificial membranes. These membranes provide a valuable means of isolating or identifying the roles of topography in infection structure induction. Scratches or precisely defined ridges on artificial substrates have induced appressorium formation in many indirect-penetrating rust fungi, apparently by mimicking the stomatal ridges present on the host guard cells (e.g. Allen *et al.*, 1991). Dickinson (e.g. 1949, 1971) was the first to utilise artificial substrates in this context.

Generally, infection structures produced by rust fungi on artificial substrates, have an identical morphology to those produced on/in the leaf, and nuclear behaviour appears to correlate in most cases (Mendgen and Deising, 1993). However, Mendgen and Deising (1993) advise caution in the interpretation of *in vitro* results, as there are fungi (e.g. *Colletotrichum lindemuthianum* (Sacc. & Magn.) Br. & Cav.) that produce abnormal structures on artificial substrates.

Bourett, Hoch and Staples (1987) suggested that because appressoria can be induced by both ridges and furrows, the signal required for induction may be a combination of angular changes in topography or elevation.

Virtually all studies in this area of research have focused on the indirect-penetrating species, where thigmodifferentiation forms a major part of the infection process. However, Allen *et al.* (1991) conducted a survey of the responses of 27 rust species to ridges of various heights on artificial membranes, and this included an investigation of two of the direct-penetrating rust species, i.e. *Physopella zeae* (Mains) Cumm. & Ramachar and *P. pachyrhizi*.

P. zeae was found to form appressoria in response to ridges of 0.11 µm and higher, with percentage appressorium formation increasing with ridge height, up to about 2.24 µm. At ridge heights of 0.8 µm and higher, this fungus tended to form appressoria beside the ridges, as opposed to on top of them. Furthermore, 94% differentiation of appressoria occurred in response to scratched polystyrene membranes.

The second direct penetrator studied by Allen *et al.* (1991), *P. pachyrhizi*, formed appressoria readily on ridges of all heights tested (90-100%), as well as on both smooth polystyrene membranes (75%) and silane-treated glass (97%). Appressoria formed on smooth areas between, beside and on ridges in approximately equal proportions. The authors concluded that differentiation occurred 'in loose association with the ridges'. This is in contrast to the findings of Koch and Hoppe (1988), who reported no significant difference between the numbers of *P. pachyrhizi* appressoria developing on scratched and unscratched dialysis membranes, and who found no indication of specific germ tube differentiation in contact with grooves of scratched membranes.

When Allen *et al.* (1991) germinated urediospores of *P. pachyrhizi* on polystyrene replicas of both *Glycine* and *Phaseolus* leaf surfaces, urediospore germ tubes formed appressoria at the junctions between the epidermal cells.

The fact that *P. pachyrhizi* forms appressoria readily on both ridged and unridged topographies, would suggest that appressoria form primarily in response to an unspecific surface contact stimulus, but the preference for epidermal cell junctions would suggest that it does sense surface topography to some extent.

Koch and Hoppe (1987) described how they had previously demonstrated the lack of host specificity in the induction stimuli required for differentiation of *P. pachyrhizi* germlings, when they found high numbers of appressoria forming on non-hosts such as wheat, lettuce and potato.

It would seem that the critical and possibly most specific stage of infection by *P. pachyrhizi* is the development of the penetration hypha, as this occurs at very low rates on non-host surfaces (Koch and Hoppe, 1987). Koch and Hoppe (1988) suggested that a second stimulus is required in *P. pachyrhizi* to trigger the formation of the penetration hypha.

The induction of infection structure formation by basidiospore germlings *in vitro* may depend greatly on the hardness of the substrate (Gold and Mendgen, 1991). However, Hoch and Staples (1987) thought it unlikely that fungi have mechanisms to detect differences in substrate hardness, and suggested that factors such as rigidity, porosity of the substrate to ions and metabolites, and surface energy, i.e. hydrophobicity of the surface, may play a greater role in determining the inductive properties of a substrate to appressorium formation. Porosity may be of importance in terms of the diffusion of fungus-leaked compounds. Accumulation of these compounds may signal appressorium formation.

Infection structures from basidiospores can form on a wide range of artificial membranes, indicating that surface recognition in the haploid stage is not very specific (Gold and Mendgen, 1991).

Aeciospore germlings of *A. peckianus* would also appear to have very unspecific thigmotropic requirements for appressorium differentiation, i.e. the signal would simply seem to be contact of the germ tube tip with a solid surface (Swann and Mims, 1991).

The apparently wide range of inducive thigmotropic stimuli that are detected by many of the direct penetrators, may have significance in terms of their adaptation to a wider host range than those rusts which only produce appressoria in response to a very specific and narrow range of conditions (Allen *et al.*, 1991). Perhaps this is exemplified in *P. pachyrhizi*, which, according to Keogh[5] (Bonde *et al.*, 1976), is known to infect many leguminous species, in numerous orders of the family Leguminosae.

Exactly how a germ tube can sense minute changes in surface topography has not been clearly elucidated. However, the mechanisms involved may include the different components of the cytoskeleton and/or an ionic or electric change mediated by mechanosensitive channels (Zhou, Stumpf, Hoch and Kung, 1991; Read *et al.*, 1992).

Sensing and recognition of the host plant appears to take place at the hyphal tip (Hoch and Staples, 1991), although this may vary between different rust species (Allen *et al.*, 1991). Within the apical dome, a cluster of vesicles occurs in an area devoid of other organelles (Mendgen and Deising, 1993). The tips of fungal hyphae are very sensitive to disruptions, and upon detecting these, they immediately cease growth and vesicles associated with the tip disperse (Heath, 1987).

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5 Keogh, R.C. 1974. Studies on *Phakopsora pachyrhizi* Syd.: The causal agent of soybean rust. M.S. Thesis, The University of Sydney, Australia. 95 p.

In urediospore germ tubes of *Uromyces appendiculatus*, the vesicles concentrate near the substrate in a position where they can be influenced by minor irregularities in surface topography (Kwon, Hoch and Aist, 1991). The dispersed vesicles become distributed peripherally in the general region of the cell tip, resulting in the ballooning out of the hyphal apex, essentially as a result of lateral growth from the fusion of vesicles with the plasmalemma (Staples and Macko, 1984). The redistribution of vesicles may be guided by the microtubule and/or F-actin microtubule cytoskeleton (Kwon *et al.*, 1991).

The microtubule and microfilament cytoskeleton would seem to play an important part in the sensing of topography by the germling, and also in the differentiation process. During the early stages of appressorium development in *U. appendiculatus*, many of the individual elements become oriented parallel to the inducing signal (eg. a microfabricated ridge on an artificial substrate), and when germlings were treated with microtubule-depolymerizing chemicals, griseofulvin, nocodozole or vincristine sulfate at the time of signal reception, appressorium formation was prevented (Hoch, Bourett and Staples, 1986). The F-actin component of the cytoskeleton does not appear to play a direct role in signal reception for appressorium differentiation, but it is central in the processes leading to septum formation, and hence delimitation of the appressorium (Tucker, Hoch and Staples, 1986), and it may play a secondary role in signal perception (Hoch and Staples, 1991).

Generally, the entire process from cessation of germ tube growth to completion of the appressorium (septum formation and nuclear division) occurs within about 50-60 minutes, depending on environmental conditions, and the appressorium 'matures' for another 30-60 minutes before penetration peg formation is initiated (Hoch and Staples, 1991). The exact time taken for basidiospore appressorium formation and maturation does not appear to have been investigated. Possibly, development is a quicker process in appressoria which are not delimited by a septum.

According to Hoch and Staples (1991), development of appressoria in most fungi is similar with respect to the initial changes involved in growth cessation and apical swelling of the germ tube. It is in the subsequent morphological, pigmentation and functional specialisations that the processes differ between species.

1.2.3.3.2 *Chemical induction of differentiation*

Some chemical stimuli which have been discovered to play a role in induction of appressorium formation include:

- metabolites extracted from urediospores (Macko, Renwick and Rissler, 1978);
- volatiles from the host plant (Grambow, 1977);
- ions, such as K^+ and Ca^+ (Kaminsky and Day, 1984; Hoch and Staples, 1987; Stumpf, Leinhos, Staples and Hoch, 1991);
- sucrose (Kaminsky and Day, 1984; Hoch and Staples, 1987; Stumpf *et al.*, 1991);
- cyclic nucleotides or stimulants of adenylate cyclase (Hoch and Staples, 1983).

Potassium ions can stimulate the formation of appressoria at pH 7.0, even when the germ tubes grow away from the substrate (Hoch, Staples and Bourett, 1987a).

Even though, through the use of inductive artificial substrates, it is known that the germlings of many rust fungi can differentiate appressoria in the absence of the host, Allen (1957) and Grambow (1977) have provided strong evidence to suggest that in some cases, e.g. *Puccinia graminis*, the chemical environment around the stoma may be equally as effective at inducing appressorium formation as the thigmotropic stimulus of the guard cell lip.

1.2.3.3.3 *Environmental factors affecting differentiation*

Environmental factors including temperature, light etc. may play a greater or lesser role in appressorium differentiation, depending on the species of rust fungus involved. In some species, appressorium formation is only slightly influenced by environment, while in others, there is a need for a fairly well-defined conducive environment (Emmett and Parbery, 1975).

Heat shock has been demonstrated to induce appressorium differentiation in some rust fungi (Maheshwari, Allen and Hildebrand, 1967; Staples, Hoch, Freve and Bourett, 1989).

1.2.3.4 The function of appressoria

There do not appear to be any definite theories as to the exact function of appressoria. In some direct-penetrating fungi, such as many of the anthracnose fungi and *Magnaporthe grisea* (Herbert) Barr (rice blast fungus), the appressoria become melanised. It has been experimentally demonstrated that the melanin enables great turgor pressure to be built up in the appressorium, and this presumably facilitates the application of mechanical pressure on the penetration site (Howard and Ferrari, 1989; Howard, Ferrari, Roach and Money, 1991).

Perhaps the accumulation of the spore cytoplasm over a small surface area above the penetration site, in the form of an appressorium, can be seen as a 'focusing' or 'pooling' of the germling's activity, creating an increased inoculum potential. Concentration of the cytoplasm may bring all the organelles, elements of endoplasmic reticulum, etc. closer to the penetration site, facilitating the rapid transport of enzymes, wall materials and other substances that are needed for penetration, as opposed to moving them along the length of the germ tube. Presumably this would be an energy-saving strategy.

Emmett and Parbery (1975) believed that appressoria play a very important role in the survival of the fungus on the leaf surface. They regarded this to be an auxiliary role to infection, and not a secondary one. This aspect is of greater significance in some direct-penetrating fungi such as the anthracnose fungi, where the appressorium may remain attached to the substrate for many weeks prior to infection. In rust fungi, appressoria are not long-term survival structures, but are sometimes able to endure conditions of light intensity, desiccation or antagonism potentially lethal to unprotected germ tubes or hyphae during penetration (Emmett and Parbery, 1975). This may be a result of the decreased surface area to volume ratio of the appressorium, as compared to a germ tube.

1.2.4 The development of infection structures within the host

In the indirect-penetrating species of rust fungus, host invasion from urediospores and aeciospores can be summarised as follows: a spore germinates to produce a long and usually unbranched germ tube which swells apically to form an appressorium over a stoma on the host leaf; a short hypha develops from the base of the appressorium, and this penetrates between the guard cells of the stoma, enlarging to form a substomatal vesicle in the chamber beneath the stomatal pore; primary and secondary hyphae grow out into the leaf tissues and, upon contact with the mesophyll cells, the tips of the intercellular hyphae form haustorial mother cells which are delimited by a septum; an infection peg develops from the base of each haustorial mother cell, and penetrates the host cell wall; each establishes an haustorium in a leaf cell (Littlefield and Heath, 1979; Mendgen and Deising, 1993).

Therefore, in the indirect-penetrators, intracellular structures are only formed at a relatively late stage in the infection process, following haustorial mother-cell formation. In direct-penetrating rust fungi however, the first structures formed after penetration are intracellular structures. The morphology and sequence of development of these structures, as well as the subsequent colonisation of the leaf tissue, varies with both the species and ontogenetical stage of the rust fungus.

1.2.4.1 Direct penetration from basidiospores

As previously mentioned, basidiospore-derived infections have recently received less attention than those involving urediospores. Gold and Mendgen (1991) felt that this may be at least partly due to the difficulties experienced in inducing teliospore germination, and therefore basidiospore formation, in the economically important rusts. Gold and Mendgen (1984) found 18 reports of direct penetration from the basidiospores of different rust species. All except one occurred on angiosperms. They found five reports of indirect penetration from basidiospores, and all but one species (*Puccinia arenariae* (Schum.) Wint.), produced infections on gymnosperms. An example of a more recent report on the development and nuclear behaviour of basidiospore germlings, was by Heath, Xu and Eilam (1996) who investigated and compared the urediospore and basidiospore germlings of the cowpea rust fungus, *Uromyces vignae* Barclay.

In direct-penetrating species, a basidiospore germ tube usually gives rise to an appressorium under suitable conditions, and penetration commences from the base of this structure. In indirect-penetrating species, which typically infect gymnosperm hosts, no appressoria are formed, and the germ tube grows through the stomatal pore (Gold and Mendgen, 1991), thereby avoiding the need to penetrate the thick cuticle and cell walls of the epidermis. The hypodermis, a characteristic of the tough gymnosperm leaves, is discontinuous beneath stomatal openings.

Rust fungi with basidiospore germlings that have been reported to penetrate the host cuticle directly, include:

- *Puccinia coronata* Corda (Allen, 1932b);
- *Puccinia graminis* Pers.:Pers. (Waterhouse, 1921; Allen, 1930);
- *Puccinia triticina* Eriks (Allen, 1932a);
- *Puccinia xanthii* Schw. (Morin *et al.*, 1992);
- *Melampsora lini* (Ehrenb.) Desmaz. (Allen, 1934);
- *Puccinia malvacearum* Bert. (Allen, 1935);
- *Gymnosporangium fuscum* DC. (Metzler, 1982)
- *Cronartium quercuum* (Berk.) Miyabe ex Shirai f.sp. *fusiforme* (Gray *et al.*, 1983);
- *Uromyces appendiculatus* (Pers.) Unger (Gold and Mendgen, 1984);

- *Gymnosporangium juniperi-virginianae* Schw. (Mims and Richardson, 1989);
- *Uromyces vignae* Barclay (Heath *et al.*, 1996).

In most cases, direct penetration from basidiospores is effected from an appressorium, which is differentiated from a usually short germ tube. As discussed earlier, production of a mucilagenous adhesive material around the appressoria and germs tubes has been reported for many basidiospore germlings. The appressoria produced by basidiospore germlings are often no more than slight swellings of the germ tube tip, and in no cases has a septum been observed to cut off an appressorium from the germ tube. Appressoria usually range from fairly well defined structures to virtually unenlarged germ tube ends, e.g. *P. xanthii* (Morin *et al.*, 1992) and *U. appendiculatus* var. *appendiculatus* (Gold and Mendgen, 1984). The appressoria of *Cronartium quercuum* germlings are irregular in size and are often barely differentiated from the germ tube apex, with Gray *et al.* (1983) describing them as being 'either undifferentiated from germ tubes or moderately expanded.'

Two apparent exceptions to this production of a germ tube and an appressorium from the basidiospore, have been described by Waterhouse (1921) and Allen (1932a) respectively. Waterhouse (1921), in his classical study on the infection of *Berberis vulgaris* L. from basidiospores of *P. graminis*, observed that infection took place either from the end of a germ tube, or from a short, beak-like outgrowth of the basidiospore. This beak-like structure was observed to press closely to the cuticle, and in some cases, caused an indentation on its surface. Growth of the beak-like hypha was also observed to force the end of the basidiospore away from the leaf surface. Infection was effected by 'a very fine style-like infection hypha', which reminded Waterhouse (1921) of the proboscis of an insect. The style pushed through the cuticle and underlying cellulose layers of the epidermal cell wall. Many of Waterhouse's (1921) findings were later confirmed by Allen (1930).

Allen (1932a) found that basidiospores of *Puccinia triticina*, on *Thalictrum flavum* L., fail to produce germ tubes and appressoria. Instead, penetration occurs directly from the basidiospore, although *P. triticina* does not seem to form a beak-like structure equivalent to that observed in *P. graminis*.

It is possible, based on the present author's examination of Allen's paper (1932a), that a cone-like structure forms in the base of the basidiospores of *P. triticina*, at the site of penetration peg emergence. It is also possible that Waterhouse (1921) inadvertently recorded the presence of an appressorial cone in one of his drawings of a *P. graminis* basidiospore penetrating the epidermis from one of the 'beak-like' structures described above. An electron microscope study of the 'beak-like' structure that Waterhouse (1921) refers to, would be very interesting, and could verify or disprove the presence of an appressorial cone. Production of an appressorial cone in the appressoria of *Gymnosporangium juniperi-virginianae* has also been reported (Mims and Richardson, 1989).

Following penetration of the epidermal cell wall, the basidiospore germling forms an expanded, ovate vesicle in the lumen of the host epidermal cell. This appears to be a development common to all the rust fungi under discussion here, viz. *P. graminis* (Waterhouse, 1921; Allen, 1930), *P. triticina* (Allen, 1932a), *P. xanthii* (Morin *et al.*, 1992), *C. quercuum* (Gray *et al.*, 1983), *U. appendiculatus* var. *appendiculatus* (Gold and Mendgen, 1984), *G. juniperi-virginianae* (Mims and Richardson, 1989), and *U. vignae* (Heath, 1989; Xu and Mendgen, 1991).

From the intraepidermal vesicle a primary hypha develops. A term sometimes used for the vesicle and the primary hypha together, is the invasion hypha (Heath, 1989; Xu and Mendgen, 1991). In some cases, the primary hypha is septate and hence multicellular, e.g. in *P. graminis* (Waterhouse, 1921; Allen, 1930), *P. triticina* (Allen, 1932a), and in other cases, it is cut off from the vesicle by a septum, e.g. in *P. xanthii* (Morin *et al.*, 1992), *U. appendiculatus* var. *appendiculatus* (Gold and Mendgen, 1984).

In basidiospore germlings of *Cronartium quercuum*, the penetration or infection hypha extends a short distance into the epidermal cell, before the formation of the vesicle, which Gray *et al.* (1983) call an expanded body. The infection hypha is then delimited from the vesicle by a septum. Sometimes the expanded body also contains a septum. The primary hypha that develops from the expanded body is not delimited by a septum, and does not branch prior to exiting the epidermal cell (Gray *et al.*, 1983).

In germlings of *P. graminis*, a secondary hypha grows from each segment of the multicellular primary hypha (Waterhouse, 1921). These secondary hyphae then exit the epidermal cell, where the mycelium proliferates and effects single-celled haustorium formation. A very similar pattern of development was observed by Allen (1932a) in germlings of *P. triticina*. The secondary hyphae produced by *P. triticina* usually passed directly into the intercellular spaces of the spongy mesophyll, but occasionally they first entered a palisade cell.

In cases where the primary hypha is aseptate, branches also develop, which then exit the epidermal cell directly into adjacent cells, or into the intercellular space of the mesophyll prior to M-haustorium formation. *C. quercuum*, in addition to forming M-haustoria, also forms intracellular hypha (Gray *et al.*, 1983).

Infection structures similar to those found in found in *C. quercuum* f.sp. *fusiforme* have been found in the direct-penetrating (basidiospore) stages of *Gymnosporangium juniperi-virginianae* Schw. (Nusbaum, 1935) and *Puccinia malvacearum* Bert. (Allen, 1935).

To summarize the morphological development of infection structures as typically occurs from basidiospores:

- an often poorly defined appressorium develops at the tip of the germ tube, and is not delimited from the germ tube by a septum;

- a penetation hypha develops from the base of the appressorium and penetrates directly into an epidermal cell;

• an elliptical vesicle develops inside the epidermal cell;

• a primary hypha develops from the vesicle, and extends futher into the epidermal cell;

• secondary hyphae develop from the primary hypha, and exit the epidermal cell, into the intercellular sapce of the mesophyll, or into adjacent pallisade cells;

• M-haustoria are formed from the intercellular mycelium.

1.2.4.2 Direct penetration from pycniospores

According to Hoch and Staples (1991), pycniospore germlings of *Melampsora lini* and *P. malvacearum* have been reported to penetrate the epidermal cells of hosts that these two fungi usually infect from basidiospores (Allen, 1934; Allen, 1935). No appressoria were reported to be involved, and the germ tubes apparently penetrated directly through the cuticle and epidermis, usually near junctions between epidermal cell walls. Occasionally they penetrated the guard cells.

This is a most peculiar report as, typically, pycniospores fuse with the flexuous hyphae in pycnia produced by a fungus of the opposite mating strain, and do not initiate penetration of a host leaf.

1.2.4.3 Direct penetration from aeciospores

To the present author's knowledge, only one species of rust fungus has been reported to penetrate the host epidermis directly from aeciospore-derived infection structures, viz. *Arthuriomyces peckianus*.

Pady's (1935) account of penetration from aeciospores of *Gymnoconia interstitialis* (Schl.) Lagerh. (*A. peckianus*), on *Rubus* sp., was one of the earliest reports of direct, cuticular penetration from a dikaryotic rust spore stage. Well defined appressoria are delimited from the germ tube by a septum. A short penetration peg develops from the base of the appressorium, and penetrates directly through the cuticle and epidermal cell wall. When appressoria form over a stoma, Pady (1935) observed that penetration occurred through a guard cell, rather than via the stomatal pore.

Once the penetration peg reaches the epidermal cell lumen, an intracellular penetration hypha is formed. Growth in the epidermis is limited, the hypha growing along the floor of the infected epidermal cell for a short distance, before passing through the cell wall into the intercellular spaces below. Penetration of the epidermal wall is associated with a narrowing of the penetration hypha.

At the site of hyphal emergence from the epidermal cell into the mesophyll, a short primary hypha develops. When the upper leaf surface is inoculated, the primary hypha branches into two secondary hyphae which penetrate deeper into the leaf tissue, through the palisade mesophyll layer, and into the spongy mesophyll. When the lower surface is inoculated, the mycelium spreads near the surface of the leaf in the large intercellular spaces of the spongy mesophyll. No septa appear to form until the fungus branches in the mesophyll. Pady (1935) only observed the formation of haustoria 5 dpi (days post inoculation).

Swann and Mims (1991) gave an account of their observations of the penetration processes employed by aeciosporelings of *A. peckianus* germinated on dialysis membranes.

Fully developed appressoria, which are delimited from the germ tube by a septum, are usually present 9 hpi. The region of the appressorium of *A. peckianus* appressed against the dialysis membrane substrate, apparently lacks a cell wall. This particular area of the cell wall appears to simply taper away to nothing, leaving a circular region in which the plasma membrane is in direct contact with the underlying dialysis membrane. The wall-less regions typically measure between 3 and 4 μm in diameter.

Large numbers of vesicles are present in the base of the appressoria of *A. peckianus* in the vicinity of the wall-less region (Swann and Mims, 1991). These appear to be derived from numerous simple cisternal elements, possibly representing Golgi equivalents, and many of the vesicles appear to fuse with the plasma membrane.

Other cytoplasmic components of the appressorium include numerous lipid droplets, mitochondria and microbodies, strands of endoplasmic reticulum, and aggregates of darkly staining particles which were thought to represent glycogen.

In the centre of the wall-less region, a 'curious funnel-like or cone-like structure' develops 8-9 hpi. Initially, in cross section, the appressorial cone appears as two, separate, electron-transparent plasma membrane-lined projections that extend a short distance into the cytoplasm of the appressorium. As the cone develops, microsections of the appressorium made at right angles to the membrane, reveal that the projections diverge slightly from each other as they extend further into the appressorium. Microsections made parallel to the underlying membrane, indicate that the projections are not separate, but rather part of a single cone-shaped structure.

Pady (1935) was apparently also able to observe the presence of an appressorial cone in appressoria of *A. peckianus*, as this structure is clearly visible in his drawings from the light microscope. He did not, however, comment on its presence.

Initially, in appressoria of *A. peckianus*, the plasma membrane is closely appressed to both the inner and outer surfaces of the developing cone (Swann and Mims, 1991). However, in well-developed cones, the plasmamembrane lining the inside of the cone forms extensive branched elaborations that are filled with a finely granular material of moderate electron density. This material merges with the electron-transparent material comprising the bulk of the cone. Extensive elaborations of the plasma membrane originate from the point of contact of the plasma membrane and the dialysis membrane, and extend far up into the cytoplasm of the appressorium. These membranous elaborations have not been reported in appressoria of other basidiospore germlings

(Swann and Mims, 1991). There is some evidence, however, that they occur in the appressoria of urediospore germlings of *P. pachyrhizi*.

Unfortunately, urediospores of *A. peckianus* have not yet been found. An examination of the urediospore stage would provide an interesting opportunity to make comparisons between the infection processes of two dikaryotic stages, at least one of which is a direct penetrator.

To summarize the morphological development of infection structures:

- an appressorium is cut off from the germ tube of the aeciospore by a septum;

- a short penetration peg develops from the base of the appressorium and penetrates the cuticle and epidermal cell wall beneath;

- a penetration hypha traverses the epidermal cell, and penetrates the opposing epidermal cell wall;

- a short primary hypha grows into an intercellular space in the mesophyll, and branches to form secondary hyphae;

- the fungal mycelium ramifies through the intercellular spaces of the spongy mesophyll, establishing dikaryotic haustoria in the host cells.

1.2.4.4 Direct penetration from urediospores

Hunt (1968) was probably the first to document direct appressorial penetration from urediospore germlings. To the present author's knowledge, only five rust fungi have been reported to penetrate the cuticle from urediospore-derived infection structures, although Bolley and Pritchard (Pady, 1935) apparently discovered that urediospore germlings of *Puccinia rubigo-vera* (DC.) Wint. sometimes penetrate the cuticle without the formation of appressoria. It appears that *P. rubigo-vera* was subsequently divided into a number of species, including *Puccinia recondita* (syn . *P. triticina*) and *Puccinia glumarum* (Schm.) Eriks and Henn. (syn. *P. striiformis*) (Allen, 1928). *P. triticina* is known to penetrate indirectly, through stomata, following appressorium formation in the uredial stage (e.g. Torabi and Manners, 1989). However, Allen (1928) gives an account of penetration from urediospores of *P. striiformis*, and clearly illustrates that although penetration does occur through stomata, it occurs without the formation of appressoria.

Direct penetration from urediospores has been reported for the following rust fungi:
- *Puccinia psidii* Wint. on rose apple (*Syzygium jambos)* (Hunt, 1968);
- *Ravenelia humphreyana* P. Henn. on *Caesalpinia pulcherrima* (Hunt, 1968);
- *Physopella zeae* (Mains) Cumm. & Ramachar on *Zea mays* (Bonde *et al.*, 1982);
- *Phakopsora pachyrhizi* Syd. on soybean (*Glycine max*) (Bonde *et al.*, 1976);
- *Phakopsora apoda* (Har. & Pat.) Mains on kikuyu grass (*Pennisetum clandestinum*) (Hall, 1991).

Puccinia psidii has a very unique mode of infection, remaining intercellular at all stages, except for the formation of haustoria.

At 17°C, within 6 hours of host inoculation, most spores have developed germtubes, and by 12 hpi, terminal appressoria can be observed (Hunt, 1968). Germ tubes vary in length from 15 to 400 µm, although the majority are less than two spore diameters in length (Hunt, 1968).

Following appressorium development, a narrow infection peg develops at the base of the appressorium, growing down between two epidermal cells, or less commonly, at the junction between three epidermal cells (Hunt, 1968). The appressorium is larger than the average epidermal cell, and therefore at least part of the appressorial base is always positioned above the anticlinal walls of two or more epidermal cells.

Whilst penetrating the cuticle, the penetration peg has a very slender appearance, and is circular in cross-section. However, whilst growing intercellularly between the epidermal cells, it enlarges, adopting an ellipsoidal cross-section if between two epidermal cells, or a roughly triangular shape when between three cells (Hunt, 1968).

Once the epidermis has been traversed, the hypha usually continues growing deeper into the leaf tissue, pushing its way intercellularly, in as near a straight line as the cell arrangement will allow. Occasionally, especially if penetration occurs near a stoma (e.g. between a guard cell and a subsidiary cell), the penetration hypha may grow para-epidermally, between the mesophyll and epidermis, often entering a substomatal chamber before penetrating deeper into the tissues. During this process, the cell contents move out of the appressorium and into the infection hypha, with the development of a septum within the depth of the epidermis, cutting off the empty appressorium.

Germ tubes have been observed to grow directly across stomatal pores, forming an appressorium partially over a guard cell. In these instances, the infection peg penetrates between the guard cell and an adjacent epidermal cell.

In Hunt's (1968) study, few infections led to the formation of haustoria within 72 hpi, but those that did develop in the mesophyll cells, were lobate.

To summarize the infection process initiated by urediospores of *Puccinia psidii* at a morphological level:

- an appressorium is delimited from the germ tube by a septum;

- a penetration peg develops from the base of the appressorium and penetrates through the cuticle, and between two/three epidermal cells;

• the penetration hypha enlarges slightly in the epidermal cell layer, and forms a septum within the depth of the epidermis;

• once in the mesophyll, the hypha branches intercellularly and haustoria develop from the mycelium.

Unlike *P. psidii*, ***Ravenelia humphreyana*** only produces intracellular fungal structures.

Urediospores of this rust fungus germinate more rapidly than those of *P. psidii*, with penetration occurring twelve hours after inoculation (Hunt, 1968).

A germ tube is typically short or absent, with appressoria frequently appearing to be sessile on the spore (Hunt, 1968). In those cases where a germ tube is absent, septum formation may occur in the constricted region of the germ pore (Hunt, 1968).

It is interesting to note that Hunt (1968) described the attachment of the various infection structures of of *R. humphreyana*, both to each other and to the cuticle, as being very fragile, as evidenced by the abundance of appressoria without spores and infection pegs without appressoria in his preparations of infected leaf material. This does not entirely correspond to the idea that attachment is a very important part of the penetration process, especially in the direct-penetrating rust fungi.

An infection peg develops from the base of the appressorium, and penetrates the outer wall of the underlying epidermal cell, entering the cell lumen. Even though Hunt (1968) does not comment on its presence, appressoria of *R. humphreyana* would appear to produce cone-like structures in the cytoplasm, associated with the development of a penetration peg. Appressorial cones have been clearly drawn into several of Hunt's (1968) illustrations of germlings penetrating the host leaf.

Hunt (1968) observed a host reaction similar to papilla formation in the powdery mildews (i.e. an inner thickening of the host cell wall e.g. Bushnell, 1972) at the site of penetration by the infection peg. In some cases, the infection peg failed to penetrate through to the cell lumen, and Hunt (1968) suggested that this may have been directly related to the increased thickness of the cell wall in a resistant plant reaction. In a susceptible plant however, the penetration peg usually continues to grow through the thickened wall, becoming larger in diameter, until it breaks into the cell lumen. Here it enlarges into a 'vesicular haustorium'. Within 24 hours, further irregularly lobed vesicles and finger-like processes grow out from the primary lobe. Upon contact with a host wall, these 'processes' swell slightly, and a fine peg penetrates through the wall of the infected epidermal cell, and directly into the lumen of an adjacent cell. In this way, adjacent mesophyll and epidermal cells become colonised.

In cells penetrated at a later stage (especially palisade cells), the fungus appears to be more hyphal than vesicular in form. Septa are relatively infrequent.

The fungus apparently remains permanently intracellular. Presumably, the closely packed nature of the epidermal and palisade cells of the leaves of the host plant, *Caesalpinia pulcherrima*, allows for the continuity of the intracellular fungal growth.

To summarize the infection process initiated by urediospores of *Ravenelia humphreyana* at a morphological level:

- a well defined appressorium is delimited from the germ tube by a septum;

- a penetration peg emerges from the base of the appressorium, and penetrates through the cuticle and cell wall beneath;

- the penetration peg enlarges in the cell lumen to form a vesicular haustorium;

- further lobes develop from the primary lobe, and these exit the epidermal cell and penetrate adjacent epidermal and mesophyll cells, thereby establishing the fungal mycelium.

According to Bonde *et al.* (1982), urediospores of ***Physopella zeae*** often produce very short germ tubes, with the formation of appressoria over the junctions between anticlinal walls of adjacent epidermal cells. They described the penetration process as follows.

A penetration peg develops from the underside of an appressorium, and enters the leaf at the junctions between epidermal cells. The peg proceeds to enter one of the epidermal cells through an anticlinal wall, and forms a primary hypha (usually elliptical in profile) within the host cell. Occasionally, the fungus penetrates an epidermal cell wall at the leaf surface, rather than first penetrating between adjacent epidermal cells, and then entering via an anticlinal wall.

Secondary, intracellular hyphae develop from the sides or tip of the primary hypha, and colonise adjacent epidermal and mesophyll cells, establishing the fungal mycelium in the leaf tissue. No intercellular hyphae were observed by Bonde *et al.* (1982).

To summarize the infection process as initiated by urediospores of *Physopella zeae*:

- an appressorium is delimited from the germ tube by a septum;

- a penetration peg develops from the base of the appressorium and penetrates between epidermal cells, entering one of the cells via the anticlinal wall;

- an elliptical primary hypha develops in the epidermal cell lumen;

- secondary, intracellular hyphae develop, which exit the epidermal cell, and colonise adjacent epidermal and mesophyll cells.

Phakopsora pachyrhizi has been studied more extensively than any other direct-penetrating rust fungus, as it is a significant pathogen of the important soybean crop.

Appressoria begin to develop within 2 hpi, and after five hours, are nearly the size of the parent spores (Bonde *et al.*, 1976). They generally develop over anticlinal walls as mentioned previously, but can also form in the centre of epidermal cells or, more rarely, over stomata, in which case a guard cell is penetrated (Koch *et al.*, 1983). Many appressoria are sessile to their parent spores. When germ tubes are present, they vary in length from a few to more than 320 µm (Bonde, *et al.*, 1976; Koch *et al.*, 1983).

Bonde *et al.* (1976) observed two types of direct penetration from appressoria over anticlinal walls. More frequently, a hypha-shaped structure penetrated the outer epidermal cell wall directly into the cell lumen. Occasionally, this structure penetrated between adjacent epidermal cells and then through the anticlinal wall of one of the cells. This latter mode of penetration was also observed by Pua and Ilag (Koch *et al.*, 1983). Initially, in both types of penetration, the hypha-shaped structure is approximately the same diameter as the germ tube, but after 40-90 hours it increases in diameter, frequently to 8 µm.
Koch *et al.* (1983) did not observe any penetration between adjacent epidermal cells and subsequent entry into the epidermal cell through the anticlinal wall.

The hypha-shaped penetrating structure that develops within the epidermal cell was first observed by Keogh (Bonde *et al.*, 1976), who called it a 'transepidermal vesicle'. He concluded that this structure had 'no close counterpart in the structures elaborated in penetration by commonly studied rust species'. Koch *et al.* (1983) felt that this term was misleading, and suggested that the transepidermal vesicle be called the 'penetration hypha'.

There is no clearly defined penetration peg at the site of entry, that remains morphologically distinct from the rest of the transepidermal vesicle (Bonde *et al.*, 1976). Instead, there is a constricted region between the penetration hypha and the appressorium, and this is not always easily discernible with the light microscope (Bonde and Brown, 1980).

According to Bonde *et al.* (1976), after 22 hpi, a primary hypha has usually developed from the distal end of the penetration hypha, which is in contact with or nearly touching, the inner wall of the invaded epidermal cell. A septum forms which separates the penetration hypha from the primary hypha. The primary hypha then penetrates through the inner epidermal cell wall, into the mesophyll tissue. Immediately adjacent to the penetration hypha, the primary hypha is usually relatively narrow (3 µm diameter), but once it emerges from the epidermal cell, it forms an intercellular, 'bag-like' structure (average maximum diameter of 8 µm) in the mesophyll tissue (Bonde *et al.*, 1976).

This description of the penetration process conflicts with observations made by Koch *et al.* (1983). According to their model, it is the penetration hypha that extends from the epidermal cell into the mesophyll tissue, and the first septum, delimiting the penetration hypha from the primary hypha, is only formed once the spongy or palisade mesophyll has been reached. In some cases, where infection occurs on the adaxial leaf surface, the infection hypha may penetrate through and traverse the epidermal cell, continuing its growth into an underlying palisade cell. In this situation, the first septum forms in an intercellular space beyond the palisade cell layer.

The primary hypha branches intercellularly to form secondary hyphae (3-5 µm in diameter), which ramify through the leaf tissues, establishing haustoria in the mesophyll and epidermal cells (Bonde *et al.*, 1976; Koch *et al.*, 1983).

Bonde *et al.* (1976) found no differences between the infection processes observed on the upper and the lower leaf surfaces.

Koch and Hoppe (1988) observed the development of germtubes and appressoria from urediospores germinated on artificial membranes such as dialysis tubing. By 12 hpi, some appressoria have produced an infection hypha which appears to originate from a cone-like structure in the appressorium. This funnel-shaped structure has also been observed 12 hpi in appressoria formed on the host (Koch *et al.*, 1983). It appears to arise within the appressorium as a branched cell wall-like structure (Koch *et al.*, 1983).

Near the site of penetration, the cell wall becomes thinned and electron dense (Koch *et al.*, 1983; Ebrahim-Nesbat, Hoppe and Rohringer, 1985), and abundant mitochondria are present between the appressorial cone and the cell wall (Koch *et al.*, 1983).

On artificial membranes, the penetration peg emerges from the floor of the appressorium, at the point of contact with the membrane surface. This would appear to correspond with the 'directional peg emergence' observed in the stoma-entering rusts (Koch and Hoppe, 1988). The percentage of appressoria observed to produce infection hyphae on dialysis membrane is often small, but sometimes one or two very thin, thread-like structures emerge from the floor of the appressorium. Koch and Hoppe (1988) felt that these threads represented the first step of penetration hypha formation. The penetration peg wall has been shown to be continuous with an inner cone wall layer (Ebrahim-Nesbat *et al.*, 1985).

The development of infection structures up to the formation of the primary hypha has been observed on the host leaf surface in cases where penetration into the host has failed (Koch and Hoppe, 1988).

Infection hyphae of *P. pachyrhizi* have been reported to be able to penetrate thin collodion membranes, with the development of branched primary hyphae in the underlying water agar substrate (Koch and Hoppe, 1988). This ability to penetrate thin membranes suggests that there may be a mechanical component of the direct penetration process, as opposed to there being purely enzymatic processes. However, as Koch and Hoppe (1988) pointed out, penetration by infection pegs and development of vesicles beneath membranes has also been reported by Dickinson (1949), for *P. triticina*, an indirect penetrator.

Penetration of the host by *P. pachyrhizi* results in yellow or brown discolouration of the penetrated epidermal cell. The contents of the cell become granular, and within 24 hours the infected cell has often collapsed completely (Keogh, Deverall and McLeod, 1980; Koch *et al.*, 1983). Collapse of the epidermal cell has been observed to coincide with an increase in size of the penetration hyphae and penetration pores in the epidermal cell walls.

To summarize the morphological development of infection structures initiated from urediospores of *Phakopsora pachyrhizi*:

- an appressorium is delimited from the germ tube by a septum;

- a penetration peg emerges from the base of the appressorium, and penetrates through the cuticle and epidermal cell wall beneath;

- an intracellular penetration hypha traverses the epidermal cell and emerges into the mesophyll tissue on the other side;

- a septum delimits a primary hypha from the penetration hypha;

- the primary hypha branches to form intercellular, secondary hyphae, leading to the establishment of haustoria and mycelium in the leaf tissue.

In his dissertation, Hall (1991) describes direct penetration from urediospore-derived infection structures of a rust fungus on kikuyu grass (*Pennisetum clandestinum*). As discussed previously, this fungus has been identified as ***Phakopsora apoda*** (Adendorff and Rijkenberg, 1995a).

According to Hall (1991), the majority of germ tubes grow parallel to the longitudinal axis of the leaf. Generally, germ tubes are shorter than 2 spore widths, and very often appressoria appear to be sessile on the parent spore. Penetration does not occur through stomata, although the guard cells of some stomata are penetrated. Hall (1991) goes so far as to say that stomata are actually avoided by the germlings.

A mucilaginous material is produced in association with the appressorium, and when appressoria are stripped from the leaf surface, the wax layer beneath is removed.

From the base of the appressorium, a cylindrical penetration peg emerges. Very often, this is produced 'off-centre', especially when the appressorium forms in the junction between two epidermal cells. This facilitates penetration through the epidermal cell wall, as opposed to penetration between the cells.

The infection processes following peg emergence and penetration, were not observed by Hall (1991).

Preliminary studies by the present author (Adendorff and Rijkenberg, 1995b; 1996) indicated that host leaf penetration by *P. apoda* urediospore germlings is very similar to that of germlings of *P. pachyrhizi*, at least at the morphological level.

1.2.5 The appressorial cone

Cones or cone-like structures have been observed in a number of fungi, but whether they should be regarded as homologous from species to species, is uncertain. To date, these cone-like structures have only been reported in appressoria of direct penetrators, but are not present in the appressoria of all direct-penetrating fungi. For example, no appressorial cones were observed by Gray *et al.* (1983) in basidiospore germlings of *Cronartium quercuum*, and *Colletotrichum truncatum* (Schw.) Andrus and Moore, *Colletotrichum graminicola* (Ces.) Wilson and *Magnaporthe grisea* produce a 'pore wall overlay', rather than a well-defined cone, from which the penetration hypha develops (Politis and Wheeler, 1973; Bourett and Howard, 1990; Howard *et al.*, 1991; Van Dyke and Mims, 1991). Howard and Ferrari (1989) suggested that this 'pore wall overlay' may be analogous to appressorial cones in other direct-penetrating fungi.

Cone-like structures have been found in the following fungi:

- basidiospore germlings of *G. juniperi-virginianae* (Mims and Richardson, 1989), *G. fuscum* (Metzler, 1982), *Uromyces appendiculatus* (Gold and Mendgen, 1984) and possibly *Puccinia graminis* (Waterhouse, 1921; Allen, 1930) and *Puccinia triticina* (Allen, 1932a);

- aeciospore germlings of *Arthuriomyces peckianus* (Pady, 1935; Swann and Mims, 1991);

- urediospore germlings of *Ravenelia humphreyana* (Hunt, 1968) and *Phakopsora pachyrhizi* (Koch *et al.*, 1983);

- in appressoria of some anthracnose fungi, e.g. *Colletotrichum lindemuthianum* (Landes and Hoffmann, 1979), *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Brown, 1977), *Colletotrichum lagenarium* (Pass.) Ell. & Halst. (Xuei, Järlfors and Kuc, 1988), *Colletotrichum trifolii* Bain & Essary (Mould, Boland and Robb, 1991) and *Gloeosporium platani* (Seifers and Amon, 1980);

- in appressoria produced by *Spilocaea pomi* Fr. (Corlett and Chong, 1977).

According to Koch *et al.* (1983), there are obvious morphological differences between the cone-like structures found in *Colletotrichum* spp., and those found in *P. pachyrhizi*. In the former, the appressorial wall appears to be invaginated, forming a collar around the appressorial pore. This collar is directly attached to the appressorial wall. In the case of *P. pachyrhizi* however, it is not clear whether the cone is actually connected directly to the appressorial wall.

Corlett and Chong (1977) reported the presence of an 'infection sac' in appressoria of *S. pomi*. They describe this feature as a cup-shaped structure, enclosed by a membrane. Between the membranes, a wall layer is laid down, and the wall of the infection hypha which emerges from the base of the appressorium, is continuous with the wall of the infection sac.

The fact that both Hunt (1968), Pady (1935) and possibly Waterhouse (1921) and Allen (1930; 1932a), documented appressorial cones in their drawings made with the aid of a light microscope, even though they did not find them noteworthy enough to mention in the text, is admirable. This highlights the benefits of detailed drawings made with the aid of a *camera lucida*, as opposed to the more selective light micrographs, where only one plane is usually in focus.

In the case of *P. graminis* and *P. triticina*, if Allen (1930, 1932a) and Waterhouse (1921) did in fact see cones associated with the penetration pegs emerging from basidiospores, these would represent the only records known to the present author, of a cone-like structure being found inside a spore, as opposed to an appressorium.

It would appear that little is known about the development or significance of appressorial cones in the penetration process. In view of the convoluted nature of the plasma membrane in association with the cone, it may well be involved in the transfer of materials and enzymes to and/or from the host surface (Metzler, 1982). Swann and Mims (1991) noted the similarity between the extensive membrane elaborations of the cones in appressoria of *A. peckianus*, and the irregular outgrowths of wall material found in transfer cells of higher plants. In plants, these outgrowths are involved in the short-distance transfer of solutes (Gunning and Pate, 1969).

It has been suggested that the cones produced in appressoria of *Colletotrichum* spp. act to focus hydrostatic pressure to the site of penetration (Mercer, Wood and Greenwood, 1971; Landes and Hoffmann, 1979).

In appressoria that develop these structures, it seems that the wall of the penetration hypha is continuous with the cone. This has been observed in appressoria formed by *G. juniperi-virginianae* (Mims and Richardson, 1989), *U. appendiculatus* basidiosporelings (Gold and Mendgen, 1984), *P. pachyrhizi* (Koch *et al.*, 1983), and in the appressoria of four species of *Colletotrichum* (Brown, 1977; Landes and Hoffmann, 1979; Xuei *et al.*, 1988; Mould *et al.*, 1991). Ebrahim-Nesbat *et al.* (1985) conducted lectin-binding experiments which confirmed that the appressorial cone of *P. pachyrhizi* is continuous with the penetration hypha. This indicates that the penetration hypha is attached to the appressorium by the appressorial cone, and is possibly formed via elongation of the cone (Koch *et al.*, 1983).

1.2.6 The penetration peg

In a successful infection, the penetration peg always emerges from the base of the appressorium, as opposed to the top or sides, and Wynn and Staples (1981) suggested that a contact or chemical stimulus must be involved.

Thinning of the basal wall of the appressorium, at the site of contact with the substrate, has been observed in many direct-penetrating fungi. Wynn (1981) and Mendgen and Deising (1993) suggested that the wall-less region in the appressorial bases of many direct-penetrating fungi, allows for effective focusing of pressure in this part of the appressorium. In addition, Wynn and Staples (1981) postulated that this thinning of the wall, which is probably a contact response, favours the development of the penetration hypha within that region, i.e. the emergence of the penetration peg is controlled by the location of a weak point in the appressorium floor. The emergence of the peg from the centre of the appressorial base in many direct-penetrating fungi, is given by Wynn and Staples (1981) as evidence supporting this hypothesis.

This does not hold in the case of *P. apoda* however, where peg emergence often occurs 'off-centre', especially where the appressorium has formed directly over an anticlinal wall or a stoma and the fungus penetrates the epidermal cell wall slightly to one side. In the case of *P. psidii*, an infection peg develops directly over an epidermal cell junction, even though the appressorium may span the width of more than one epidermal cell. This would suggest that a very specific and sensitive contact stimulus may be involved, enabling the fungus to select a penetration site within the infection court itself.

In many cases, the wall of the penetration hypha is not continuous with the appressorial wall, and originates from within the appressorium, as discussed above with regard to the formation of appressorial cones. However, according to Gray *et al.* (1983), the penetration peg that develops from basidiospore germlings of *C. quercuum*, is continuous with the thinned basal wall of the appressorium.

Very little appears to be known about the cytoskeletal behaviour in appressoria during penetration peg development. Mendgen and Deising (1993) contemplated the possibility of similarities between peg emergence and the budding site in yeasts, where a ring of filaments can be observed (Kim, Haarer and Pringle, 1991). In appressoria of *Gymnosporangium juniperi-virginianae* (Mims and Richardson, 1989) and *Arthuriomyces peckianus*, as the appressorium matures, numerous vesicles of different sizes, and of unknown origin, gather at the penetration pore, where the penetration hypha will develop (Mims and Richardson, 1989; Swann and Mims, 1991).

Vesicles are often produced at the tip of the penetration hypha. Mendgen and Deising (1993) suggested that these may contain cuticle and wall-degrading enzymes because smooth membranous elements similar to Golgi equivalents pinch off such vesicles (Hoch, 1991).

Many fungi appear to define the diameter and length of their penetration hyphae, without the influence of the host, as evidenced by the formation of identical structures *in vivo* and *in vitro* (Mendgen and Deising, 1993).

1.2.7 Enzymes or mechanical pressure?

As discussed by Mendgen and Deising (1993), the question as to whether penetration is facilitated by enzymes or mechanical forces, or both, has been a source of speculation for many years, especially in the obligately biotrophic fungi.

The cuticle is the first barrier confronting a direct-penetrating fungus. According to Köller (1991), there is little evidence that the mere physical strength of the cuticle is a major factor in plant defense against pathogens, and only a few cases have been recorded of cuticle thickness correlating with increased passive resistance to fungal attack (eg. Corner, 1935; Matta, 1971). Martin (1964) gave evidence for and against the involvement of the cuticle as a defense mechanism against pathogens. One has to bear in mind that although the cuticle must contribute to the defense mechanisms of the leaf, its primary function is that of water conservation (Raven, Evert and Eichhorn, 1986).

Most of the evidence presented to support either enzymatic or mechanical mechanisms of cuticular penetration, has been based on the appearance of the penetration pores in electron micrographs. Round and smooth penetration holes have been assumed to result from chemical dissolution of the cuticle, as opposed to a mechanical mode of penetration where irregularities and signs of tearing would be expected around the penetration hole (Köller, 1991). The disadvantage of making these assumptions, is that processing of the specimen could well lead to alterations in the appearance of the hole.

Other evidence that has been cited in favour of a mechanical mechanism, includes the tight adherence of the infection structures to the cuticle, the ability of some fungi to penetrate inert, artificial membranes, and that a strengthening of appressoria by melanin is sometimes a prerequisite for penetration (Köller, 1991).

The presence of cutinase genes and cutinase production has been demonstrated in a number of fungi, but the exact role of cutinase in the penetration process has not yet been elucidated (for a review see Kolattukudy, 1985).

According to Mendgen and Deising (1993), plant cell wall-degrading enzymes have been detected in a number of direct-penetrating fungi belonging to different systemic and tropic groups. They proposed that plant cell wall-degrading enzymes are of greater importance in those fungi that do not differentiate appressoria, as they may have a reduced ability to effect mechanical pressure on the penetration site. If this theory is true, then enzymes may play a very great role in penetration from basidiospore germlings, which may not even develop a detectable appressorial swelling.

1.2.8 Nuclear behaviour of the pathogen during cuticular penetration

Hoch and Staples (1987) described the development of appressoria in *Uromyces appendiculatus* urediospore germlings as occurring 'in concert with mitotic divisions', with septum formation and mitosis being associated with appressorium differentiation. A strict correlation between nuclear division and appressorium development has also been observed in urediospore germlings of *Uromyces viciae-fabae* (Kapooria and Mendgen, 1985) and *P. pachyrhizi* (Koch and Hoppe, 1988), where mitosis occurs after the entry of the two nuclei into the appressorial swelling and the formation of a septum between the appressorium and germ tube.

Pady (1935) and Swann and Mims (1991), found the same pattern of development in aeciospore germlings of *Arthuriomyces peckianus*. The two nuclei move into the developing appressorium, and both undergo mitosis, sometimes even before septum formation between the germ tube and appressorium, resulting in the presence of four nuclei in the mature appressorium.

Swann and Mims (1991) suggested that these events may be linked to the dikaryotic condition of the urediospore and aeciospore germlings, as opposed to the situation in the monokaryotic germlings of some rust fungi. For example, in the basidiospore germlings of *Gymnosporangium juniperi-virginianae*, neither mitosis or septum formation occurs during the differentiation of appressoria (Mims and Richardson, 1989). As described previously, nuclei were not observed by Mims and Richardson (1989) in appressoria formed on leaf or membrane surfaces, even after the penetration peg had entered the leaf, and they surmised that nuclear migration from the basidiospore occurs later in the infection process.

Typically, basidiospores contain two haploid, homokaryotic nuclei at maturity, although heterokaryotic and quadrinucleate basidiospores have been found in some species (Gold and Mendgen, 1991). In the basidiospore stage of *U. appendiculatus*, the spores, infection structures and intra-epidermal vesicles are all binucleate. During the early development of primary hyphae, two to four nuclei are commonly observed, but upon further growth and septation, a reversion to the uninucleate or binucleate condition takes place (Gold and Mendgen, 1984).

Generally, nuclear behaviour during penetration has been regarded as an aside, and apart from some of the earlier, descriptive papers (e.g. those published by Allen in the 1930s), nuclear observations have seldom been more than attempts to determine the ploidy of the fungal structures involved. A notable exception, as has been mentioned, is the recent study by Heath *et al.* (1996), of the nuclear behaviour of both the urediospore and basidiospore germlings of *Uromyces vignae*.

The nuclear condition of the pathogen would seem to have a very profound effect on the type of penetration effected by the fungus. In the discussion, the significance of fungal ploidy is considered with respect to the different penetration strategies adopted by different spore types of the same species of fungus.

1.2.9 Host response to penetration

Rust fungi are obligately biotrophic organisms, which need to co-exist with the host plant for a substantial part of their life-cycles. The survival strategy of the rust fungi would seem to involve the maintenance of close contact with the host whilst causing the minimum of disruption to host tissue. The typical, intercellular growth of hyphae, with the development of relatively small ingrowths (haustoria) as the only intrusive stage, probably contributes greatly to the reduction of host cell disruption. Perhaps stomatal penetration represents the most sophisticated form of this 'minimum invasive' infection, as the only intracellular phase is the haustorium. Direct penetrators on the other hand, usually have to come into immediate contact with the host's defense mechanisms during the penetration of the epidermal cell, and unlike any of the indirect-penetrators that have been studied, at least two direct penetrators (Hunt, 1968) have adopted an almost completely intracellular mode of parasitism.

Host resistance is generally not a factor in determining whether appressoria form and often, appressoria form on physiologically resistant hosts, as well as on non-host plants (Hoch and Staples, 1987). Recently however, host avoidance mechanisms in two plants have been demonstrated to reduce the number of appressoria formed by invading rust fungi, apparently by preventing stomatal recognition (Broers and López-Atilano, 1996; Rubiales and Niks, 1996). In the direct penetrators, such mechanisms would be unlikely to have any effect, as these fungi tend to require non-specific, contact stimuli for appressorium induction. In the direct-penetrators, it would seem that a host response is elicited later in the infection process, once penetration itself is under way.

Bonde *et al.* (1976) reported that the epidermal cells initially invaded by germlings of *Phakopsora pachyrhizi* became necrotic from 28 hpi onwards. Collapse of some of the mesophyll cells in the immediate vicinity was common within 40 hpi. After 6 days, mesophyll cells near many penetration sites had become hypertrophied. Hypertrophied cells were also observed near hyphae some distance from the penetration site. Deverall, Keogh and McLeod (1977) documented the soybean host reaction to infection from urediospores of *P. pachyrhizi*, in terms of the staining properties of the surrounding host tissues. When infected leaf material was stained with trypan blue in lactophenol, clusters of two or three palisade cells immediately beneath penetration sites exhibited distinctive staining properties. The penetration/primary hyphae of the fungus only passed between these cells, and yet still elicited some sort of host response. According to Deverall *et al.* (1977), the apparent incompatibility between the infection hypha and the host cells which resulted in the different staining properties of the host cells, initially only occurred in the first host cells contacted. No further, visible responses occurred until later in the rust development.

In a later study, Keogh *et al.* (1980) studied this staining response further in resistant and susceptible varieties of soybean. They attributed the increased stain retention to a change in physiology, and not necessarily necrosis, of the cells surrounding infection sites. This change in physiology was more rapid and widespread in a resistant cultivar, where it occurred in all cells in contact with, and in advance of, infection hyphae. Another reaction that was observed was the deposition of material in the intercellular spaces at the junctions of the epidermis and the palisade around infection sites in both the susceptible and resistant cultivars. Sectioning and histochemical evidence would seem to indicate that these deposits are phenolic in nature.

This detection of the invading fungus in the absence of actual penetration, has been observed in other host/parasitic fungus associations. It is a common response to fungal invasion, for the host plants to form cytoplasmic aggregates against the wall in contact with the fungus (Littlefield and Heath, 1979). In bean epidermal cells infected by basidiospores of *U. appendiculatus*, the cytoplasm aggregates around the developing intra-epidermal vesicle. The nucleus is often closely appressed to the vesicle or apex of the primary hypha (Gold and Mendgen, 1984). Cytoplasmic aggregation is usually followed by the deposition of a papilla directly beneath the site of fungal penetration, e.g. as occurs in response to penetration from basidiospores of *Uromyces appendiculatus* (Gold and Mendgen, 1984). Often, the papillae do not actually prevent penetration of the fungus, and seem to form in both resistant and susceptible hosts.

Allen (1932a) observed a fascinating host response to infection by urediospore germlings of *Puccinia triticina*. Apparently the intracellular, primary hypha that develops during the infection process, gradually becomes coated in a sheath of material with staining properties similar to the host cell walls. This sheath persists for some time after the primary hypha has disintegrated. The same ensheathing process was observed in older haustoria. Allen (1932a) interprets this as a defence mechanism on the part of the host against the invading fungus.

1.2.10 Discussion

An important advantage of obligate parasitism is that the fungus can proliferate and reproduce on a susceptible host for extended periods of time. However, this mode of parasitism has meant that the fungus has had to evolve very sophisticated mechanisms of host penetration and nutrient extraction. Unlike the necrotrophic fungi, which adopt rather less subtle means of breaking down the barriers presented by the host, the rust fungi have had to adopt strategies that keep disturbance of the host tissues to a minimum, at least until the fungus has been able to reproduce.

The result of the co-evolution between rust fungi and their respective hosts, is a variety of infection techniques, each accommodating the features peculiar to a particular host leaf.

Indirect-penetrating rusts require longer germ tubes to find specialised points of entry on the leaf surface, i.e. stomata. Although a large amount of energy is spent on finding a point of entry, energy is saved on the enzymes and other processes required for penetrating the cuticle. In direct-penetrating rusts, where the requirements for site selection are less specific, e.g. any of the abundant shallow depressions between cells, the fungus can economise on germ tube growth and focus its energy on the actual penetration process. Rust fungi have therefore had to find a balance between behavioural and physical energy expenditure to optimise the efficiency of the infection process. Why some fungi find it more efficient to penetrate a particular host indirectly, and others prefer direct penetration, is not understood. For example, *Puccinia sorghi* penetrates maize indirectly from urediospores, and *Physopella zeae* penetrates maize directly from urediospores.

Koch *et al.* (1983) suggested that the apparent lack of specificity in the host surface signals required for the induction of infection structure formation in *Phakopsora pachyrhizi*, could account for the ability of this fungus to infect 87 plant species (Sinclair, 1982). Certainly, by avoiding the need for exact morphological cues on the leaf surface, the fungus is able to form pre-infection structures on a wider variety of surfaces. However, it is tempting to think of the infection process in purely morphological terms, and to forget that there are many other host-specific barriers for the fungus to overcome, such as the resistance mechanisms that are only mobilised once the fungus has been detected by the host.

The ability of fungi such as *P. pachyrhizi* to develop penetration pegs and primary and secondary hyphae on artificial membranes would seem to indicate that the infection process in some direct-penetrating rust fungi could follow a predetermined sequence of morphological development, triggered by surface stimuli, until the point of haustorium formation, as occurs in indirect- penetrators. The sequence followed by stoma-penetrators is well-documented, and it is clear why such a sequence would be required. The fungus, upon reaching and penetrating a stoma, does not have further host contact to guide its progress through the substomatal cavity. Following a pre-programmed growth pattern until well into the mesophyll tissue, provides the fungus with the most efficient and direct route into the leaf tissue, without having to rely on further thigmotropic effects. In direct penetrators, which often need to traverse the epidermal cell immediately after infection, a pre-programmed growth pattern might allow them to enter the mesophyll via the most direct route to establish haustoria and commence intercellular growth.

Aside from noting the role of the stoma in the indirect-penetration process, and observing host responses to infection, surprisingly little effort has been made to understand exactly what the fungus has to overcome to infect a host successfully. Generally, penetration is discussed in terms of a standardised model of a host leaf. The present author believes that to understand each unique plant-pathogen interaction, the host must be studied and understood. For example, how thick is the epidermal cell wall in hosts of direct-penetrating rusts, as opposed to those of the indirect penetrators? At what stage in the plant's life does the most significant amount of infection occur, and is it the older or younger leaves that are most successfully infected? What is the structure of the epidermal layer in terms of numbers and arrangement of stomata, especially in hosts of those fungi opting for direct penetration? Why have no rust fungi been found on the non-vascular plants, since direct penetration is an option, and since these plants lack sophisticated barriers such as a cuticle? The morphology of the leaf surface and the spatial arrangement of tissues must have influenced the development of infection strategies by rust fungi, and yet the host morphology is often not studied in any detail.

Leaf age has been found to influence the ability of some rust species to penetrate the host through the stomata. In younger leaves, the stomata may not yet be sufficiently developed to allow for penetration (eg. Coutinho, 1990). Leaf age may well affect direct-penetrating rusts as well, as suggested by Hunt (1968). Cuticular penetration may be restricted to immature leaves in some hosts, by virtue of increased mechanical or chemical barriers associated with a mature cuticle. Perhaps there is a broad correlation between the spring germination of teliospores and subsequent release of basidiospores, and the abundance of young, tender shoots of prospective hosts. The soft young tissues would probably allow for easier direct penetration. The cuticles of grasses are known to be toughened by deposits of siliceous and other materials. Maybe this factor has played a role in determining the penetration strategies employed by the cereal rusts, the vast majority of which are indirect-penetrators. Hunt (1968) thought it probable that all rusts of grasses penetrate through stomata, but studies of *P. zeae* on maize (Bonde *et al.*, 1982) and *P. apoda* on kikuyu grass (Hall, 1991) indicate that there are at least two exceptions.

Why would some rust fungi initially penetrate between epidermal cells, only entering the cell lumen via the penetration of side walls or mesophyll/palisade cells? This mode of penetration was observed in *P. zeae* (Bonde *et al.*, 1982) and *P. pachyrhizi* (Bonde *et al.*, 1976; Koch *et al.*, 1983). In many plants, the outer epidermal wall is significantly thicker than the transverse walls, probably to conserve moisture as well as to give protection from mechanical or biological factors. These outer walls are presumably more difficult to penetrate than the side walls. A fungus penetrating an epidermal cell may therefore require less energy for the penetration process if the thinner transverse wall is penetrated. However, this intercellular penetration would still require energy expenditure, in the form of enzyme production and/or mechanical force, to overcome the barrier presented by the middle lamella between the epidermal cells.

The role of enzymes and mechanical pressure in the direct penetration process is poorly understood in the rust fungi. A greater understanding of the structures formed during penetration may well give some clues as to the mechanisms involved.

The appressorial cone probably plays a key role in the penetration process. The large surface area of the invaginated plasmamembrane, covering the surface of the cone, may allow for the rapid, simultaneous release of vesicle contents in enzymatic processes during the penetration of the cuticle and host wall. At any one time, a larger number of vesicles could fuse with the plasmamembrane, and hence release enzymes or other required products. The cone would appear to be closely associated with the ER system, and may provide an important channel for products directly to the site of activity, or the cone itself may be a site of production for various enzymes and other substances required during penetration.

It is interesting that most direct-penetrating rust fungi require an appressorium and in some cases, elaborate cone-like structures, to penetrate from the leaf surface into an epidermal cell, and yet they can exit the host epidermal cell with little more than a restriction in hyphal diameter within the breadth of the wall. Transcellular penetration pores are characteristically larger than the initial penetration pore into the epidermis. What is the difference between the outer and inner cell walls?

Perhaps a major function of the appressorium is related to breaching the cuticle. In many of the accounts given of direct penetration, penetration pegs appear to have a smaller diameter in the cuticle than in the host cell wall. This may be further evidence that the cuticle is more difficult to penetrate than the host wall. An alternative theory is that the small diameter of the penetration peg in the outer region of the host cell wall, represents a mechanical penetration technique, where pressure is exerted over a very small area to increase the attainable pressure levels. The tube may widen when penetrating the wall, because the process becomes enzymatic in nature.

Of great significance is the fact that it is possible for some germlings to penetrate the host cuticle without forming an appressorium at all. As mentioned before, Pady (1935) observed an aeciospore germling that had penetrated through the cuticle without forming an appressorium, and Allen (1930; 1932a) and Waterhouse (1921) reported direct penetration from basidiospores of *P. graminis* and *P. triticina* without appressorium formation. If it is indeed possible for a fungal germling to penetrate without forming an appressorium, then why do most rusts produce these structures?

Strangely, these observations of penetration directly from basidiospores, in the absence of appressoria, have not even been mentioned in many of the publications on host penetration by rust fungi. Although the papers themselves have been regularly cited, this mode of penetration seems to have been largely ignored. Studies of cuticular penetration without the formation of an appressorium or even a germ tube, could give insight as to the purpose of pre-infection structures. Perhaps the spore saves energy by using the spore structure in the same way as other fungi use an appressorium - the thin basidiospore walls may make this feasible.

A most intriguing concept, is that genetic information governing different modes of penetration from different structures, can be present in the same organism. What are the evolutionary implications of this concept? In the words of Hunt (1968), 'since the germ tube from a monokaryotic basidiospore has the capacity to penetrate through the cuticle, there would seem to be no inherent reason why the dikaryotic aecidiospores or urediospores could not behave similarly'. In effect, the genetic material which controls several penetrating procedures is present within each rust propagule, and a change in the penetration mechanisms within a 'stage' of the rust life-cycle would probably not require elaborate mutations of the physiological and morphological nature of an entire organism, but rather the differential expression of existing genes.

Gold and Mendgen (1991) suggested that when complementary nuclei are brought together in the same cytoplasm, different parts of the fungal genome are expressed. A genetic and physiological comparison between urediospore and basidiospore propagules of the same species could be very fascinating.

But which came first in the rust fungi - direct penetration or indirect penetration? Did those exceptional rust fungi that penetrate directly in the urediospore stage, evolve first as indirect penetrators, or did they follow a path of evolution different from that of the stomatal penetrators from the beginning? Staples and Hoch (1987) contemplated whether the contact response of *P. pachyrhizi* to form appressoria from urediospores represents a 'phylogenetic degeneration from the more sophisticated stomate recognition response'. But can one really regard the infection process in this fungus as having 'degenerated' from a more sophisticated mode of penetration, when *P. pachyrhizi* performs well enough to cause huge economic losses every year? And in a purely phylogenetic sense, how can we be sure that the morphologically and behaviourally more sophisticated indirect penetration, evolved after direct penetration from urediospores? Perhaps direct penetration from urediospores evolved from indirect-penetrating ancestors, and represents an example of convergent evolution with other apparently less sophisticated, direct-penetrating fungi.

At least two direct penetrators exist intracellularly within the host. Perhaps they have evolved specialised mechanisms to establish this more intimate relationship with the host, whilst maintaining their status as obligate parasites, as opposed to the minimal-invasive strategy utilised by the indirect-penetrating, intercellular fungi. Perhaps *R. humphreyana* evolved its intracellular mechanism of host colonisation because of the compact nature of the host leaf tissue. The small cell size may imply that intercellular space in the leaf is limited. On the other hand, the direct penetrating, intracellular *Physopella zeae* infects maize, which is happily colonised intercellularly by *Puccinia sorghi*.

Research to date has been very biased with respect to the sample of rust fungus species that have been selected for study. The majority have been rust fungi attacking graminaceous hosts. Bonde *et al.* (1982) knew of only four rust pathogens that penetrated the host directly from urediospores, with *P. zeae* being the only species infecting a monocotyledonous species. However, they recognised that further research could reveal that direct penetration from urediospores is not uncommon for pathogens of either monocotyledonous plants or dicotyledonous plants. It is interesting to note that of four Jamaican rusts studied by Hunt (1968), two were found to penetrate their host directly. Hunt (1968) pointed out that very few rust fungi that are parasitic on tropical or dicotyledonous hosts have been examined in this respect, and also shares the opinion that the phenomenon of cuticular penetration may be fairly common.

As discussed by Hoch and Staples (1987), understanding the nature of the signals for appressorium initiation, and how this signal is perceived and mediated by rust fungi, could inspire the development of novel approaches to disease control. An understanding of the cell biology of appressorium formation, could lead to methods of manipulating appressorium formation in inappropriate places or at unsuitable times, or could prevent appressorium formation altogether. An even more exciting alternative, is the breeding of plants with resistance mechanisms aimed at thwarting the fungus at the pre-penetration or penetration stages. Perhaps a plant with a particularly thick cuticle or cell wall, could reduce the number of successful fungal infections. However, if Köller (1991) was correct in his statement that increased cuticle thickness is seldom correlated with increased resistance, then this may not be a viable option. Perhaps the cuticle would have to be so thick to be effective, that the host plant would be adversely affected. This aspect of resistance has not been sufficiently investigated, and could very well make a contribution to plant resistance in the future.

Wynn and Staples (1981) emphasised that creating conditions on a plant surface that are unfavourable for penetration by fungi, will not result in 100% control of a disease. As with most resistance mechanisms, chances are good that a few individuals will be able to overcome the obstacles presented by the modified surface. Since only the pre-infection stages are targeted, subsequent development from a successful infection will continue as normal. Perhaps the future for pre-infection resistance lies in its integration with post-infection resistance, contributing to a multifaceted system that reduces both incidence and severity of disease (Russell, 1978; Wynn and Staples, 1981). Combining a pre-infection resistance mechanism, which is more likely to be a horizontal resistance mechanism, with the more conventionally used vertical resistance, may assist greatly in increasing the length of time that the vertical resistance is effective.

Unfortunately, pre-infection control of direct penetrators could be more difficult than in the case of indirect penetrators. The reduced specificity in penetration site selection, could mean that the resistance mechanisms are challenged to a greater extent than in fungi requiring more specialised sites of infection. In other words, when virtually every cell represents a potential infection site, the resistance mechanism involved would have to have a very broad effect. In the case of indirect penetrators, it is likely that resistance mechanisms would target interactions involving the stomata in particular, e.g. as has been illustrated by Rubiales and Niks (1996), where the germ tubes of various rust fungi failed to recognise the stomata on rust-resistant plants.

Studies of rust fungi have become progressively more and more specialised. Increasingly, researchers have focused on narrow issues pertaining to a single aspect, of a single stage, of a single rust species. No recent publications can compare with for example, Allen 's (1928, 1930, 1932a, 1932b, 1934, 1935) early papers in terms of scope and detail. In many ways, a great deal of perspective is lost during the course of specialisation. This is especially apparent in the rust fungi, where the temptation exists to regard each spore stage of a particular fungus, as a separate species, ignoring what happens during a different part of the life-cycle. As a result, there are large, frustrating gaps in the literature. Maybe if we tried to understand rust fungi in a more holistic manner, we would stand a greater chance of discouraging their proliferation.

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CHAPTER 2 THE DEVELOPMENT OF INFECTION STRUCTURES FROM UREDIOSPORES OF *PHAKOPSORA APODA* ON ARTIFICIAL MEMBRANES

ABSTRACT

Urediospores of the direct-penetrating rust fungus *Phakopsora apoda* (Har. & Pat.) Mains were germinated on various artificial membranes to elucidate the morphological development and nuclear behaviour of the fungus during the development of infection structures. The effects of topography on appressorium induction were examined with the aid of smooth artificial membranes and those that bore microfabricated ridges. It was determined that appressoria form freely on smooth membranes, and germlings do not demonstrate a clear preference for appressorium formation in association with the microfabricated ridges. Morphological development involves the formation of a short germ tube, an appressorium which is cut off from the germ tube by a septum (6 hours post inoculation [hpi]), a penetration pore which is surrounded by a cone-like structure within the appressorium (8 hpi), a twisted penetration hypha which develops from the base of the mature appressorium (12 hpi) and a primary hypha (18 hpi) which is delimited from the penetration hypha by a septum and which branches to form two secondary hyphae. The two nuclei present in the urediospore migrate into the appressorium where they undergo mitosis, resulting in the presence of four nuclei (6 hpi). These nuclei move into the penetration and primary hyphae, where at least one further round of mitotic division occurs (22 hpi).

2.1 INTRODUCTION

Hall (1991) was the first to investigate the infection of kikuyu grass (*Pennisetum clandestinum* (Hochst. ex Chiov.)), an important lawn and pasture grass, by a rust fungus which he tentatively identified as *Puccinia stenotaphri* (Hall and Rijkenberg, 1989). This fungus was subsequently identified as *Phakopsora apoda* (Har. & Pat.) Mains (Adendorff and Rijkenberg, 1995a). Hall's (1991) studies revealed that this rust fungus is unusual in a number of respects. Of primary interest is its mode of penetration into the host leaf. Hall (1991) found that the urediospore germlings penetrate the host leaf directly, through the cuticle, from urediospore-produced infection structures, as opposed to the more conventional stomatal-type of penetration which has been so extensively dealt with in the literature (Littlefield and Heath, 1979; Hoch and Staples, 1987; Hoch and Staples, 1991). In view of this, the current study was undertaken to investigate the morphological development of, and nuclear behaviour in, the infection structures of *P. apoda* germlings, as well as to examine the possible involvement of the leaf topography in appressorium induction.

Artificial membranes have been used in numerous studies of fungal germlings, in to replicate certain aspects of the generally hydrophobic leaf surface (e.g. Young and Klauss, 1984; Epstein, Laccetti, Staples, Hoch and Hoose, 1985; Epstein, Laccetti, Staples, and Hoch, 1987; Hamer, Howard, Chumley and Valent, 1988; Jones and Epstein, 1989). As discussed by Nicholson and Epstein (1991), the use of artificial membranes as physical substrates for fungus germling studies generally simplifies microscopy and quantification of these structures, as the germlings are easier to locate on a membrane than on a host, and details of cell structure are not obscured by underlying host tissues. Furthermore, the effects of topography on the fungal germling can be studied through the use of membranes with microfabricated surfaces, without the interference of host leaf chemistry.

A great deal of research has been directed towards the mechanisms involved in the sensing of host topography by fungal germlings, and the subsequent differentiation of infection structures. Staples and Macko (1984) introduced the term thigmodifferentiation, to describe the inducive properties of topography in appressorium formation, as opposed to chemodifferentiation, the response to chemical signals. In the case of rust fungus germlings, artificial membranes have proven to be extremely useful in investigations of the thigmotropic stimuli required for the differentiation of infection structures (particularly appressoria) on the leaf surface. Dickinson (1949, 1971) was probably the first to utilise artificial substrates in this context and, since then, considerable research on this aspect of pathogenesis has been conducted on numerous indirect-penetrating rust fungi (e.g. Wynn and Staples, 1981; Staples and Hoch, 1984; Staples and Macko, 1984; Staples, Hoch, Epstein, Laccetti and Hassouna, 1985; Hoch, Staples, Whitehead, Comeau and Wolf, 1987; Allen, Hazen, Hoch, Kwon, Leinhos, Staples, Stumpf and Terhune, 1991a).

Scratches or precisely defined ridges on artificial substrates have induced appressorium formation in many indirect-penetrating rust fungi, apparently by mimicking the stomatal topography (e.g. Hoch *et al.*, 1987; Allen *et al.*, 1991a; Allen, Hoch, Stavely and Steadman, 1991b). Initially it was thought that a sequence of topographical signals was required to trigger differentiation (Wynn and Staples, 1981), but Staples *et al.* (1985) demonstrated that only a single scratch in the substrate was required. Hoch *et al.* (1987) found that signalling for both growth orientation and differentiation was equally efficient for grooves as for ridges in the topography. The important parameters were the depth or height of the feature, and its distance from neighbouring signals.

Unfortunately, in comparison to the vast amount of information available on appressorium induction in the indirect-penetrating fungi, relatively little work has been carried out on those factors leading to the differentiation of appressoria in basidiospore and direct-penetrating urediospore and aeciospore germlings. This has mainly been due to the preponderance of indirect-penetrators that cause economically important rusts on graminaceous hosts, and which have therefore been more extensively studied.

The only direct-penetrator that has been studied in any detail, is *Phakopsora pachyrhizi* Syd., which causes rust on soybean (Bonde, Melching and Bromfield, 1976; Keogh, Deverall and McLeod, 1980; Koch, Ebrahim-Nesbat and Hoppe, 1983; Ebrahim-Nesbat, Hoppe and Rohringer, 1985; Allen *et al.*, 1991a). Allen *et al.* (1991a) found that topography does play a role in the development of infection structures by *P. pachyrhizi*, and another direct penetrator, *Physopella zeae* (Mains) Cumm. & Ramachar on maize.

They found that both these fungi formed appressoria in association with ridges and scratches in plastic membranes.

During the course of the present study, microfabricated membranes were used to determine the role of topography in the induction of appressoria of *P. apoda*. A further aim was to determine the sequential development of infection structures from the urediospores of *P. apoda*, both at a morphological level, and also in terms of the nuclear behaviour during each stage of development. In a previous study, some similarities were found between the infection structures produced by *P. apoda* (Adendorff and Rijkenberg, 1995b) and *P. pachyrhizi* (Koch and Hoppe, 1988), and throughout this investigation, comparisons have been made between the two fungi.

2.2 MATERIALS AND METHODS

Several plastic substrates were tested for their ability to induce infection structure formation whilst maintaining high levels of spore germination, viz. smooth and scratched polyethylene (commercially available 'freezer bag'); smooth and scratched polypropylene (Panbro; Natal, SA); microfabricated polystyrene (ridged); microfabricated polycarbonate (ridged); teflon 50LP and mylar #3517[1], and dialysis tubing.

Polyethylene and polypropylene membranes were exposed to various treatments in order to create a series of grooves in the membrane surface. For example, they were scratched with a copper brush, various grades of sand paper and fine needles.

Ridged polystyrene and polycarbonate membranes were cast from a laser-etched, silicon wafer, using liquid solutions of polystyrene (expanded polystyrene balls, commonly used as a packing material) in methylene chloride, and polycarbonate (corrugated polycarbonate sheeting, commonly used in greenhouse construction) in

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1Samples kindly provided by R.J. Howard at DuPont Central Research and Development, Wilmington, Delaware, U.S.A.

chloroform respectively, as described by Hoch *et al.* (1987), Allen *et al.* (1991a) and Kwon and Hoch (1991). The silicon wafer was obtained from Prof. H.C. Hoch[2]. The ridges on the membranes cast from the wafer were 2.0 µm wide and 0.9 - 1.1 µm high, and were spaced 60 µm apart, in a grid pattern.

All the plastics were boiled (for 1 to 3 minutes) first in a 0.01M solution of NaOH, and then a 0.01M solution of HCl, to remove any substances which may interfere with germination of the urediospores. The membranes were rinsed in distilled water between treatments, and afterwards they were boiled for 2 minutes in distilled water. The membranes were cut into squares (1 cm x 1 cm) and spores were scraped, using a scalpel, from uredia onto these plastic squares. Exact spore concentrations were not calculated, due to the difficulties experienced in obtaining even inoculation over the membrane surface (the spores had a strong tendency to 'clump'), but the estimated concentration was, on average, 100 spores/mm^2 of membrane.

The inoculated membranes were floated, spore-side up, on distilled water in petri dishes, and were lightly misted with distilled water, using a hand-held sprayer. The petri dish lids were also misted with distilled water and the closed dishes were placed (in black plastic bags to exclude light and to maintain high levels of humidity), into an incubator at 19 °C.

At least 3 pieces (1 cm x 1 cm) of each type of plastic were examined at 6, 10 and 24 hours post-inoculation (hpi), to assess spore germination rates and the inducivenes of each membrane to appressorium formation. Each membrane was mounted in distilled water, beneath a glass coverslip, prior to viewing under a Zeiss Axiophot light microscope.

The number of appressoria that formed over ridges on the microfabricated polystyrene membranes, were counted, as well as the number formed on smooth membrane between the ridges. These figures were compared with the ratio of membrane area

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2 Dept. Plant Pathology, Cornell University, NYSAES, Geneva, New York.

designated as 'ridge-associated' versus the area designated as 'smooth'. Ridge-associated area was defined as the area of membrane available for appressorium formation, such that each appressorium (with an average width of 16 µm) would overlap a ridge by at least 1 µm. Smooth membrane represented the area of membrane between the ridges.

On polyethylene (which was found to be the best membrane all-round for the examination of infection structures), specimens were viewed and described at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hpi. A minimum of 20 000 propagules was viewed at each of these times post-inoculation.

Germlings were stained with 4',6-diamidino-2-phenylindole nuclear stain (DAPI; Sigma, St. Louis, MO) to view the nuclear behaviour at various stages during the development of infection structures on polyethylene. A specimen was first mounted in distilled water for viewing under the light microscope, and once suitable infection structures had been found, the slide was irrigated with DAPI (0.01 µg/ml) and viewed under ultraviolet light. Specimens were viewed and photographed with a Zeiss Axiophot microscope.

Preparation of membranes and host material (inoculated and incubated as for the membranes) for viewing with the scanning electron microscope followed standard procedures. The specimens were fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer overnight, washed twice in the same buffer for 30 minutes and postfixed for 3 hours in 2% osmium tetroxide in 0.05M sodium cacodylate buffer. This was followed by two 30-minute washes in 0.05M sodium cacodylate buffer, and then the specimens were passed through a graded ethanol series. Following dehydration, the material was critical-point dried with carbon dioxide as a transition fluid.

The critical-point dried specimens were mounted onto brass stubs with double-sided tape, and were gold/palladium sputter-coated with an E5110 S.E.M. coating unit (Polaron Equipment Ltd.) before viewing with a Hitachi S-570 scanning electron microscope.

2.3 RESULTS

2.3.1. Induction of germling infection structures on various plastic membranes

Urediospore germination and germling development on each type of artificial membrane that was tested, was highly variable. Polyethylene membranes proved to be the most suitable for use in the investigation of the morphological and nuclear development of infection structures from urediospores of *P. apoda*.

Urediospore germlings on microfabricated polystyrene developed appressoria on, against and between the microfabricated ridges (Figs 1 and 2). The proportion of 'ridge associated' area on the artificial membrane was 0.445, based on an average appressorium diameter[3] of 16 µm, and the ratio of the number of appressoria observed to differentiate within this area (i.e. were overlapping a ridge), was 0.490 (n = 542). This difference was found, through a statistical test of proportions, to be significant at the 5%, but not the 1% level of significance.

Generally, membranes scratched manually with various implements did not appear to be more inducive than smooth, unscratched membranes. The grooves were coarse and wide, with poor resolution, and the germ tubes seldom acknowledged their presence with appressorium formation (Fig. 3).

2.3.2. Development of infection structures of *P. apoda* on polyethylene membranes over a 24-hour period

The developmental sequence of *Phakopsora apoda* on polyethylene membranes, as observed under the light microscope, and after staining with DAPI (a UV-fluorescent stain of nuclei), can be summarised as follows:

- the binucleate urediospore (Fig. 4) germinated to form a germ tube;

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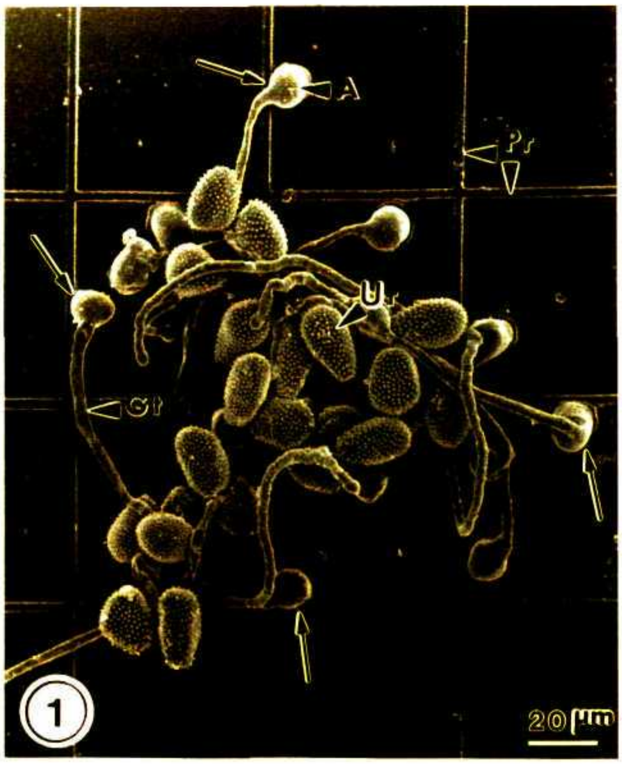
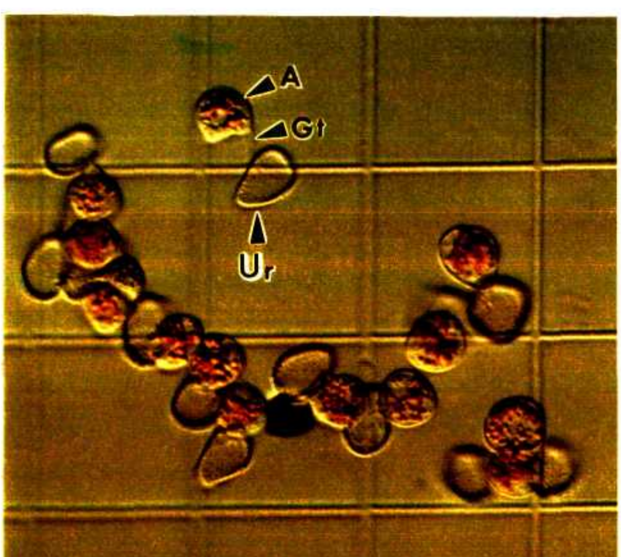
3 Please see the section on Methodology, in the general discussion, for clarification on how the 'ridge associated' area was calculated.

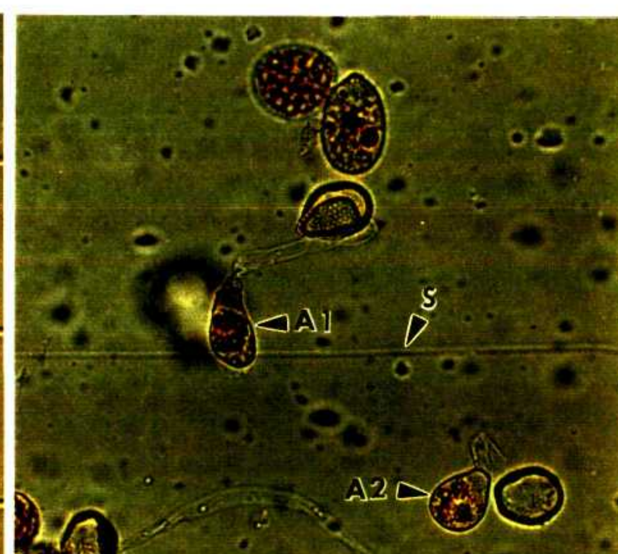
Figure 1: Scanning electron micrograph of urediospore (Ur) germlings on microfabricated, polystyrene membranes, with germtubes (Gt) of varying length (**10 hpi**). Some appressoria (A) have been produced upon contact with the polystyrene ridges (Pr)(arrows).

Figure 2: Light micrograph of urediospores (Ur) on ridged polystyrene. The germ tubes (Gt) are very short, the appressoria (A) sometimes appearing to be sessile to the urediospores. In some cases appressoria have developed immediately adjacent to the ridges (Pr), whilst others have developed in the smooth areas between ridges (**10 hpi**).

Figure 3: Urediospore germlings on polypropylene. An appressorium (A1) has apparently formed in response to a random scratch (s) on the membrane surface. An adjacent appressorium (A2), appears to have been induced in the absence of a visible topographical stimulus (**10 hpi**).


A
Pr
Ur
Gt
1
20 µm


A
Gt
Ur
Pr
2
20 µm


A1
S
A2
3
20 µm

- the two nuclei in the urediospore migrated into the germ tube (Fig 5);
- a swelling of the tip of the germ tube developed, and the nuclei migrated into the developing appressorium, which was subsequently cut off from the germ tube by a septum (Fig. 6 (i));
- once the septum formed, the nuclei underwent mitosis, resulting in the presence of four nuclei in the appressorium (Fig. 6 (ii));
- at a point of contact between the membrane and base of the appressorium, a clear, circular region developed in the wall of the appressorium. Around this basal pore, the wall appeared to be altered in some way (Fig. 6 (i) inset). Later in the development of the germling, once the cytoplasm had moved out of the appressorium, a cone-like structure could be seen lining the periphery of the pore (Figs 7 (i) and 9). Often the cones were partially obscured, and yet more clearly outlined, by aggregates of cytoplasmic material remaining in the appressorium;
- from the pore, a hypha developed (Fig. 7 (i));
- the four nuclei in the appressorium often arranged themselves in a fairly straight line at the entrance of the penetration hypha, and proceeded to enter the hypha in 'single-file'. In Fig. 7 (ii) there are two germlings in which the first nucleus has moved into the penetration hypha. Sometimes, one or two nuclei remained in the appressorium. These would presumably enter the hypha at a later stage (Fig. 8 (ii)). Nuclear division occurred again in the penetration hypha (Fig. 8 (ii));
- the penetration hypha continued to elongate, and often adopted a 'corkscrew' growth habit (Fig. 9);
- a septum formed and a primary hypha elongated further before branching into two secondary hyphae (Fig. 9).

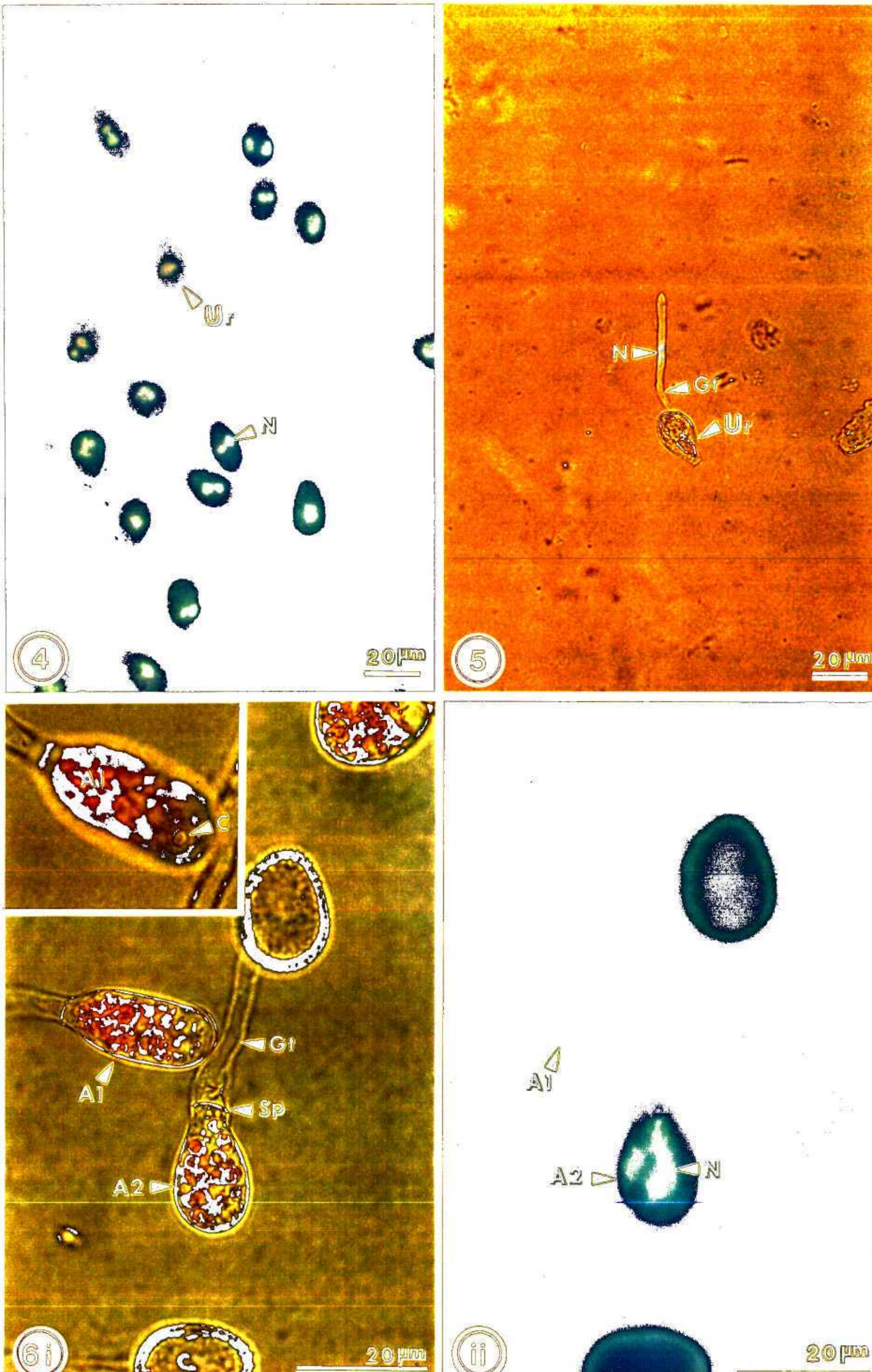
Nuclear behaviour in the primary hypha was not clearly observed. In some instances, two nuclei were left behind the septum delimiting the primary hypha from the penetration hypha, and even in the appressorium. No more than two nuclei were ever observed to be left behind. After 24 hours, up to 9 nuclei were observed in a single germling.

Figure 4: Urediospores (Ur) of *P. apoda* after staining with DAPI fluorescent nuclear stain, and viewed under UV light. Two nuclei (N) are visible in each spore (**2 hpi**).

Figure 5: A urediospore (Ur) has germinated to produce a germ tube (Gt). Two nuclei (N) have migrated from the urediospore and are visible in the germ tube (the specimen was illuminated with both visible and UV light when this micrograph was taken) (**4 hpi**).

Figure 6: (i) Two appressoria (A1 and A2), each delimited from their germ tubes (Gt) by a well-defined septum (Sp in the latter germling) (**8 hpi**).
Inset: Appressorium 1 photographed on a different focal plane (closer to the membrane). A clear, circular region faintly surrounded by a ring of wall material with slightly different optical properties (C), is visible in the base of the appressorium. The faint ring around the hole could represent an appressorial cone.

(ii) A1 and A2 stained with DAPI and viewed under UV light. Four nuclei (N) are clearly visible in the appressorium. These nuclei are fairly large and diffuse in appearance.



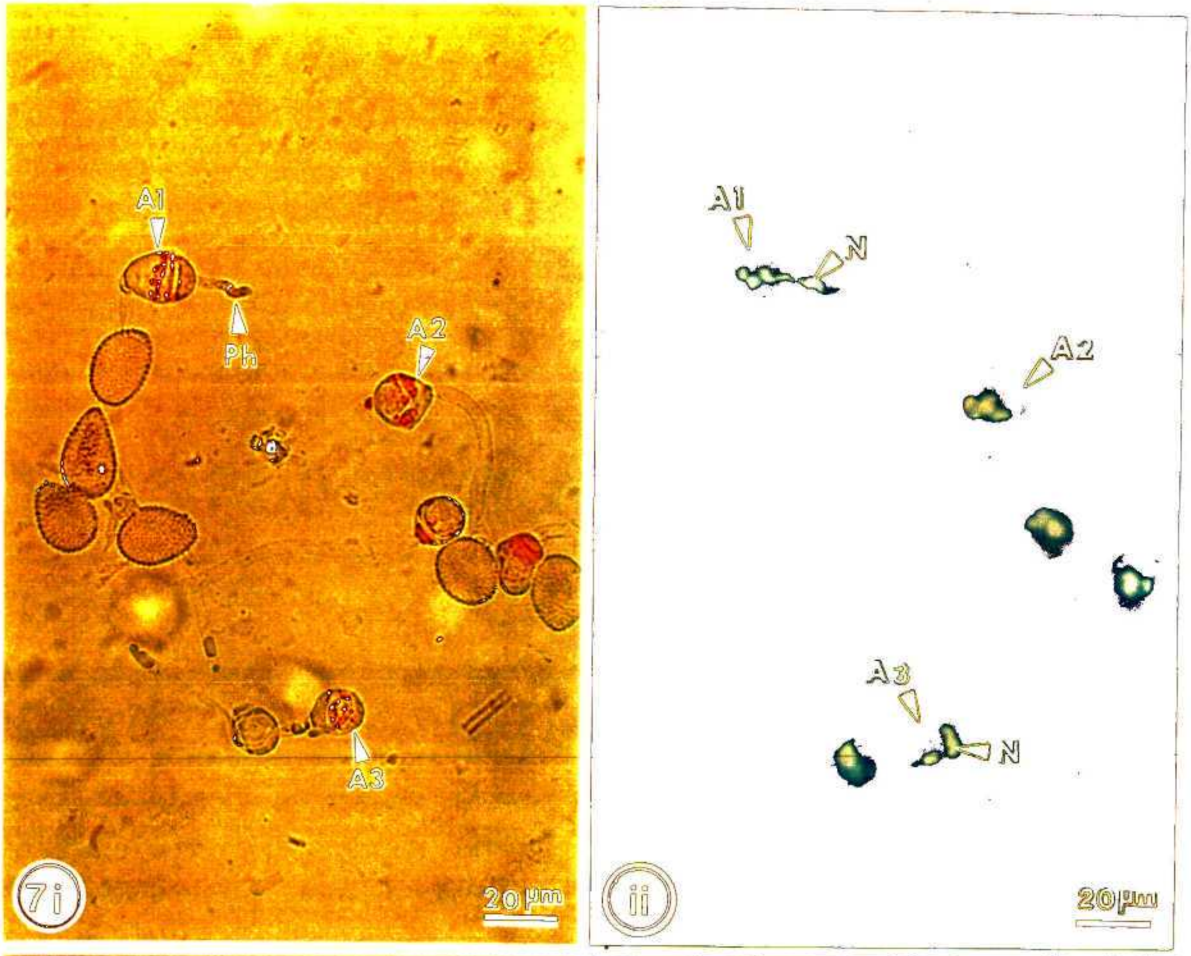
4
Ur
N
20 µm
5
N
Gt
Ur
20 µm
6 i
A1
C
Gt
A1
Sp
A2
C
20 µm
ii
A1
A2
N
20 µm

Figure 7: (i) Three appressoria (A1, 2, 3) have each produced a penetration peg/penetration hypha (Ph) (**24 hpi**).

(ii) In appressoria 1 and 3, one nucleus (N) has entered the penetration peg, and the remaining three in the appressorium are lined up, in 'single file', ready to follow. The nuclei appear to be more compact (and fluoresced more brightly) than prior to penetration peg development (Fig. 6).

Figure 8: (i) Two appressoria (A1, 2) have each given rise to a penetration hypha (Ph) (**24 hpi**).

(ii) In A1, it would appear that two nuclei (N) (one of which is possibly dividing) are entering the penetration hypha (Ph), and two (one of which appears to be dividing) have already migrated some distance in the penetration hypha.


A1
Ph
A2
A3
7i
20 µm
A1
N
A2
A3
N
ii
20 µm

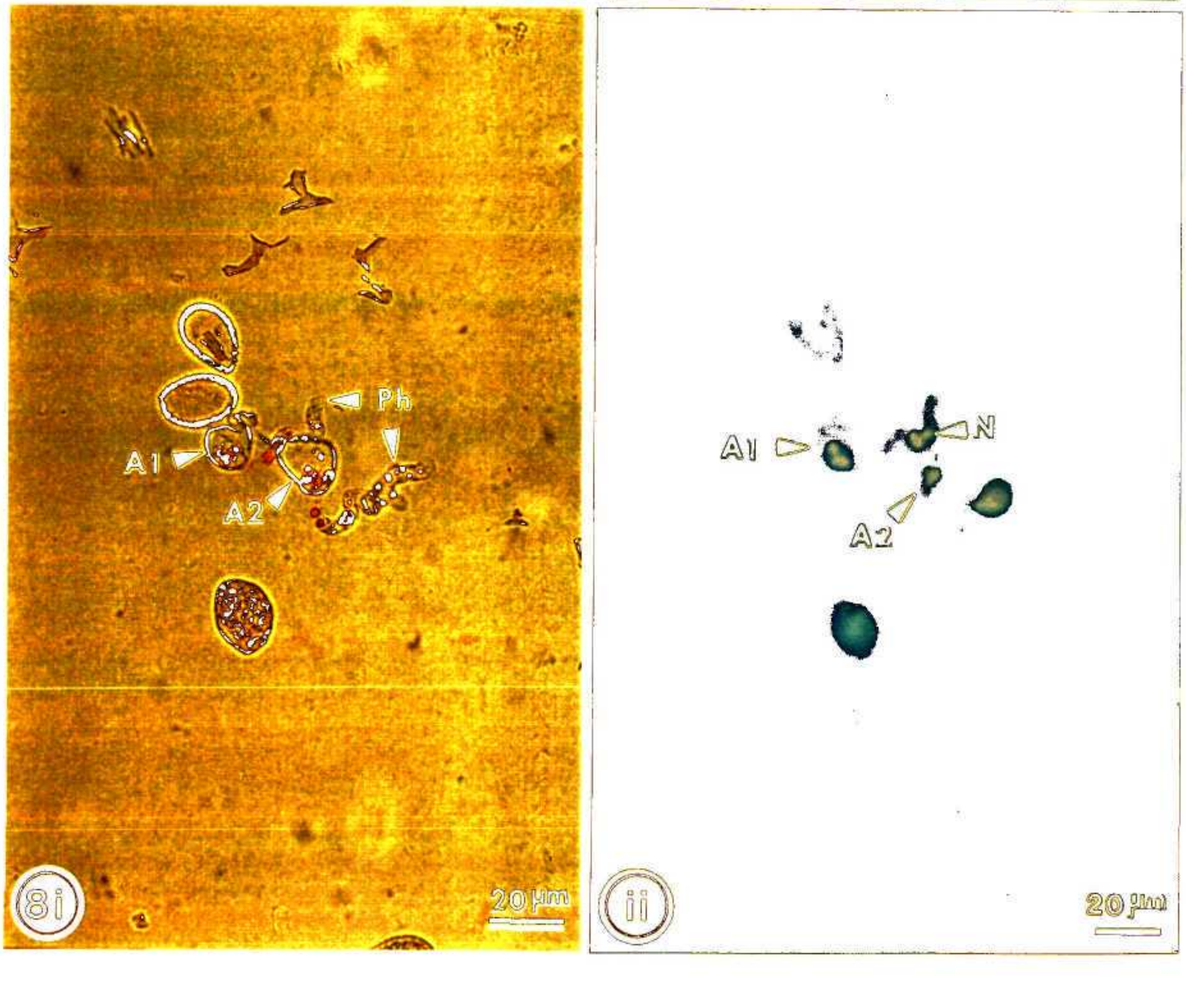
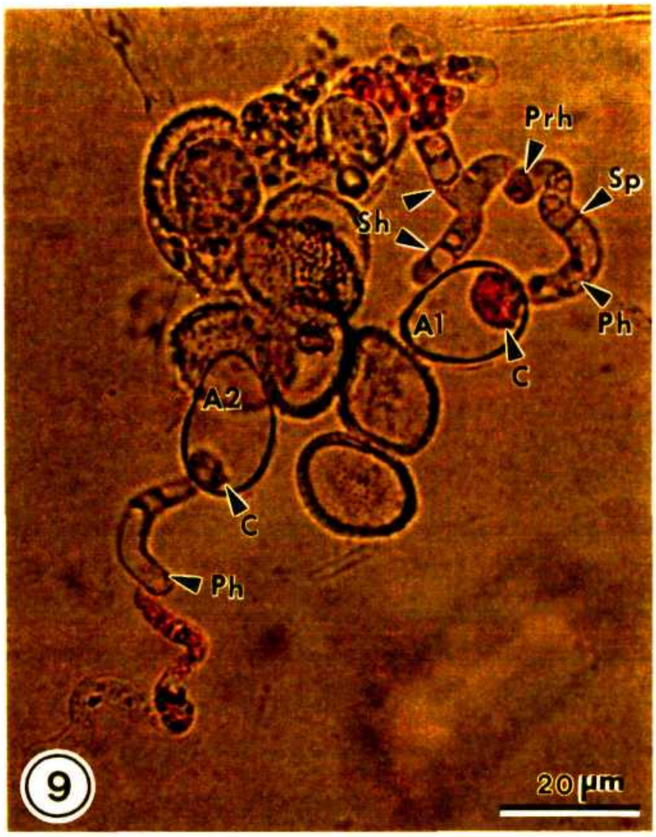

Ph
A1
A2
8i
20 µm
A1
N
A2
ii
20 µm

Figure 9: Two germlings have reached advanced stages of infection structure development. In the upper germling, a penetration hypha (Ph) has developed from a cone-like structure (C) in the base of the appressorium (A1). This penetration hypha is fairly short. A septum (Sp) delimits the penetration hypha from the primary hypha (Prh), which has extended in a twisted, cork-screw fashion, before branching to produce secondary hyphae (Sh).
In the lower germling, the appressorium (A2) has also produced a penetration hypha (Ph). Either this penetration hypha is very long, or the septum delimiting it from the primary hypha has been obscured. In the base of the appressorium, where the penetration peg has emerged, a very clear cone-like structure (C) can be seen. Often this cone is obscured by the cytoplasmic contents in the appressorium, but here, all the cytoplasm has moved into the penetration hypha (**20 hpi**).

Figure 10: In this scanning electron micrograph, two appressoria are clearly visible on the host leaf surface (A1, 2). A third appressorium (A3) is present beneath the two urediospores on the right. Note how A2 has formed in the valley at the junction between two epidermal cells (arrows). Either A1 or A3 has failed to effect penetration of the leaf, and the infection structures have developed on the leaf surface. Note the cork-screw growth habit of the penetration/primary hypha (Ph), and the terminal branching into secondary hyphae (Sh). No septum is visible delimiting the penetration and primary hyphae (**10 hpi**).


Prh
Sp
Sh
Ph
A1
C
A2
C
Ph
9
20 μm

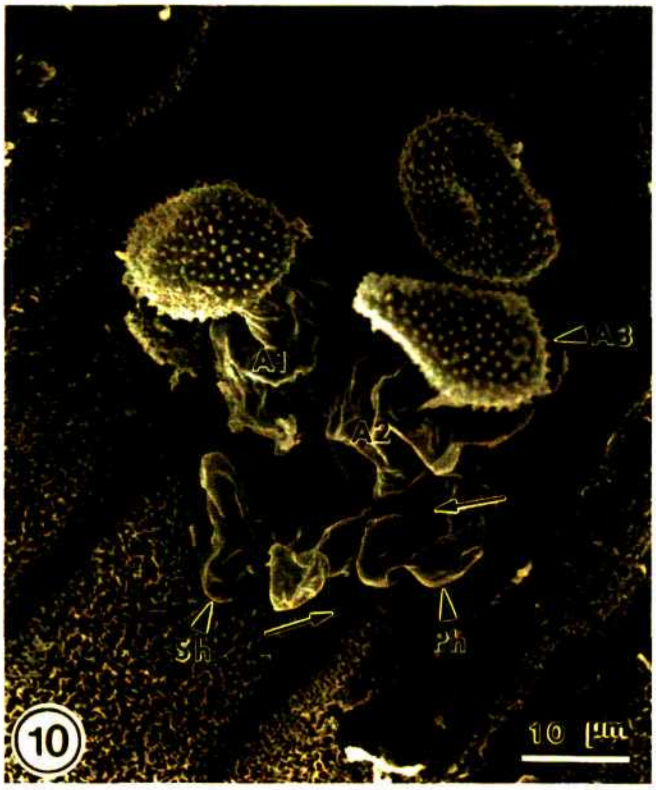

A1
A3
A2
Sh
Ph
10
10 μm

Figure 11: Development of infection structures from urediospores of *P. apoda*, on polyethylene membranes, over a 24 hour period.

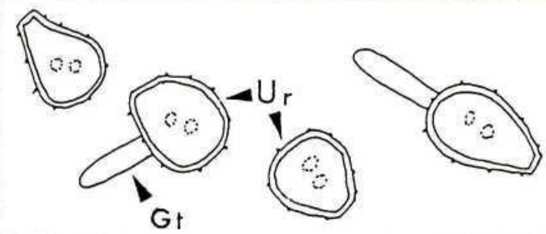
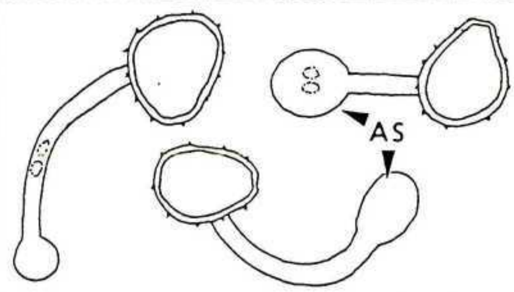
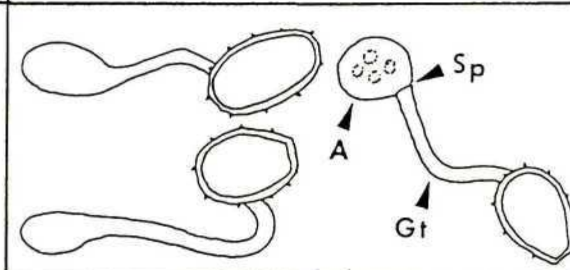
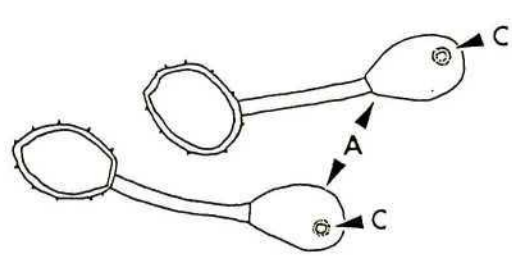
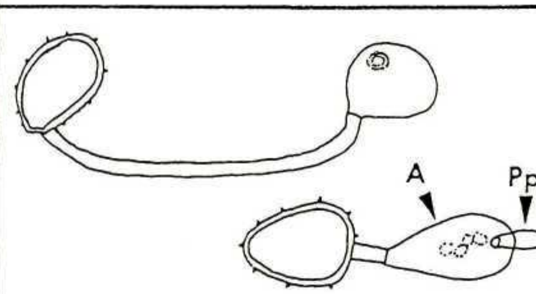
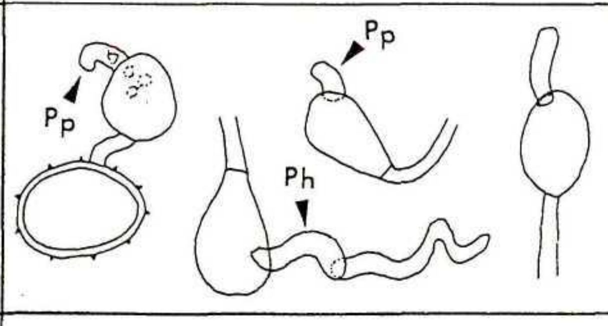
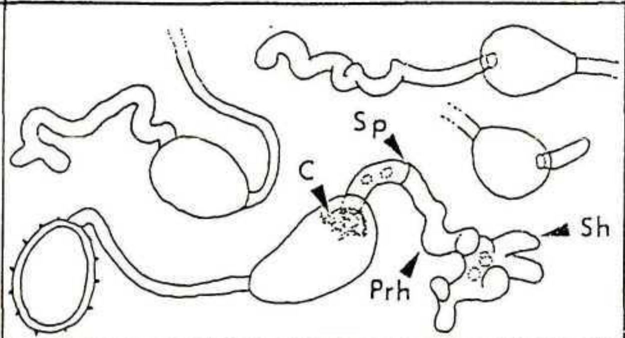
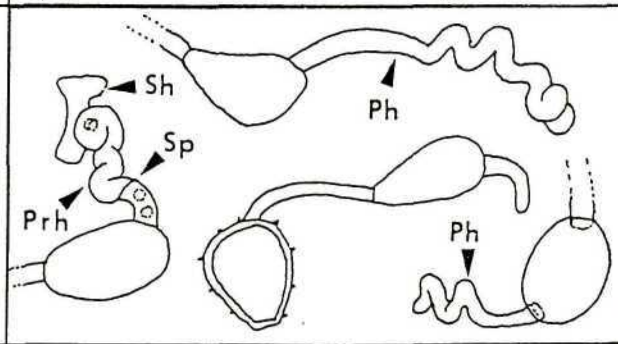
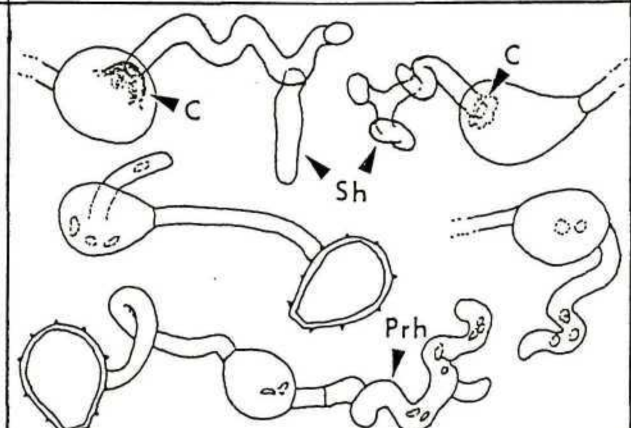
HOURS POST INOCULA -TION	DESCRIPTION OF PREDOMINANT STAGES OF GERMLING DEVELOPMENT	DIAGRAMS OF TYPICAL GERMLINGS OBSERVED AT SPECIFIED TIMES AFTER INOCULATION
2 hpi	Short germ tubes (Gt) developing from some of the urediospores (Ur); many spores ungerminated.	
4 hpi	Longer germ tubes, appressorial swellings (AS) developing at their tips; 2 nuclei present in some terminal swellings.	
6 hpi	Septum (Sp) delimits mature appressorium (A) from germ tube (Gt).	
8 hpi and 10 hpi	First appearance of a wall-less region and possibly a cone (C) in base of appressorium (A).	
12 hpi and 14 hpi	First sign of penetration peg (Pp) emergence from base of appressorium (A).	

Figure 11: (cont.)

HOURS POST INOCULA -TION	DESCRIPTION OF PREDOMINANT STAGES OF GERMLING DEVELOPMENT	DIAGRAMS OF TYPICAL GERMLINGS OBSERVED AT SPECIFIED TIMES AFTER INOCULATION
16 hpi	Penetration pegs (Pp) more numerous; first twisted penetration hyphae (Ph).	
18 hpi	Septum (Sp) formation; first primary hyphae (Prh); first secondary hyphae (Sh); clearly defined cones (C).	
20 hpi	Long penetration hyphae (Ph); some septa (Sp) and primary (Prh), secondary (Sh) hyphae.	
22 hpi and 24 hpi	Excellent visibility of cones (C); long penetration hyphae (Ph); primary (Prh) and secondary (Sh) hyphae.	

On the leaf surface, infection structures that were apparently morphologically identical to those produced on the membranes, were occasionally observed. In Fig. 10, the 'corkscrew' penetration/primary hypha can be seen, with the terminal branching of the primary hypha into two secondary hyphae. Note the similarities to germlings in Fig. 9. Fig. 11 provides illustrations of the various stages of germling development with reference to a 24 hour time frame. Examples of typical germlings seen at various time intervals following inoculation of polyethylene membranes are shown.

2.4 DISCUSSION

The relatively tough, hairy leaves of kikuyu grass made direct observation of the infection process with light microscopy, very difficult. The epidermal layer was not easily stripped from the fresh leaf, and hairs often obscured the infection structures on the leaf surface. Furthermore, staining infected host tissue with DAPI stain proved to be futile, as the host nuclei were so large and fluoresced so brightly under UV light, that the fungal nuclei were obscured. The use of membranes solved the problems of reduced germling visibility on the host, and also facilitated the investigation of the role of topography in the positioning of appressoria on the host leaf surface.

2.4.1. Adhesion and differentiation of germlings on artificial membranes

Rust fungi are notoriously sensitive to the environment - perhaps due to the obligate nature of their interactions with the host -and it is sometimes very difficult to determine why germlings develop better on some membranes than others.

Close contact with the substrate is very important for the induction of appressoria. Adhesion of fungal germlings to artificial membranes has been well documented (e.g. Koch and Hoppe, 1988; Swann and Mims, 1991). The strength of adhesion and subsequent closeness of the germling to the substrate probably plays an important part in determining whether the fungus will differentiate infection structures or not, especially in the indirect penetrators. The mechanisms by which germling adhesion is

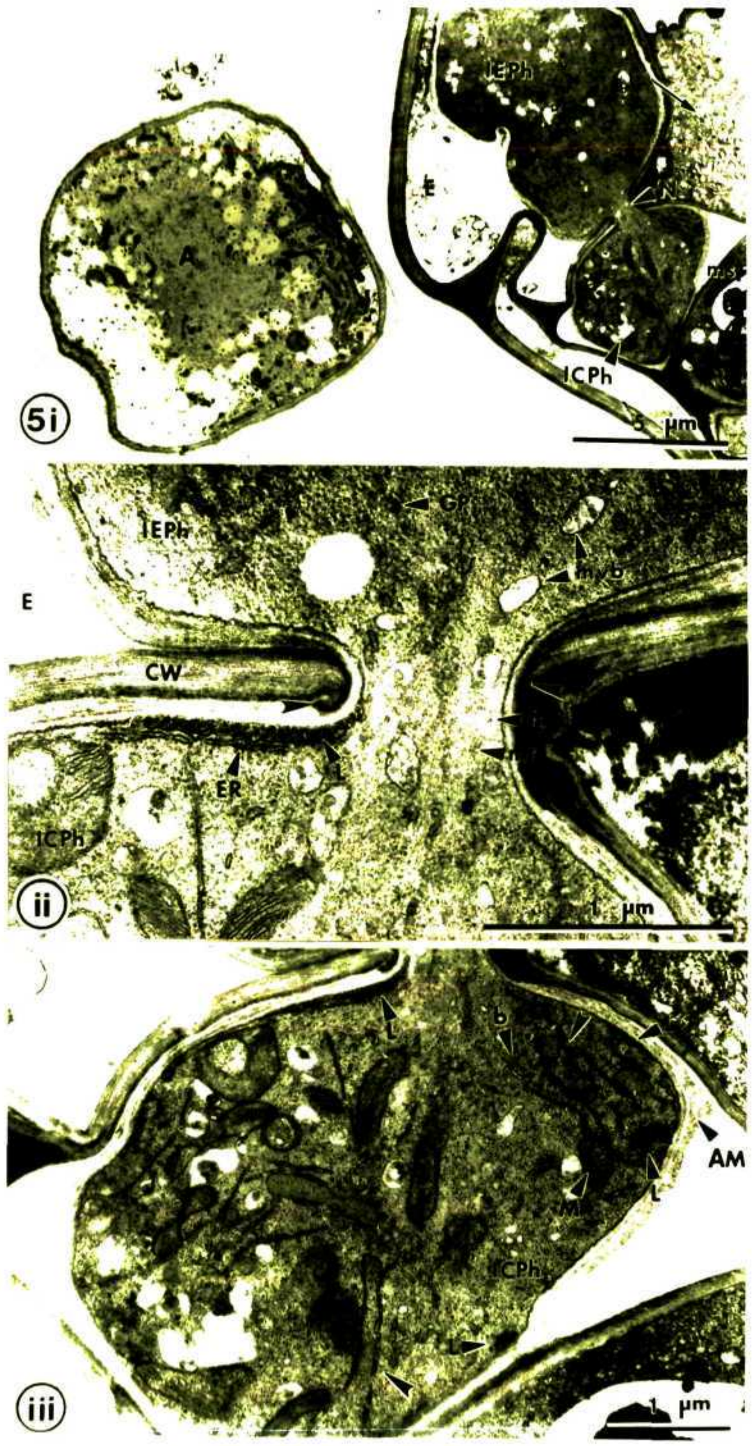
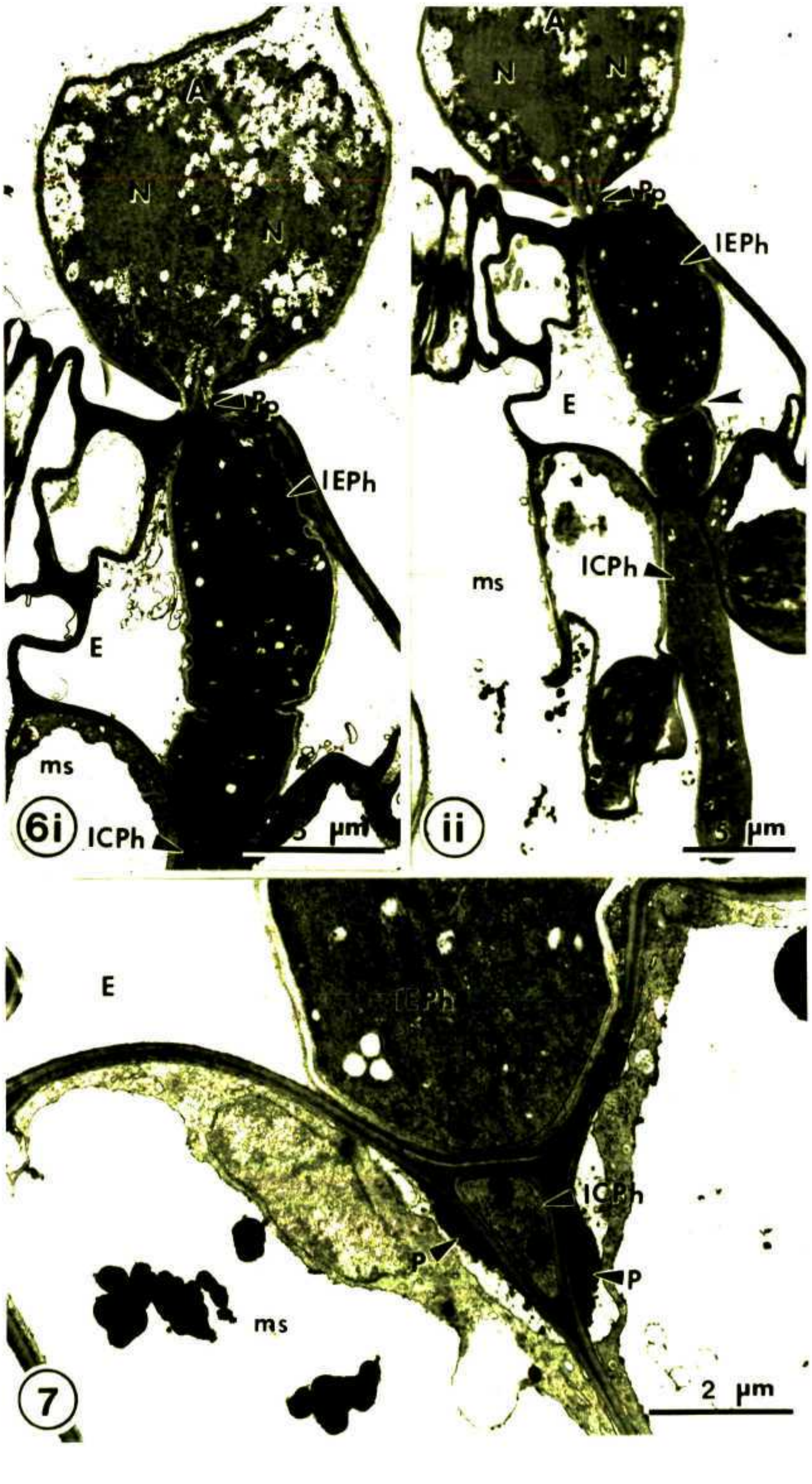

5i
IEPh
A
ICPh
5 µm
ii
IEPh
E
Gr
mvb
CW
ER
L
ICPh
1 µm
iii
L
b
AM
L
ICPh
1 µm

Figure 6: (i) and (ii) A view of the complete penetration process, with the appressorium (A) (containing at least two nuclei (N)), penetration peg (Pp), Intraepidermal penetration hypha (IEPh), and intercellular penetration hypha (ICPh). The break in the intraepidermal penetration hypha in (ii) (arrow) is due an infolding of the wall. In (i) the aseptate, continuous penetration hypha can be seen. No septum formation is visible in the intercellular penetration hypha. E - epidermis; ms - mesophyll.

Figure 7: Host reaction to the penetration hypha: the host cytoplasm, in mesophyll cells adjacent to the infection site (ms), has formed aggregates adjacent to the epidermal cell (E) (containing an intraepidermal penetration hypha (IEPh)), and the intercellular penetration hypha (ICPh). The electron-dense areas, between the host plasmalemma and the host wall, adjacent to the penetration hypha, probably represent papillae (P).


A
N
N
Pp
IEPh
E
ms
6i
ICPh
μm
ii
5 μm
E
IEPh
ICPh
P
P
ms
7
2 μm

mediated are poorly understood but, very often, adherence is determined by surface characteristics of the substrate (Mendgen and Deising, 1993). The ability of fungal germlings to adhere to plastics such as polyethylene and teflon, has been cited as evidence of the involvement of hydrophobic bonds in the attachment process (Nicholson and Epstein, 1991). The general response of some rusts of graminaceous hosts is that they adhere more tightly to hydrophobic, rather than hydrophilic surfaces (Wynn and Staples, 1981; Hoch and Staples, 1987; Hamer *et al.*, 1988). If hydrophobicity and inducive potential of membranes are positively correlated, then this could explain why teflon, which is very hydrophobic, is more inducive of appressorium formation than polyethylene.

Koch and Hoppe (1988) made an interesting observation: the more inducive the membrane to appressorium formation, the lower the rate of penetration hypha formation. This corresponds to some extent with the present author's observations, i.e. teflon, mylar, and polypropylene were highly inducive of appressorium formation, but penetration hyphae were extremely rare on these membranes. On the other hand, appressorium formation on polyethylene was often low, but most appressoria that developed, produced penetration pegs. Perhaps this is related to how well the appressorium adheres to the membrane surface. Since induction of infection structures and close adhesion to the membrane go hand in hand, one would expect there to be tight adhesion on highly inducive membranes. Perhaps, when adhesion is very tight, this prevents the penetration peg from effecting its directional peg emergence, especially if penetration is effected by enzymatic, rather than mechanical means. When the adhesion of the appressorium to the substrate is weaker, this may allow the penetration peg to lift the appressorium slightly, and grow out sideways from beneath the appressorium, onto the surface of the membrane.

Manual scratching of membranes with various implements did not produce good results in terms of increasing the inducive properties of the membranes for appressorium formation. Often, the scratches appeared to be too wide and too irregular, to elicit a response from the germ tubes, which would simply grow over the grooves.

Microfabricated polystyrene membranes, with their well-defined topographical features, were more useful in quantifying the response of the fungal germlings to abrupt changes in elevation.

2.4.2. The role of topography in appressorium induction

Germlings of *P. apoda* appear to respond to both leaf and artificial topographies in a manner similar to that of *P. pachyrhizi*. Germlings of both fungi form appressoria on, between and beside ridges on artificial, microfabricated membranes, as well as on smooth surfaces. This would seem to indicate that a non-specific contact stimulus is required for the induction of appressorium differentiation, as suggested by Koch and Hoppe (1988) and Kitani and Inoue (Allen *et al.*, 1991a). However, Allen *et al.* (1991a) concluded that differentiation of appressoria of *P. pachyrhizi* occurred 'in loose association with the ridges', and in the case of *P. apoda*, although appressoria also formed on smooth surfaces, when a germ tube did encounter a ridge during the course of its short meanderings, it was usually induced to form an appressorium. This may be related to the fact that both *P. pachyrhizi* (Koch and Hoppe, 1988) and *P. apoda* (Hall, 1991; Adendorff and Rijkenberg, 1995b; Fig. 10) appear to form appressoria preferentially over the depressions formed at the junctions between epidermal cells. In the case of *P. pachyrhizi*, the same preference has been observed on leaf replicas (Allen *et al.*, 1991a).

Allen *et al.* (1991a) reasoned that if the percentages of appressorium formation by *P. pachyrhizi* germlings on and beside ridges were pooled and considered to be a thigmotropic response, then the appressorium formation in association with ridges (63%) would approximate the proportion of appressoria observed to form over epidermal cell junctions. In other words although *P. pachyrhizi* and *P. apoda* are capable of forming appressoria in the absence of a specific thigmotropic stimulus, it is possible that the surface topography aids in the location of optimal penetration sites. Penetration at epidermal cell junctions could perhaps be seen as being advantageous, but not essential.

Many direct-penetrating fungi respond to the indentations between epidermal cells, and often form appressoria preferentially at or near these epidermal cell junctions (Hunt, 1968; Bonde *et al.*, 1976; Wynn and Staples, 1981; Bonde, Bromfield and Melching, 1982; Gold and Mendgen, 1984; Staples and Macko, 1984).

Bonde *et al.* (1976), found that approximately 85% of the *Phakopsora pachyrhizi* appressoria they observed, developed over the anticlinal walls separating adjacent epidermal cells. According to Wynn and Staples (1981), at least 22 species of pathogenic fungi in 14 genera have been documented to form appressoria frequently at cell junctions, and they regarded this to be a generalised phenomenon amongst the direct-penetrating fungi.

Cutler, Alvin and Price (1982) suggested that this site preference may be related to the greater availability of nutrients and moisture due to a higher rate of exosmosis in these areas and/or the chemical composition and physical structure of the wax layer that affects both the pH and wettability of the leaf surface. The increased 'leakiness' of these areas to exudates from within the leaf, may result from the fact that cuticular waxes are often thinner towards the epidermal cell junctions (Schönherr and Bukovac, 1970), and that the anticlinal walls serve as a wick for the exudates (Hoch and Staples, 1991).

The relatively unspecific thigmotropic sensitivity exhibited by *P. apoda* and many of the direct penetrators may have significance in terms of giving these fungi the flexibility to explore a wider host range, and to adapt to variable characters within a particular host genus, in contrast to those rust fungi which only produce appressoria in response to a very specific and narrow range of stimuli (Allen *et al.*, 1991a). Perhaps this is best illustrated in *P. pachyrhizi* which, according to Keogh (Bonde *et. al.*, 1976), is known to infect many legume species, in numerous orders of the family Leguminosae. According to Sinclair (Koch *et al.*, 1983), soybean rust is able to penetrate into at least 87 different plant species, and can reproduce in 31 species in 17 genera of the leguminous plants (Ono, Buritica and Hennen, 1992). *P. apoda* is known to infect a number of grass species, including *Setaria glauca* (Dingley, 1977), *Pennisetum pedicellatum* Trin. (Cummins, 1971), *Pennisetum polystachyon* (L.) Schult. (Cummins, 1971), and *Pennisetum setosum* (Sw.) Rich. (Mains, 1938; Cummins, 1971).

2.4.3. Development of infection structures on polyethylene membranes

The infection structures formed by *P. apoda* on artificial membranes closely resembled those produced by *Phakopsora pachyrhizi* (Koch and Hoppe, 1988). A similar time scale in terms of development was also noted. In both fungi, germ tube tips began to swell between 2 and 4 hpi, and penetration pegs were first observed 12 hpi.

In Fig. 10, a germling has failed to penetrate the leaf surface, and instead, has produced its infection structures on the leaf surface. The fact that these structures (as well as those that have been observed previously within the host (Adendorff and Rijkenberg, 1995b) are morphologically identical to those observed on membranes, is encouraging. This suggests that the artificial conditions provided by the membranes do not affect the morphological development of the fungal germlings to a great extent.

Koch and Hoppe (1988) also observed the development of appressoria, penetration hyphae and primary hyphae of *Phakopsora pachyrhizi* on the surface of the host leaf (*Glycine max* (L.) Merr.), apparently as a result of the formation of papillae in the host epidermal cells directly beneath the proposed penetration sites - presumably a host resistance reaction.

The first visible sign of infection structure development from the appressorium of *P. apoda*, is the development of a clear, circular region in the base of the appressorium. This pore-like region is probably the equivalent of the wall-less region that develops at the site of penetration peg emergence in many direct-penetrating fungi, including *P. pachyrhizi* (Littlefield and Heath, 1979; Wynn, 1981; Koch *et al.*, 1983; Swann and Mims, 1991; Mendgen and Deising, 1993).

In any successful infection from an appressorium, the penetration peg always emerges from the base of the appressorium, as opposed to the top or sides - the 'directional peg emergence' discussed by Staples and Macko (1984) for the stoma-penetrating rust germlings. Wynn and Staples (1981) suggested that a contact or chemical stimulus may be involved, leading to the development of the wall-less region. This thinning of the wall may favour the development of the penetration hypha within that area, i.e. the emergence of the penetration peg may be controlled through the creation of a weak or chemically altered area in the base of the appressorium. Peg emergence often occurs 'off-centre' in appressoria of *P. apoda*, as is evident in Figs 7 and 8.

In *P. pachyrhizi*, a cone of wall material and plasmamembrane was observed in association with the wall-less region (Koch *et al.*, 1983). The funnel-shaped structure was observed 12 hpi in appressoria formed on the host, and it appears to originate within the appressorium as a branched cell wall-like structure (Koch *et al.*, 1983). Although very difficult to see under the light microscope, such cone-like structures were observed in appressoria of *P. apoda*, as illustrated in Figs 6 and 9.

Cones or cone-like structures have been observed in a number of fungi, but it is not known whether these can be regarded as homologous from species to species. To date, these cone-like structures have only been reported in appressoria of some direct penetrators, e.g. in urediospore germlings of *R. humphreyana* (Hunt, 1968), basidiospore germlings of *Gymnosporangium juniperi-virginianae* Schw. (Mims and Richardson, 1989), aeciospore germlings of *Arthuriomyces peckianus* (Howe in Peck) Cumm. Hirat. (Swann and Mims, 1991), several species of *Colletotrichum* (Mercer, Wood and Greenwood, 1971; Landes and Hoffmann, 1979), and in *Spilocaea pomi* Fr. (Corlett and Chong, 1977).

On artificial membranes the hypha that develops from the pore-like zone in appressoria of *P. apoda*, is probably analogous to the penetration peg that penetrates the cuticle and epidermal cell wall of the host. Upon elongation of this hypha, it would probably be more correct to regard it as the equivalent of the penetration hypha that transverses the epidermal cell lumen, and emerges into the intercellular spaces of the mesophyll tissue in the host. Koch *et al.* (1983) found no morphological difference between the penetration peg of *P. pachyrhizi* and the penetration hypha that traversed the epidermis, except that the peg was smaller in diameter, as fungal hyphae often are when they pass through a host cell wall. It would seem that in both *P. apoda* and *P. pachyrhizi*, the penetration peg and penetration hypha can be distinguished on a functional rather than a morphological basis, and the distinction between the two in the absence of the host, is rather arbitrary.

The factors leading to primary hypha formation on artificial membranes are not understood, and generally the penetration hyphae of *P. apoda* produced on membranes were a good deal longer than the depth of the host epidermal cell layer. Many other fungi, however, appear to define the diameter and length of their penetration hyphae in the absence of the host, as evidenced by the formation of similar structures *in vivo* and *in vitro* (Mendgen and Deising, 1993).

In the case of *P. pachyrhizi*, it would seem that the development of the infection hypha is probably the most critical and specific stage of infection as, according to Hoppe and Koch (Koch and Hoppe, 1988), this occurs at very low rates on non-host surfaces. Koch and Hoppe (1988) suggested that a second stimulus is required in *P. pachyrhizi* to trigger the formation of the penetration hypha. The low rates of penetration hypha formation by *P. apoda* on most of the membranes used in this study, would seem to indicate that it is also a critical stage in the development of this fungus, but whether certain membranes inhibit penetration hypha formation, or whether certain membranes actually provide a particular stimulus inducing penetration hypha formation, is not clear.

A septum was observed to develop in the penetration hypha of *P. apoda* on polyethylene membranes. In *P. pachyrhizi*, an apparently equivalent septum forms in the penetration hyphae on both artificial membranes (Koch and Hoppe, 1988), and also in the host, once the penetration hypha reaches the intercellular spaces of the host mesophyll after traversing the epidermal layer (Koch *et al.*, 1983).

The penetration and primary hyphae of germlings of *P. apoda* often grew in a spiral configuration. This 'corkscrew' growth habit of the penetration and primary hyphae was also observed by Koch and Hoppe (1988), in germlings of *P. pachyrhizi* grown on artificial membranes.

The most advanced stage of germling development that was observed on membranes after 24 hpi, was the growth of secondary and tertiary hyphae, and no structures resembling haustorial mother cells were produced by *P. apoda* on any of the artificial membranes used during the course of this study. Haustorial mother cells were not observed for *P. pachyrhizi* on artificial membranes either (Koch and Hoppe, 1988). As a general rule, for the induction of haustorial mother cell formation to occur, rust fungus germlings would seem to require additional stimuli, or perhaps certain nutrients usually derived from the host, which are not provided by artificial membranes. Apparently, an exception is *Uromyces viciae-fabae* (Pers.) Schroet. which develops infection structures up to and including the stage of the haustorial mother cell, on polyethylene membranes scratched with brass brushes (Deising, Jungblut and Mendgen, 1991).

2.4.4. Nuclear behaviour in germlings of *P. apoda* on artificial membranes

When infected kikuyu grass leaf samples were stained with DAPI, the host nuclei were bigger and brighter than those of the fungus, and they obscured the nuclei in the infection structures. Using membranes facilitated clear observation and photography of nuclear behaviour, as seen in Figs 4, 5, 6, 7 and 8.

Nuclear staining of the infection structures revealed a pattern of nuclear behaviour similar to that which has been observed in other rust urediospore germlings, including those of *P. pachyrhizi*.

The fact that the nuclei entered the penetration peg in an ordered fashion, lining up in 'single file', is further evidence to substantiate the theory that movement of the cytoplasm within the infection structures is tightly controlled, and not a random streaming event. Researchers have implicated the involvement of the microtubule/filament cytoskeleton in the positioning of organelles during the migration of cytoplasm during the infection process. It is reasonable to assume that such a skeleton is responsible for the orderly, planned, lining up of nuclei prior to their entry into the infection peg.

In *P. apoda*, mitosis occurred after the entry of the two nuclei into the appressorial swelling and the formation of a septum between the appressorium and germ tube. An identical pattern of nuclear behaviour was observed in *P. pachyrhizi* (Koch and Hoppe, 1988). A strict correlation between nuclear division and appressorium development has been observed in urediospore germlings of several rust fungi (Hoch and Staples, 1987).

Nuclear behaviour in the later stages of infection structure development in *P. apoda* was unclear. The nuclei were frequently grouped closely together, or were at different focal planes within the germlings. In some cases, structures other than nuclei were stained (e.g. spore walls), and sometimes the nuclei within spores and the germlings did not stain at all. It was important to make observations quickly once the specimen had been exposed to UV light, as the fluorescence faded very rapidly. No definite patterns in the numbers and movement of nuclei were established after the entry of the nuclei into the infection hypha. In some cases, as seen in Table 1, nuclei were even observed to have been left behind the septum that forms in the penetration hypha, delimiting it from the primary hypha. It is possible that the artificial environment on the membranes elicited an 'unnatural' response in some cases.

It would seem that the development and behaviour of *P. apoda* germlings on artificial membranes is virtually identical to that observed in *P. pachyrhizi*. The use of artificial membranes allowed for the examination of the infection structures under ideal viewing conditions, enabling the elucidation of early nuclear behaviour, and the morphological development of the infection structures until the stage of infection hypha formation. Apparently, the early stages of *P. apoda* germling development are relatively unaffected by their presence on an artificial surface. Inconsistencies in nuclear behaviour may have been an indication of abnormal germling development, especially in the later stages of growth, although variable nuclear numbers in infection structures have been reported in other urediospore germlings. For example, Heath, Xu and Eilam (1996) attributed irregularities in nuclear numbers in *Uromyces vignae* urediospore germlings to nuclear degradation and asynchronous nuclear divisions, partly as a result of a lack of tight control over nuclear positioning in relation to septum development. Such disorganisation in rust fungus germlings in their natural state is difficult to accept when the infection process itself appears to be so well orchestrated.

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CHAPTER 3 SCANNING ELECTRON MICROSCOPY OF DIRECT HOST LEAF PENETRATION BY UREDIOSPORE-PRODUCED INFECTION STRUCTURES OF *PHAKOPSORA APODA*

ABSTRACT

Phakopsora apoda (Har. & Pat.) Mains is a rust fungus on kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chiov.) which penetrates the host leaf directly, through the cuticle, as opposed to the more conventional stomatal penetration employed by many rust fungi in the uredial stage. A urediospore germinates on the leaf surface to form a short germ tube which terminates with the formation of an appressorium. The majority of germlings develop appressoria at the junctions between epidermal cells. Appressoria are often sessile to the urediospores. From the base of the appressorium, a penetration peg emerges, which penetrates through the host cuticle and epidermal cell wall. Penetration of the epidermal cell wall occurs approximately 6 hours post inoculation. Once inside the epidermal cell, the penetration peg expands to form a penetration hypha, which traverses the epidermal cell and emerges into the intercellular spaces of the mesophyll tissue. A septum is formed in the intercellular portion of the penetration hypha, delimiting a primary hypha which extends further into the mesophyll, before branching to form two secondary hyphae. This penetration process, on a monocotyledonous host, would appear to be very similar to that of *Phakopsora pachyrhizi* on soybean.

3.1 INTRODUCTION

Most recent rust research, on the host penetration mechanisms employed by urediospore germlings, has focused on a relatively small group of economically important rust fungi, all of which have been shown to penetrate the host epidermal layer via the stomatal pores. These 'indirect penetrators' have been studied in some detail, regarding the mechanisms they use to locate and enter the stomata, and the structures they form during the penetration process (e.g. Wynn and Staples, 1981; Hoch and

Staples, 1987; Hoch and Staples, 1991). 'Direct penetration' in the rust fungi, which is effected through the cuticle of the host leaf, and not through a stoma, has typically been associated with basidiospore germlings (Littlefield and Heath, 1979), and has been studied in considerably less detail than indirect penetration. It would seem that only five rust fungi have been reported to penetrate the host leaf through the cuticle from urediospores, viz. ***Puccinia psidii*** Wint. on *Syzygium jambos* (L.) Alston (Hunt, 1968), ***Ravenelia humphreyana*** P. Henn. on *Caesalpinia pulcherrima* (L.) Sw. (Hunt, 1968), ***Physopella zeae*** (Mains) Cumm. & Ramachar on *Zea mays* L. (Bonde, Bromfield and Melching, 1982), ***Phakopsora pachyrhizi*** Sydow on *Glycine max* (L.) Merrill (soybean) (Bonde, Melching and Bromfield, 1976) and ***Phakopsora apoda*** (Har. & Pat.) Mains on *Pennisetum clandestinum* Hochst. ex Chiov. (kikuyu grass) (Hall[1], 1991).

Phakopsora pachyrhizi, the causal agent of rust on soybean, is the only one of these direct penetrators that has been studied in any detail, in terms of the development of urediospore-derived infection structures (Bonde *et al.*, 1976; Bonde and Brown, 1980; Keogh, Deverall and McLeod, 1980; Koch, Ebrahim-Nesbat and Hoppe, 1983; Ebrahim-Nesbat, Hoppe and Rohringer, 1985; Koch and Hoppe, 1987; Koch and Hoppe, 1988). A urediospore germling of this fungus penetrates the host leaf by means of a penetration peg, which emerges from the base of the appressorium. A penetration hypha traverses the host epidermal cell, and emerges into the intercellular spaces of the mesophyll, where a septum is formed. A primary hypha grows further into the mesophyll, before branching to form secondary hyphae.

It would seem that the only work that has been conducted on the direct penetrator *Phakopsora apoda*, was a preliminary study conducted by Hall (1991). Hall (1991) found that the appressorium seldom formed over a host stoma, and when it did, penetration was observed to occur directly through one of the guard cells, and not through the stomatal pore, as occurs in indirect-penetrating rust fungi. An

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1 Hall (1991) tentatively identified this fungus as *Puccinia stenotaphri* - subsequent investigations revealed that it is *Phakopsora apoda* (Har. & Pat.) Mains (Adendorff and Rijkenberg, 1995).

appressorium, which was delimited from the germ tube by a septum, usually formed at the junction between host epidermal cells. A mucilaginous material was produced in association with the appressoria of *P. apoda*, and when the appressoria were stripped from the leaf surface, the wax layer beneath was removed. Hall (1991) illustrated the emergence of a cylindrical penetration peg from the base of the appressorium. Very often, the peg was produced 'off-centre', especially when the appressorium formed in the junction between two epidermal cells. This facilitated penetration through the epidermal cell wall, as opposed to penetration between the cells. The infection process after peg emergence and penetration, was not observed by Hall (1991).

From the evidence presented in Hall's (1991) work, and from initial studies of the infection process from urediospores of *P. apoda* on artificial membranes (Adendorff and Rijkenberg, 1996), it would seem that the infection structures produced by this fungus are very similar to those of *P. pachyrhizi*.

The present study is largely an elaboration and continuation of Hall's (1991) preliminary investigations. It is primarily a scanning electron microscope examination of the infection structures produced by urediospore germlings of *P. apoda*, from the point of appressorium formation, to entry of the fungus into the mesophyll tissue of the host leaf. Haustorial development has not been included. Rather, the emphasis has been placed on the actual penetration of the host leaf epidermis and early development in the leaf.

The terminology adopted by Koch *et al.* (1983) has been followed during the course of this investigation, due to many of the similarities that have been found between *P. pachyrhizi* and *P. apoda*.

3.2 MATERIALS AND METHODS

Fresh leaf material bearing uredia was collected as needed from Hilton, on the outskirts of Pietermaritzburg in the KwaZulu-Natal Midlands. Spores were scraped from the uredia with a scalpel and were applied evenly to either the abaxial or adaxial surfaces of uninfected kikuyu grass segments (approximately 2 cm long). When severely infected material was used as a source of inoculum, the infected leaf was carefully pressed and rubbed against the uninfected leaf pieces to facilitate spore transfer. This latter means of inoculation helped to minimise damage to the host leaf surface. Conventional means of inoculation, for example dusting the spores onto the leaf with a camel hair brush, were not practical due to the small size of the uredia and the minute quantities of spores that were produced per uredium.

The inoculated leaf pieces were lightly sprayed with distilled water (using an atomising hand spray), and were floated spore-side up on distilled water in a petri dish. The inside of the lid was sprayed with distilled water, and the closed petri dish was placed in a sealed, black, plastic bag (also sprayed with distilled water), in an incubator at 19 °C.

Leaf material was removed from the incubator at 4, 5, 6, 8, 12 and 24 hours post inoculation (hpi), and was cut with a sharp razor blade into small pieces (approximately 2 x 2 mm) whilst submerged in a solution of 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, at room temperature. The specimens were fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer overnight, washed twice in the same buffer for 30 minutes and post-fixed for 3 hours in 2% osmium tetroxide in 0.05M sodium cacodylate buffer. This was followed by two 30-minute washes in 0.05M sodium cacodylate buffer, and the specimens were then passed through a graded ethanol series. Following dehydration, the material was critical-point dried with carbon-dioxide as a transition fluid.

The critical-point dried specimens were mounted onto brass stubs with double-sided tape, and were gold/palladium sputter-coated with an E5110 S.E.M. coating unit (Polaron Equipment Ltd.) before viewing with an Hitachi S-570 scanning electron microscope.

Observations of penetration structures involved a further step prior to coating, i.e. spore/appressorium stripping (after Hughes and Rijkenberg, 1985). This was achieved by lightly pressing a small piece of double-sided tape (held with forceps or stuck to the tip of a dissecting needle) onto the critical-point dried leaf surface bearing germinated spores. The piece of double-sided tape was then lifted from the leaf, mounted on a stub and sputter-coated for viewing.

'Edge-on' views of the infection structures in the leaf tissues (i.e. cross sections of infected leaves) were obtained by gently tearing the critical-point dried leaf pieces lengthwise, parallel to the leaf venation, and mounting the halves onto stubs with double-sided tape, with the torn edges facing upwards. This was performed under a dissecting microscope, as the spores were visible on the leaf surface, and the plane of tearing could be chosen to coincide with the areas of leaf most densely covered with spores and infection structures. This greatly increased the chances of exposing infection structures within the leaf. A small cut, made with a sharp razor blade, at either end of the desired plane of tearing, increased the accuracy of this process.

Fresh material was also rapidly frozen in liquid nitrogen with a Cryo EMscope SP 2000, prior to viewing with an Hitachi S-570 scanning electron microscope.

Following the photographic development of the scanning electron micrographs, a yellow photographic dye was hand-painted onto some of the photographs to highlight the fungal infection structures, as distinct from the host tissues.

3.3 RESULTS

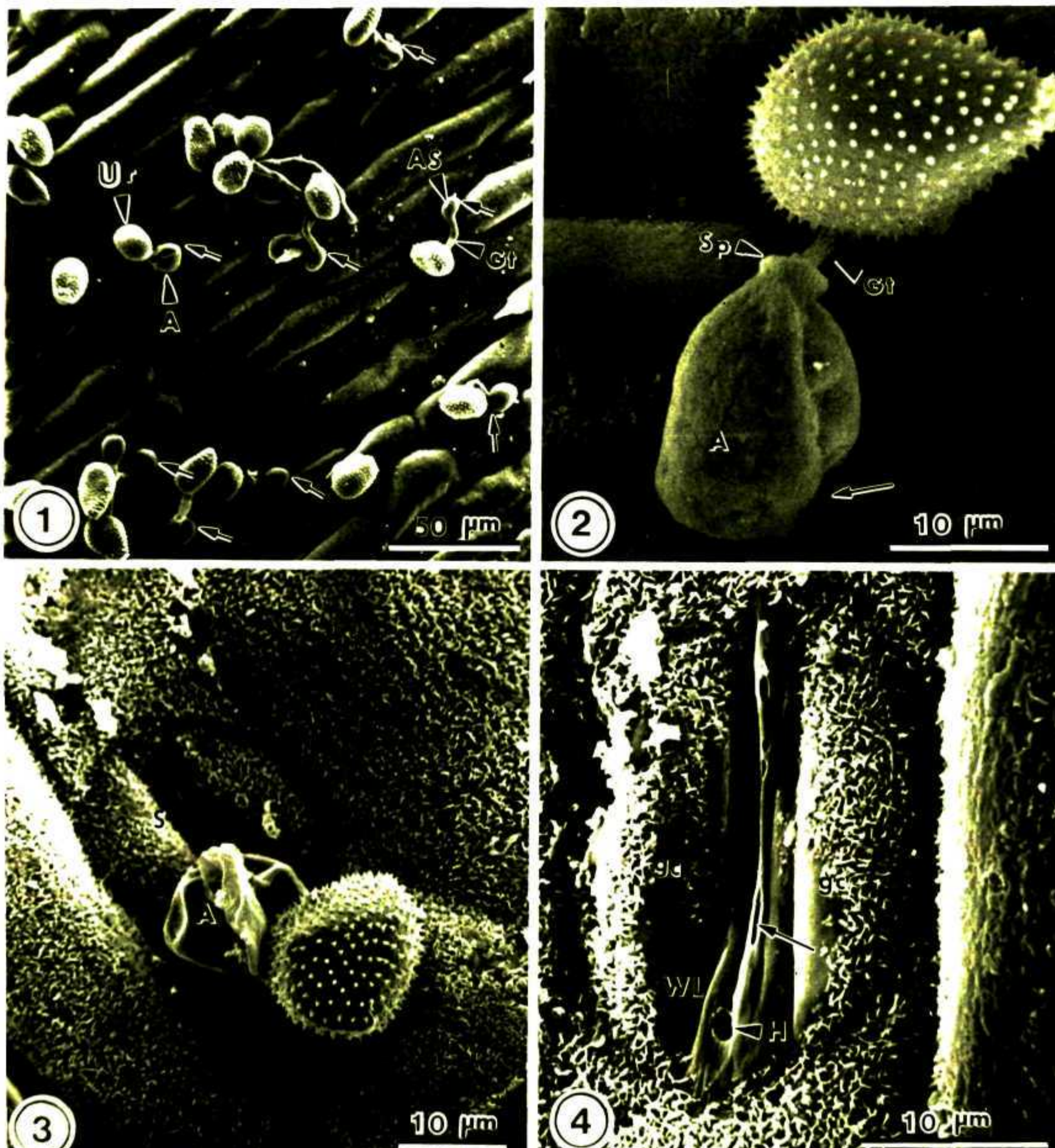
Usually, a spore germinated on the leaf surface to form a short germ tube. Once a suitable infection site was reached, the germ tube tip developed an appressorial swelling (Fig. 1). A large (approximately 13 x 16 µm), well-defined appressorium was delimited from the germ tube by a septum (Fig. 2). Often the appressoria were sessile, apparently without the formation of a germ tube. A minority of spores produced fairly

Figure 1: Echinulate urediospores (Ur) of *P. apoda* have germinated on the host leaf surface (5 hpi) to form mostly short germ tubes (Gt), each terminating in an appressorial swelling (AS), which has, in some cases developed into an appressorium (A), slightly smaller in size than the spore. Most appressoria have formed at the junctions between epidermal cells (arrows).

Figure 2: The mature appressorium (A) is clearly delimited from the short germ tube (Gt) by a septum (Sp) (6 hpi). This appressorium has formed at the junction between three epidermal cells (arrow).

Figure 3: An appressorium (A) has developed over a stoma (S) (24 hpi). The appressorium has collapsed as, by this late stage, the cytoplasm would have moved into the infection structures below.

Figure 4: A wax-less area (WL) on the epidermis indicates the prior position of an appressorium. Even though the appressorium formed over the stoma, penetration, as indicated by the round hole (H), has clearly occurred directly into one of the guard cells (gc), and not via the stomatal pore (arrow) (24 hpi).



Us
A
AS
Gt
1
50 µm
Sp
Gt
A
2
10 µm
S
A
3
10 µm
gc
gc
WL
H
4
10 µm

long germ tubes (sometimes more than 10 times the diameter of a urediospore) prior to appressorium formation (Fig. 1). Most appressoria (80,7 %, n = 200) were formed at the junctions between epidermal cells (Fig. 1), although actual penetration was never observed to occur between adjacent epidermal cells. Appressorium formation over the middle of epidermal cells, occurred to a lesser extent (19,3 %).

Included in the percentage of appressoria that formed at epidermal cell junctions, were the 10,9% that formed over stomata or at guard cell junctions (Fig. 3). When this occurred, penetration occurred directly through one of the guard cells, and not through the stomatal pore, as indicated by the presence of a penetration hole to the side of the stomatal opening (e.g. Fig. 4).

Wherever an appressorium had lifted away from the epidermis, it left a bare, wax-less area on the surface of the leaf (Fig. 5). These wax-less circles on the leaf were used to detect the presence of penetration sites when the pre-penetration structures had been intentionally removed, or had washed off during processing. Within this wax-less area, a hole in the epidermal cell wall was usually visible. The hole, made by the penetration peg, usually had a raised rim (see Figs 6 and 7), and the remnants of the penetration peg could sometimes be seen within the hole (Fig. 6). The penetration holes were usually ellipsoid, and their average dimensions were: 1.3 µm x 1.0 µm. In several cases, there appeared to be an indentation of the leaf surface around the site of penetration. This is especially clear in Fig. 7. An unusual view of the penetration hole can be seen in Fig. 8. Here, an epidermal cell has been torn, revealing the inner surface of the outer epidermal cell wall. The penetration hole on the inside of the epidermal cell, where the penetration peg exited the cell wall into the epidermal cell lumen, would appear to be very similar to the hole seen on the leaf surface, where the peg first enters the cell wall. Both have a rim of raised material surrounding the hole (compare Fig 8 with Figs 6 and 7).

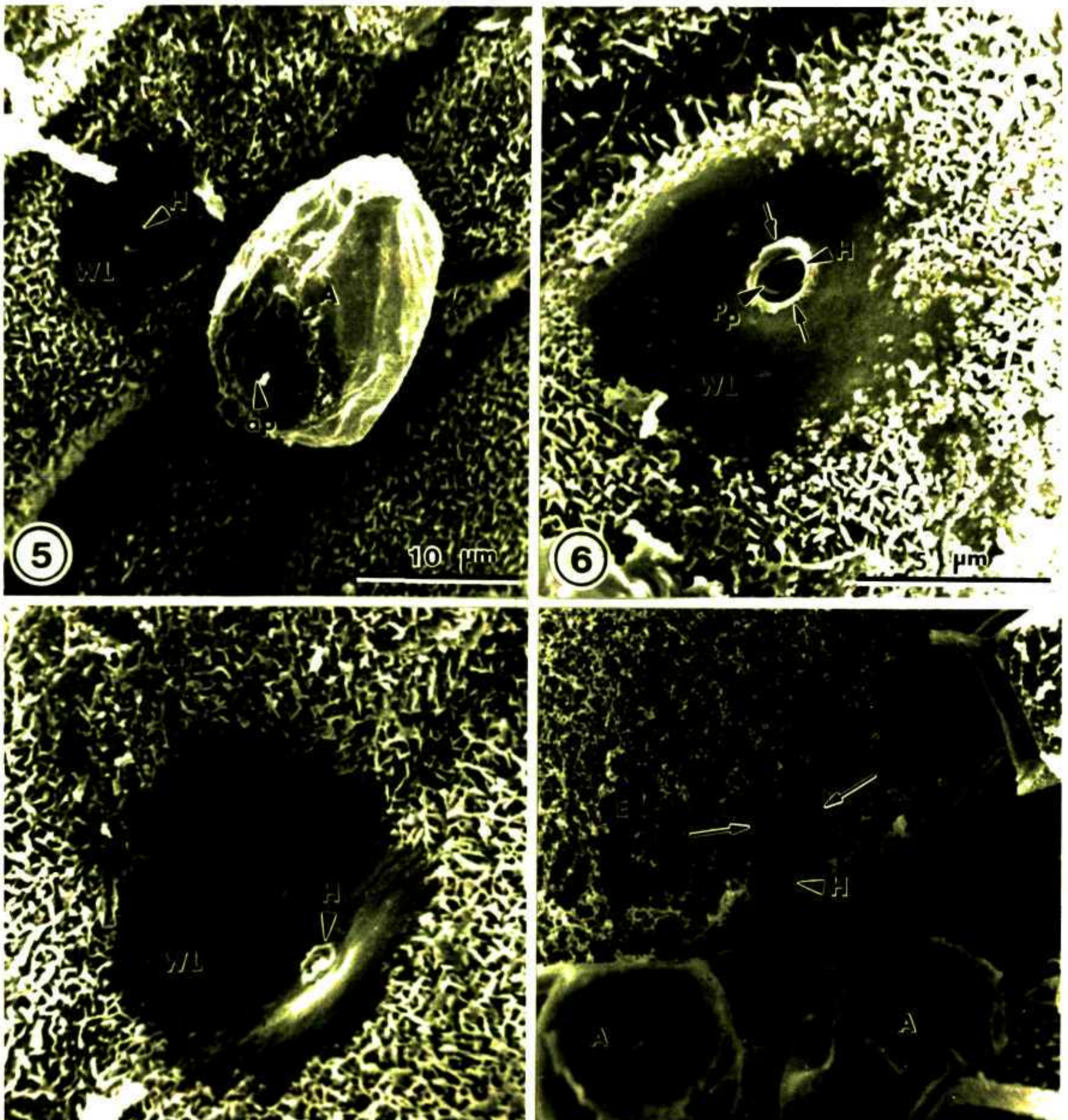
Occasionally, appressoria were found which had lifted away from the surface of the leaf, but had not been removed. In Fig. 9, the area of leaf stripped of its wax is clearly

Figure 5: An appressorium (A) that has become dislodged from its germ tube, and which has pulled away from the epidermis during preparation of the specimen. The point of peg emergence (appressorial pore) (ap) is visible on the underside of the appressorium, and on the leaf surface, the wax-less area (WL) and penetration hole (H) indicate where penetration took place (24 hpi).

Figure 6: A wax-less area (WL) on the leaf surface, with a penetration hole (H). The hollow penetration peg (Pp) is clearly visible within the hole. Note the slightly raised rim (arrows) of material around the penetration hole (24 hpi).

Figure 7: Wax-less area (WL) over the junction between two epidermal cells. The penetration hole (H) is visible to the side of the junction. Note the very clear indentation in the immediate vicinity of the penetration hole (24 hpi).

Figure 8: The side of the penetration hole (H) where the penetration peg enters the epidermal cell (E) lumen (24 hpi). Note how the cytoplasm has retained an imprint of the penetration hypha, that was removed during tearing (arrows). The appressoria (A) and Urediospores (Ur) in the foreground, were presumably detached from the host surface when the leaf tissue was torn.



H
WL
A
5
10 µm
H
Pp
WL
6
5 µm
H
WL
7
5 µm
E
H
A
A
8
10 µm

visible, as is the penetration hole. The penetration peg can be seen protruding from the underside of the appressorium, aimed directly at the hole beneath from which it has been pulled.

When appressoria were stripped from the surface of the leaf, the point of peg emergence from the base of the appressorium, was visible (see Figs 5 and 10). This pore in the basal wall was around 1.5 µm in diameter. Surrounding the pore, was a smooth ring of appressorial wall (1.5 µm in width). The ring appeared to be a region of wall that had been particularly tightly appressed to the leaf, and represented only a portion of the base that had been in direct contact with the leaf, as evidenced by the size of the wax-less areas on the leaf surface.

In order to determine when actual penetration of the epidermal cell wall occurred, both the host leaf surfaces and the undersides of appressoria were examined at 4, 5 and 6 hpi. At 4 hpi, the majority of appressorial bases and the wax-less areas on the leaf were smooth, with no sign of peg emergence or penetration hole development (Figs 11 (i) and (ii)). In a few cases, the faint outline of an appressorial pore could be seen in the base of the appressorium (Fig. 11 (ii)). After 5 hpi, there were usually no signs of penetration hole development on the leaf surface (Fig. 12 (i)), but there were areas on the appressorial bases indicative of a developing pore, prior to peg emergence. In Fig. 12 (ii), the central appressorial pore and the peripheral ring of smooth basal wall are barely discernible. By 6 hpi, penetration sites had become visible within the wax-less areas (Fig. 13 (i)). The bases of the appressoria had clearly defined penetration pores and smooth, peripheral rings (Fig. 13 (ii)). From these observations, it would seem that penetration of the leaf occurs approximately 6 hpi.

Once the fungus penetrated through the outer epidermal cell wall, it expanded to form a penetration hypha, which traversed the epidermal cell, and exited into the intercellular spaces of the mesophyll/palisade tissue. These penetration hyphae sometimes adopted a 'corkscrew' growth habit within the epidermal cell, but more commonly, they grew directly across the host cell. The distance traveled by the penetration hypha in Fig. 14

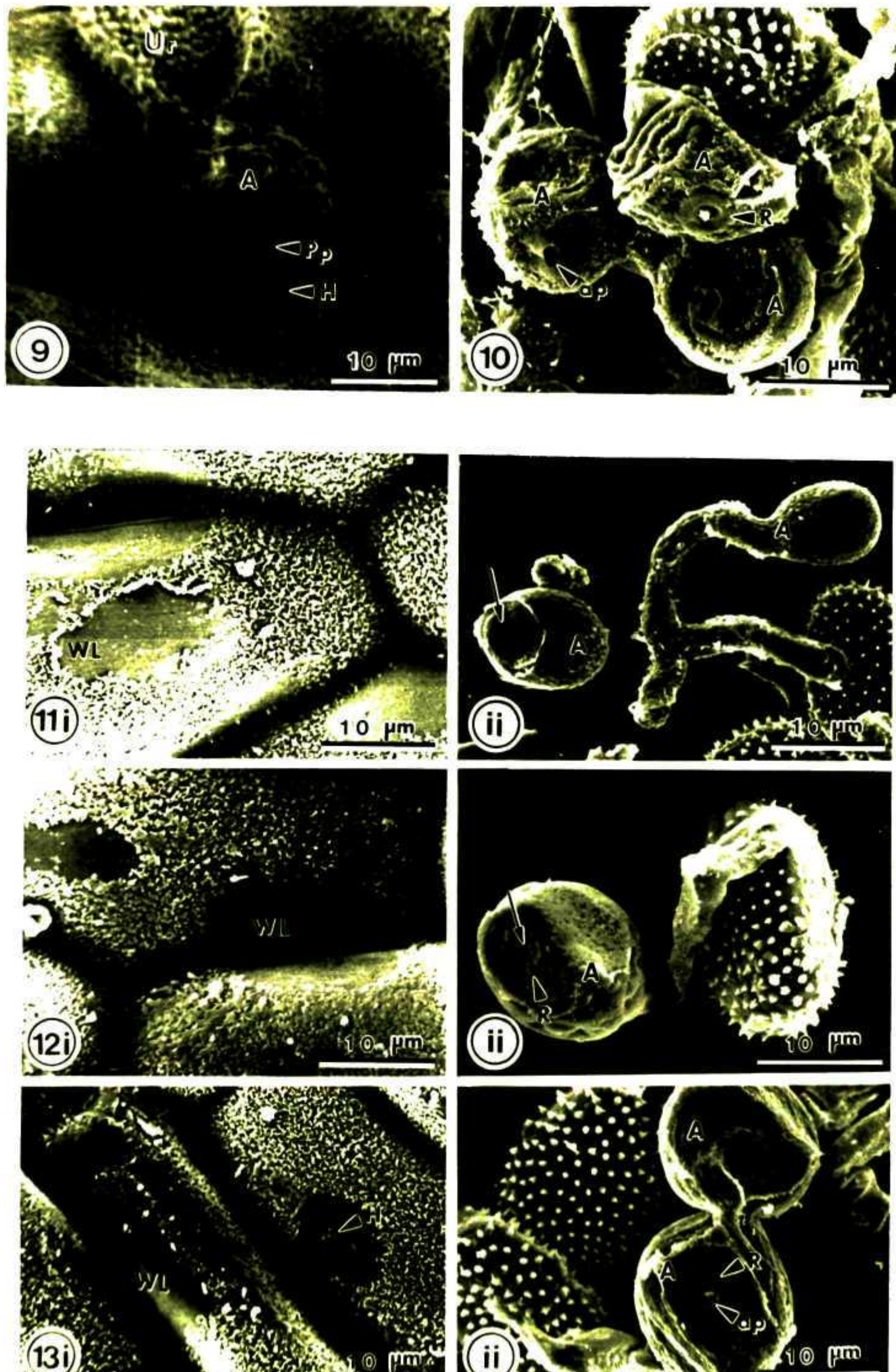
Figure 9: An unusual view of the infection process. The appressorium (A), which is apparently sessile to the urediospore (Ur), has lifted away from the host surface during the processing of the specimen, to reveal the wax-less area beneath, the penetration hole (H), and the penetration peg (Pp) still attached to the base of the appressorium (cryo-processed, 24 hpi).

Figure 10: The basal surfaces of three appressoria (A) that were stripped from the leaf epidermis (8 hpi). The base of the upper appressorium has been distorted as a result of the penetration peg being pulled away during stripping. There is a ring (R) of smooth appressorium wall surrounding the appressorial pore (ap) in each case.

Figure 11: 4 hpi: **(i)** wax-less areas (WL) on the leaf, devoid of penetration holes; **(ii)** two appressoria (A) with smooth bases; there is faint evidence of appressorial pore development (arrow) in the upper appressorium.

Figure 12: 5 hpi: **(i)** wax-less areas (WL) with no evidence of penetration hole formation; **(ii)** the underside of an appressorium (A) with clear evidence of infection structure development - the developing appressorial pore is visible (arrow), and is surrounded by what appears to be the developing ring of appressed basal material (R).

Figure 13: 6 hpi: **(i)** wax-less areas (WL) on the host leaf, one of which has a developing penetration hole (H); **(ii)** the lower surfaces of two appressoria (A), one of which has an appressorial pore (ap), surrounded by a ring of smooth appressorial wall (R).



9
Ur
A
Pp
H
10 µm
10
A
A
R
dp
A
10 µm
11i
WL
10 µm
ii
A
A
10 µm
12i
WL
10 µm
ii
R
A
10 µm
13i
WL
10 µm
ii
A
A
R
dp
10 µm

(ii) was greater than in Fig. 14 (i), perhaps partly accounting for its spiralling growth habit. The average width of the penetration hyphae observed was 4.4 µm, which is approximately 1 µm wider than the average diameter observed for germ tubes (3.3 µm).

Once the penetration hypha exited the epidermal cell, it grew a short distance intercellularly, and then formed what appeared to be a septum, delimiting a primary hypha. The primary hypha grew further and then bifurcated, giving rise to two secondary hyphae (see Fig. 15).

In Fig. 16, the infection structures spanning from the penetration hole to the secondary hyphae can been seen. Within a wax-less area on the surface of the epidermal cell, the penetration hole is visible. Within the epidermal cell the partially obscured penetration hypha can be seen within the cell lumen. Where the penetration hypha exits the epidermal cell, the primary and branching secondary hyphae are visible.

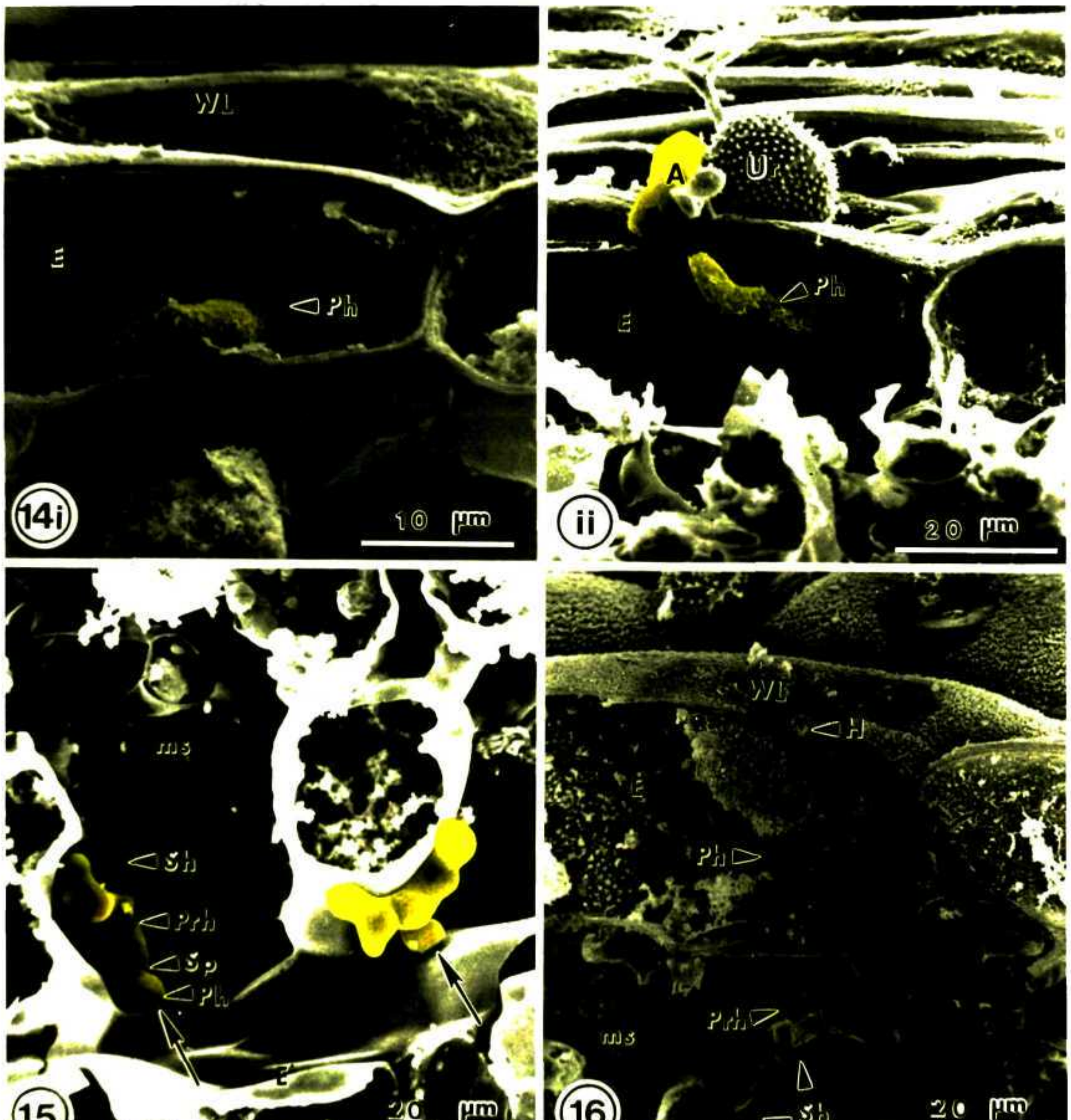
3.4 DISCUSSION

Spores of *P. apoda* tend to produce appressoria close to the parent spore, without much exploration of the host surface (Figs 1 and 2). This corresponds to the findings of Hall (1991) who observed that, generally, germ tubes of *P. apoda* were shorter than two spore widths, and very often appressoria appeared to be sessile to the parent spore. Bonde *et al.* (1976) and Koch *et al.* (1983) found that germ tubes of urediospore germlings of *P. pachyrhizi* also tended to be short and many appressoria were sessile to the urediospores. Hunt (1968) suggested that the length of urediospore germling germ tubes may be related to the mode of penetration employed, i.e. in direct penetrators, the germ tubes may be, on average, much shorter (or even absent) than those of indirect penetrating rust fungi. Presumably, because direct penetrators are less particular about penetration site selection than indirect penetrators, they are able to reduce the amount of energy spent on exploratory germ tube growth.

Figure 14: 24 hpi: **(i)** A penetration hypha (Ph) within the epidermal cell (E); note the wax-less area (WL) above, where the appressorium has been removed. **(ii)** A spore (Ur) and appressorium (A) on the leaf surface, and a penetration hypha (Ph) within the underlying epidermal cell (E). The penetration hypha has broken off at the penetration peg.

Figure 15: A cross section of a leaf infected with *P. apoda* urediospores. The epidermis (E) is visible at the very bottom of the micrograph, and there are two points (arrows) of fungal entry into the intercellular space of the mesophyll (ms). The penetration hypha (Ph) is delimited from the primary hypha (Prh) by a septum (Sp). The primary hypha has branched to form secondary hyphae (Sh) (24 hpi).

Figure 16: This micrograph illustrates the wax-less area (WL) on the host surface, a penetration hole (H), a penetration hypha (Ph) within the underlying epidermal cell (E) and a primary hypha (Prh) and secondary (Sh) hyphae within the intercellular spaces of the mesophyll (ms).



WL
E
Ph
14i
10 µm
A
U
Ph
E
ii
20 µm
ms
Sh
Prh
Sp
Ph
E'
15
20 µm
WL
H
E
Ph
Prh
ms
Sh
16
20 µm

Even though germ tubes of *P. apoda* appear capable of forming appressoria anywhere on the leaf surface, there appears to be a tendency for differentiation to occur at the junctions between adjacent epidermal cells. This phenomenon was observed by Hall (1991), and can be seen in Fig. 1. As noted by Hall (1991), even though appressoria often form directly over epidermal cell junctions, the penetration peg usually emerges off-centre, avoiding penetration directly between epidermal cells or through the anticlinal walls of the epidermal cells.

The role of epidermal cell junctions as thigmotropic stimuli for the orientation of the germtubes of indirect-penetrating urediospore germlings, has been well documented, but it would seem that the germlings of many direct-penetrating fungi also respond to the indentations between epidermal cells, often forming appressoria preferentially at or near these cell junctions (e.g. Hunt, 1968; Bonde *et al.*, 1976; Wynn and Staples, 1981; Bonde *et al.*, 1982; Hau and Rush, 1982; Gold and Mendgen, 1984; Staples and Macko, 1984). This phenomenon has been observed in the direct-penetrating basidiospore germlings of several rust fungi (e.g. Gold and Mendgen, 1984). Bonde *et al.* (1976) and Koch *et al.* (1983) found that urediospore germlings of *P. pachyrhizi* also formed appressoria predominantly over anticlinal walls, although appressoria sometimes developed in the centre of epidermal cells or, more rarely, over stomata, in which case a guard cell was penetrated.

A number of theories have been proposed as to why epidermal cell junctions are often preferred sites for penetration. These relate to a potentially greater availability of nutrients and moisture in these areas, and possible differences in the leaf chemistry (Cutler, Alvin and Price, 1982; Hoch and Staples, 1991). According to Schönherr and Bukovac (1970), the cuticle is often thinner towards the anticlinal walls - a distinct advantage for a direct penetrator.

Adhesion of the infection structures to the leaf surface, is an essential stage in the infection process by fungal germlings (Kunoh, Nicholson and Kobayashi, 1991; Nicholson and Epstein, 1991). The release of enzymes or adhesives from spores or germlings may be crucial for the differentiation and growth of the infection structures, and may be associated with host recognition (Kunoh *et al.*, 1991). It is possible that if the wax crystals and other cuticular components at the site of spore germination and prospective penetration cannot be dissolved, then the close contact required for penetration and differentiation may not be achieved (Kunoh *et al.*, 1991). In the case of *P. apoda*, there is definitely either dissolution or some type of alteration of the wax layer, as evidenced by the wax-less areas left on the leaf surface when appressoria and germ tubes were removed (Figs 4, 5, 6, 7, 9, 11, 12 and 13). A wax-less area, or imprint in the cuticle, has been observed for many different fungi when their appressoria have been removed from the host leaf surface, presumably as a result of the adhesion and subsequent stripping of the underlying wax layer beneath the infection structures (e.g. Lewis and Day, 1972; Staub, Dahmen and Schwinn, 1974; Garcia-Arenal and Sagasta, 1980; Hau and Rush, 1982).

The role of the ring of smooth appressorial wall, observed around the penetration pegs of *P. apoda* germlings, is unclear. It seems likely that this feature represents an area of particularly tight adhesion to, or close association with, the host surface. Rings of this nature have not, to the present author's knowledge, been reported in any other direct penetrator.

As discussed by Mendgen and Deising (1993), the question as to whether penetration is facilitated by enzymes or mechanical forces, or both, has been a source of speculation for many years, especially in the obligately biotrophic fungi. Most of the evidence presented to support either enzymatic or mechanical mechanisms of cuticular penetration, has been based on the appearance of the penetration holes in electron micrographs. Rounded and smooth penetration holes have been assumed to result from chemical dissolution of the cuticle, as opposed to a mechanical mode of penetration where irregularities and signs of tearing would be expected around the penetration hole (Köller, 1991). The disadvantage of making these assumptions, is that preparation of the specimen could well lead to alterations in the appearance of the hole.

Penetration holes made by germlings of *P. apoda* are fairly regular, with no signs of tearing. This would suggest that there is an enzymatic mechanism involved. The raised ring or fringe of material around both sides of the hole (e.g. Figs 6 and 7), may point to enzymatic alteration of the host cell wall. The indentation created in the infection court, around the penetration hole (see Fig. 7), may be an indication that there is also a mechanical component to the penetration process.

The presence of wax-less areas on the leaf surface, where appressoria had been removed, proved to be very useful in determining the exact time of epidermal cell wall penetration by the germlings of *P. apoda*. The earliest time, following inoculation, that penetration was observed, was 6 hours, although there were signs of appressorial pore development in the bases of appressoria, as well as evidence of cuticle disruption, at 5 hpi. Bonde *et al.* (1976) reported that the earliest penetration from *P. pachyrhizi* germlings occurred 7hpi. Seven hpi probably represents the time period when most appressoria of *P. apoda* effect penetration of the host wall.

Once inside the epidermal cell, the fungus expands to form a penetration hypha. The hypha-shaped infection structure of *P. pachyrhizi* that develops within the host epidermal cell was first observed by Keogh (Bonde *et al.*, 1976), who called it a 'transepidermal vesicle'. He concluded that this structure had 'no close counterpart in the structures elaborated in penetration by commonly studied rust species'. Koch *et al.* (1983) felt that the term 'transepidermal vesicle' was misleading, and suggested that instead, it be called the 'penetration hypha'. The present author concurs with this view and this is the term that has been given to the hyphae of *P. apoda* that have been observed to traverse the epidermal cells.

The penetration hypha grows across the epidermal cell, penetrates the opposite cell wall, and exits into the intercellular spaces of the mesophyll on the other side. The morphology and lengths of the penetration hyphae observed, varied. Sometimes, the hypha adopted a corkscrew growth habit, e.g. in Fig 14 (ii). In other cases however, the hyphae were straight, without any apparent twisting or spiral growth (e.g. Figs 14 (i) and 16). The penetration hypha in Fig 14 (ii) grew diagonally across the epidermal cell, as opposed to the apparently directional growth of the penetration hypha in Fig 16, which extended straight across the epidermal cell. A corkscrew growth habit has been observed in penetration and primary hyphae of *P. apoda* on artificial membranes (Adendorff and Rijkenberg, 1996).

In Fig 14 (i), it is interesting to note that the penetration hypha has grown a short distance across the inner epidermal cell wall, and has not exited the cell immediately. Perhaps this is related to the presence of a mesophyll cell directly beneath the epidermal cell, at the site where the fungus would be expected to exit into the mesophyll. Koch *et al.* (1983) found that penetration hyphae of *P. pachyrhizi* occasionally entered a palisade cell immediately after traversing the epidermal cell, before eventually emerging into the intercellular spaces of the mesophyll. Perhaps *P. apoda* has a means of selecting its point of exit from the epidermal cell, so as to enter the intercellular cavity of the leaf, rather than to cross yet another cell prior to haustorium formation. Penetration hyphae of this fungus were not observed to enter directly into a palisade cell.

Once inside the intercellular space of the mesophyll, the penetration hypha would appear to grow a short a distance before forming a septum, delimiting the primary hypha. This septum was not always visible, and in Fig. 15, the slight constriction of the penetration hypha was interpreted as being the site of septum formation. On artificial membranes, a septum was formed in the penetration hypha, delimiting a primary hypha, and the same delimitation of the primary hypha was reported for *P. pachyrhizi* both on artificial surfaces (Koch and Hoppe, 1988) and in the host leaf (Koch *et al.*, 1983).

No haustorial mother cells or haustoria were conclusively identified 24 hpi.

As mentioned previously, the penetration processes and structures described for *P. pachyrhizi*, the causal organism of soybean rust, appear to be very similar to those of *P. apoda*, as elucidated during the course of this study. Even though these two pathogens belong to the same genus, it is surprising that their infection processes, on two such different hosts, one on a monocotyledonous and the other on a dicotyledonous host, would be so similar at the morphological level.

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CHAPTER 4 TRANSMISSION ELECTRON MICROSCOPY OF INFECTION STRUCTURE DEVELOPMENT BY UREDIOSPORES OF PHAKOPSORA APODA

ABSTRACT

Direct penetration from urediospore-derived infection structures of *Phakopsora apoda* (Har. & Pat.) Mains. on *Pennisetum clandestinum* Hochst. ex Chiov., was investigated using transmission electron microscopy. It was determined that a urediospore germinates to form a germ tube, which terminates in a well-defined appressorium. An appressorial pore develops in the basal wall of the mature appressorium, over the infection site. A cone-like structure develops around the pore. The wall of the appressorium, in the vicinity of the cone, is thinned and electron-dense, and a collar of material, similar in appearance, encircles the base of the cone. The membrane-bound cone is elaborately branched, and numerous electron-dense glycogen-like particles are associated with the cone elaborations. A penetration peg, its walls continuous with an inner wall layer of the cone, penetrates the epidermal cell wall and expands into an intracellular penetration hypha inside the host cell. The penetration hole formed by the peg, has smooth edges, and there is little deformation of the epidermal cell wall fibrils, indicating a predominantly enzymatic mode of penetration. The penetration hypha traverses the epidermal cell, penetrates the opposite cell wall, and emerges into the intercellular spaces of the mesophyll, narrowing to form a penetration neck at the exit site. Both the penetration peg and penetration neck, contain multivesicular bodies associated with parallel arrays of microtubules. The intercellular penetration hypha contains an elaborate endomembrane system, and is virtually devoid of glycogen-like particles. Lomasomes occur in the intercellular penetration hypha, near the penetration neck.

4.1 INTRODUCTION

Rust on kikuyu grass (*Pennisetum clandestinum* Hochst. ex Chiov.), was first found in South Africa in 1986 (Hall and Rijkenberg, 1989). The rust fungus responsible, was subsequently identified as *Phakopsora apoda* (Har. & Pat.) Mains (Adendorff and Rijkenberg, 1995a), and although not of great economic importance at present, this fungus has proven to be very interesting mycologically. *P. apoda* is one of the very few rust fungi known to penetrate the host leaf directly, through the cuticle, from its urediospore-derived infection structures (Hall, 1991).

Typically, direct penetration has been associated with the basidiospore stage of the rust fungus life cycle. Penetration from urediospores and aeciospores usually occurs via stomata (Littlefield and Heath, 1979; Wynn and Staples, 1981; Hoch and Staples, 1987; Hoch and Staples, 1991). The only species of rust fungus known to effect direct penetration from aeciospore germlings, is *Arthuriomyces peckianus* (Howe in Peck) Cumm. Hirat. (Swann and Mims, 1991) and apparently, only four rust fungi, other than *P. apoda*, have been reported in the literature to penetrate their respective hosts directly from urediospores, viz. ***Puccinia psidii*** Wint. on *Syzygium jambos* (L.) Alston (Hunt, 1968), ***Ravenelia humphreyana*** P. Henn. on *Caesalpinia pulcherrima* (L.) Sw. (Hunt, 1968), ***Physopella zeae*** (Mains) Cumm. & Ramachar on *Zea mays* L. (Bonde, Bromfield and Melching, 1982) and ***Phakopsora pachyrhizi*** Sydow on *Glycine max* (L.) Merrill (soybean) (Bonde, Melching and Bromfield, 1976).

P. psidii has a very unique mode of infection, remaining intercellular at all stages, except for the formation of haustoria (Hunt, 1968). The penetration peg develops from the base of the appressorium, and penetrates between the epidermal cells, and into the intercellular spaces of the mesophyll. Within the epidermal layer, a septum delimits the penetration hypha from an infection hypha, which then branches and initiates haustorium formation.

R. humphreyana only produces intracellular fungal structures (Hunt, 1968). Following penetration into an epidermal cell, a vesicular haustorium becomes established in the cell. Lobes develop from this haustorium, which exit directly into adjacent epidermal and mesophyll cells, thereby establishing the fungal mycelium.

P. zeae also appears to be an intracellular fungus (Bonde *et al.*, 1982). A penetration peg develops from the base of the appressorium and penetrates between adjacent epidermal cells, entering one of the cells via an anticlinal wall. An elliptical primary hypha develops in the lumen of the epidermal cell, and then secondary, intracellular hyphae develop, which exit the epidermal cell, and colonise adjacent epidermal and mesophyll cells.

The above observations were all made with the aid of the light microscope. The only direct penetrator, from urediospore-derived infection structures, that has been studied in detail with both light, scanning, and transmission electron microscopy, is *P. pachyrhizi* on soybean (Bonde *et al.*, 1976; Bonde and Brown, 1980; Keogh, Deverall and McLeod, 1980; Koch, Ebrahim-Nesbat and Hoppe, 1983; Ebrahim-Nesbat, Hoppe and Rohringer, 1985; Koch and Hoppe, 1987; Koch and Hoppe, 1988).

Previous studies (Adendorff and Rijkenberg, 1995b and 1996) have indicated that the penetration processes employed by *P. apoda* are very similar to those of *P. pachyrhizi*. Morphologically similar infection structures have been observed with both the scanning and light microscopes, on host and artificial surfaces. In both fungi, once a suitable infection site has been selected on the host leaf (usually the junction between epidermal cells), the mature appressorium is cut off from the germ tube by a septum. In the base of the appressorium, an appressorial pore develops, which would appear to be a wall-less area over the proposed site of penetration. Associated with this pore, is a cone-like structure, which was only observed with the light microscope in *P. apoda* (Adendorff and Rijkenberg, 1996), but which has been examined with the use of transmission electron microscopy in *P. pachyrhizi* (Koch *et al.*, 1983).

In *P. pachyrhizi*, the cone is an elaborately branched, membrane bound structure, independent of the appressorium wall, but continuous with the wall of the penetration peg. The penetration peg penetrates the cuticle and the outer epidermal cell wall. Once inside the epidermal cell, it expands to form a penetration hypha, which traverses the epidermal cell, and emerges into the intercellular spaces of the mesophyll. After extending a short distance into the mesophyll, a septum delimits a primary hypha, which grows further and branches to form secondary hyphae (Koch *et al.*, 1983). Germlings of *P. apoda* have been observed to form a penetration peg, penetration hypha and primary and secondary hyphae, in a manner similar to *P. pachyrhizi* (Adendorff and Rijkenberg, 1995b). However, these observations were made with the light and scanning electron microscopes, and the ultrastructural details of the penetration process have not yet been elucidated.

The purpose of the present study was to investigate, by means of transmission electron microscopy, the penetration of the host leaf by *P. apoda* urediospore germlings. Emphasis has been placed on the actual penetration of the epidermal cell from the appressorium, and the subsequent exit of the penetration hypha from the epidermal cell into the intercellular spaces of the mesophyll.

Since the structures formed by *P. apoda* in the host, during the early stages of infection, appear to be morphologically and functionally similar to those of *P. pachyrhizi*, similar terminology to that proposed by Koch *et al.* (1983) has been used by the present author. However, Koch *et al.* (1983) used the term 'penetration hypha', for the hypha-like structure that reaches from the appressorium to the mesophyll, and made no distinction between the penetration hypha and the constricted regions where the hypha enters and exits the epidermal cell.

In the case of *P. apoda* it was decided that, for the sake of clarity, the various regions of the penetration hypha would be referred to as follows: the '**penetration peg**' (the narrow region of hypha traversing the epidermal cell wall); the '**intraepidermal penetration hypha**' (the region of the penetration hypha within the epidermal cell), the '**penetration neck**' (the narrow region of the penetration hypha where it exits the epidermal cell), and the '**intercellular penetration hypha**' (the region of the penetration hypha in the intercellular spaces of the mesophyll). Strictly speaking, the term 'penetration peg' should perhaps only be used in reference to the transitional structure arising from the appressorium that actually penetrates the host cell wall. Once the wall has been penetrated, the region of hypha within the host wall, would more correctly be called a 'neck'. In order to reduce confusion through the use of lengthy names differentiating between the structures produced at each site of wall penetration, the term 'penetration peg' has been assigned to describe the 'neck' resulting from the penetration of the outer epidermal cell wall of the host, and the constriction resulting from penetration of the inner epidermal cell wall by the penetration hypha, has been termed the 'penetration neck'.

4.2 MATERIALS AND METHODS

Fresh leaf material bearing uredia was collected as needed from Hilton, on the outskirts of Pietermaritzburg in KwaZulu-Natal. Spores were scraped from the uredia with a scalpel, and were applied evenly to either the abaxial or adaxial surfaces of uninfected kikuyu grass segments (approximately 2 cm long), by gently brushing the flat edge of the spore-laden scalpel over the leaf surface. When severely infected leaf material was used as a source of inoculum, the infected leaves were carefully pressed and rubbed against the uninfected leaf pieces, in order to facilitate spore transferal (this latter means of inoculation helped to minimise damage to the host leaf surface). Conventional methods of dusting spores onto the leaf were not used, due to the very low spore numbers produced by the uredia, and the tendency of the spores to 'clump' together.

The inoculated leaf pieces were lightly sprayed with distilled water (using an atomising hand spray), and were floated spore-side up, on distilled water, in a petri dish. The inner surface of the lid was sprayed with distilled water, and the closed petri dish was placed in a black plastic bag. The bag was sprayed with distilled water, sealed, and placed in an incubator at 19°C.

Leaf material was removed from the incubator at 6, 8, 10 and 12 hours post-inoculation (hpi), and was cut with a sharp razor blade into small pieces (approximately 1 x 1.5 mm), whilst submerged in a solution of 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, at room temperature. The specimens were fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer overnight, were washed twice (30 minutes each) in the same buffer, and were post-fixed for 3 hours in 2% osmium tetroxide in 0.05M sodium cacodylate buffer. This was followed by two 30-minute washes in 0.05M sodium cacodylate buffer. The specimens were then stained with uranyl acetate (in the dark) for 30 minutes, and washed with distilled water for 10 minutes. The material was then taken through a graded ethanol series (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % ethanol), remaining in each solution for 10 minutes. The 100% ethanol rinse was repeated twice. Following dehydration, the specimens were embedded in Epon-Araldite. Two 30-minute washes in propylene oxide were followed by 2 hours in 25% Epon : 75% propylene oxide plus DMP (2,4,6 tri [dimethylaminomethyl] phenol); 2 hours in 50% Epon : 50% propylene oxide plus DMP; overnight in 75% Epon : 25% propylene oxide plus DMP and 100% Epon plus DMP for 24 hours. After a final change in 100% Epon plus DMP, the specimens were placed into aluminium foil embedding dishes with polypropylene-covered bases, for polymerisation. The polypropylene film ensured that the lower block faces were smooth and clear, thereby allowing the specimens to be viewed from both sides of the block with clarity.

The specimens were polymerised in an oven at 70°C for 48 hrs. The aluminium dishes were peeled away from the polymerized blocks, and the specimens were viewed under a dissecting microscope. Grass pieces bearing visible appressoria were selected for further processing. Diagrams were made, indicating the positions of infection structures and their orientation, to assist in the mounting and sectioning of individual specimens. The selected leaf pieces were sawn from the blocks, and trimmed with a razor blade. This final trimming process allowed the specimen to be mounted in such a way as to minimise the time taken to reach the specimen when sectioning, and also to ensure correct orientation of the leaf, so as to obtain exact transverse sections of the material for easier interpretation of the tissue structure.

The trimmed block was glued onto a perspex stub. Sectioning was performed on an LKB Ultratome III. Initially, monitor sections were cut with a glass knife, to locate the relevant infection structures. The monitors were approximately 2 µm thick, and were expanded in water, over a flame, prior to staining with Ladd's stain. The sections were viewing under the light microscope. Once infection structures had been reached, a diamond knife was used for ultrathin sectioning. Sections were expanded with chloroform, and were collected on flamed copper grids. They were stained for 10 minutes with Reynolds lead citrate, and were viewed and photographed either with a JEOL 100CX or a Philips CM120 Biotwin transmission electron microscope.

4.3 RESULTS

At 4hpi, appressoria (approximately 13 µm in diameter) contained numerous vesicles, mitochondria and multivesicular bodies (Fig. 1). The cytoplasm appeared to be fairly electron-dense. No more than two nuclei were observed in any single section, but serial sections suggested the presence of up to four nuclei in the appressorium. The basal wall of the appressorium appeared to be slightly less electron-dense than the remainder of the wall. An adhesive matrix was observed around the appressorium, filling the gap between the curving undersurface of the appressorium and the leaf, along the periphery of the adhesion site. The adhesive matrix was thinnest at the appressorium-leaf interface, apparently tapering away completely in the vicinity of the penetration pore (Fig. 2).

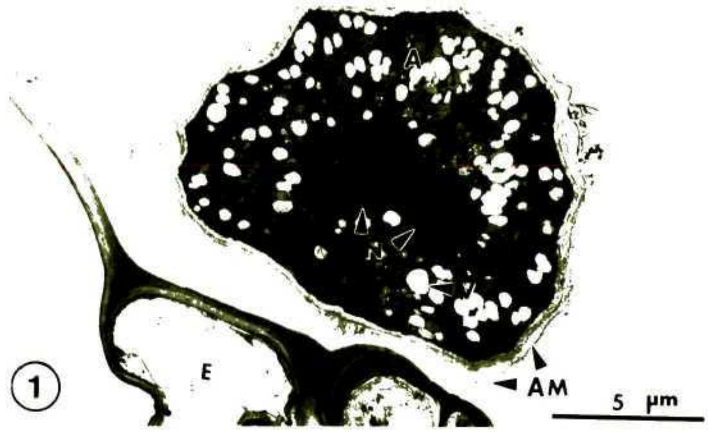
During the infection process, the appressorium (in all 11 appressoria that were examined), typically contained numerous vesicles, mitochondria, and electron-dense granules (0.03 µm in diameter) (Fig. 2). The glycogen-like granules, which did not appear to be membrane bound, were associated mainly with the cone elaborations and with vesicles in the appressorium.

By 6 hpi, a cone-like structure had developed around an appressorial pore in the bases of some appressoria. The basal appressorium wall, surrounding the appressorial pore

Figure 1: An appressorium (A), which has lifted away from the leaf epidermis (E), **4 hpi**. Note two nuclei (N) with nucleoli and numerous vesicles (V) within the cytoplasm. The adhesive matrix (AM) is visible as a layer, of variable thickness, encasing the entire appressorium.

Figure 2: An appressorium (A) which has effected penetration of the host epidermal cell (E), **12 hpi**. The adhesive matrix (AM) has lifted from the leaf surface. Numerous vesicles (V), mitochondria (M), and electron-dense glycogen-like granules are present within the appressorium. A cone-like structure (C) is clearly visible, extending from the appressorial pore (ap) in the basal wall, into the cytoplasm of the appressorium. In the vicinity of the appressorial pore, the basal wall of the appressorium is thinner and much more electron dense. A collar (clr) of wall material of the same thickness and electron density lines the appressorial pore. The cone is attached to the outer edge of the collar by means of a thin wall layer of moderate electron density (small arrows). An inner cone wall layer is contiguous with the wall of the penetration peg (Pp).

The intraepidermal penetration hypha (IEPh) appears to have three wall layers. A diffuse layer of material is visible, exterior to these layers (large arrows).
Fewer, more clearly defined vesicles (V) are present within the IEPh than in the appressorium. Glycogen-like granules are apparently evenly dispersed throughout the cytoplasm of the IEPh. Numerous elements of endoplasmic reticulum (ER) are visible, oriented parallel to the long axis of the penetration hypha. Many mitochondria (M) are also present.



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(approximately 1.5 µm in diameter), was very electron-dense and was thinner than the surrounding appressorial wall (Figs 2 and 4). The region of electron-dense wall material was approximately 4.2 µm in diameter. A collar of material apparently identical to this electron-dense wall material in texture and staining properties, surrounded the periphery of the pore, extending approximately 0.6 µm into the appressorium. The cone-like structure appeared to be attached to the appressorial wall by means of this collar. A cone wall layer of intermediate electron- density (not as dense as the collar, but more dense than the bulk of the cone wall) was attached to the collar (just beneath the outer rim of the collar), and was discernible along the outer cone wall, before merging with the less electron-dense wall layers about half-way up the cone. The cone elaborations appeared to be composed of a shell of fibrillar wall material, with more amorphous, granular material filling the central regions (Fig. 3).

The cone appeared to be lined with the plasmamembrane. In addition, the cone elaborations were often associated with elements of ER.

A thin, clearly defined, inner cone wall layer could be differentiated from the bulk of the cone wall (see Figs 2 and 4), vertically above the point where the basal wall of the appressorium became electron-dense. The wall of the penetration peg was continuous with this inner cone wall layer, and not with the basal wall of the appressorium.

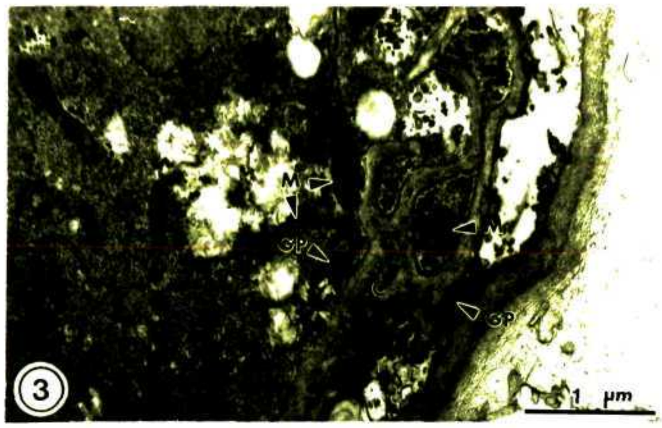
The host wall appeared to have been neatly perforated at the penetration site with very minor, or no distortion, of the wall microfibrils. In Fig. 4, the innermost host wall fibrils are curved inward slightly, in the direction of penetration. There is a thin layer of apparently amorphous, more electron-dense, host wall material lining the outer wall of the penetration peg. The penetration hole, in the epidermal cell wall, measured approximately 1.2 µm in diameter. The host epidermal cell wall was seen to be thicker at the leaf surface, thinning at the anticlinal walls (Fig. 4 and 6).

The penetration peg narrowed somewhat in diameter during the penetration of the host wall. The cytoplasm within the penetration peg, appeared to be less electron-dense

Figure 3: Detail of the cone (C): note its convoluted, branching form, and the close association of glycogen-like particles (GP) with the cone. Dense aggregates of these particles line both the inner and the outer walls of the cone (arrow). The cone appears to be lined with plasma membrane. Mitochondria (M) are abundant, in apparent association with the cone elaborations.

Figure 4: An enlargement of the penetration site in Fig. 2. The epidermal cell (E) wall (CW) does not show signs of distortion as a result of penetration, apart from a slight outward flaring of the wall fibrils towards the surface opening of the penetration hole, and a slight incurving of fibrils at the point where the penetration peg emerges into the epidermal cell lumen (small arrow). A thin layer of amorphous, slightly more electron dense material, presumably where the wall has been chemically altered, is visible on either side of the peg.

The basal appressorial wall thins where it starts to become more electron dense (large arrow). The collar (clr) is discontinuous with the basal wall, although similar in texture, thickness, and staining properties. The penetration peg (Pp) wall is continuous with an inner wall layer in the cone. Numerous multivesicular bodies (mvb) are present in the penetration peg, apparently in close association with microtubules (mt) passing through the peg area. Three wall layers of the intraepidermal penetration hypha (IEPh) are visible. The inner wall is continuous with that of the peg. Numerous strands of endoplasmic reticulum (ER) are present within the penetration hypha. Some appear to be budding vesicles from their tips (b). Glycogen-like particles (GP) are present, sometimes in apparent association with elements of ER.


M
GP
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than in the appressorium or in the penetration hypha, and contained very few glycogen-like granules. Characteristically (in all seven penetration pegs observed), there were numerous multivesicular bodies in the cytoplasm of the penetration peg. Microtubules, running parallel to the penetration peg were visible, mainly on the appressorium side of the peg (Fig. 4).

Once the fungus entered the epidermal cell, it expanded to form an intraepidermal penetration hypha (approximately 4.3 µm in diameter; 12 penetration hyphae were observed during the course of this study), which was observed to have traversed the epidermal cell at 8 hpi. At 10 hpi, the first intraepidermal penetration hyphae were observed to have emerged from the epidermal cell, into the intercellular space of the mesophyll (Fig. 6). Where the penetration hypha exited the epidermal cell, it narrowed, to form a penetration neck (approximately 0.7 µm). Once in the mesophyll, the penetration hypha was often narrower than in the epidermal cell (approximately 2.6 µm), depending on the shape of the intercellular space into which it had entered.

The wall of the penetration hypha appeared to be composed of three layers, the middle layer being thinner and more electron-dense than the outer and inner layers. The inner wall layer was continuous throughout the penetration hypha, but the outer layer was not present in the peg and neck regions. A layer of diffuse, amorphous material of varying thickness appeared to coat the intercellular penetration hypha. This material appeared to fill the gaps between the curved surfaces of the penetration hypha and the mesophyll cells (Fig. 5 (iii)), and probably represents an adhesive matrix.

The cytoplasm of the intraepidermal penetration hypha was usually more electron-dense than that of the peg and the appressorium. Glycogen-like particles were fairly evenly scattered throughout the penetration hypha, and often appeared to be associated with the numerous elements of endoplasmic reticulum that were present, running parallel to the long axis of the penetration hypha (Fig. 4). The few glycogen-like particles near the entrance of the penetration peg, appeared to be less electron-dense than those deeper in the penetration hypha.

As was the case in the penetration peg, numerous multivesicular bodies (mvbs) and microtubules were present in the penetration neck (in all 4 that were sectioned). In Fig. 5 (ii), the close association between these two structures is more clearly demonstrated. Note how the mvbs are aligned, lengthwise, in a row along the microtubules.

There was a conspicuous absence of glycogen-like particles in the intercellular penetration hypha (compare the upper and lower halves of Fig. 5 (ii)).

Numerous mitochondria were associated with prominent endomembrane elements within the intercellular penetration hypha (Fig. 5). These elements were sometimes arranged in parallel arrays, and some of the elements appeared to be budding vesicles. Elaborate, convoluted, membranous structures, which appeared to be lomasomes, were present in the penetration hypha, especially near the penetration neck (Fig. 5 (ii)). These were usually closely associated with the plasmamembrane and elements of ER.

At 12 hpi, the penetration hypha had grown at least 16 μm into the intercellular spaces of the mesophyll (Fig. 6). Nuclei were not observed in any structures other than the appressorium.

A typical host reaction to the infection process, was the development of cytoplasmic aggregates in mesophyll cells adjacent to the infected epidermal cell (Fig. 7). The aggregates developed along the host cell walls that were in contact with either the infected epidermal cell, or the intercellular penetration hypha. Sometimes papilla-like, electron-dense deposits were laid down between the host cell plasmamembrane and the cell wall in contact with the intercellular penetration hypha.

By 12 hpi, in some mesophyll cells adjacent to infection sites, the cytoplasm had become totally disorganised with associated rupturing of the organelles, and corresponding dispersal of the chloroplast lamellae (Fig. 5 (i)).

Figure 5: (i) An appressorium (A) has lifted away from the leaf surface, but the intraepidermal penetration hypha (IEPh) can been seen within the underlying epidermal cell (E), **12 hpi**. The penetration hypha has narrowed to form a neck (N) where the fungus has exited the epidermal cell. The intercellular penetration hypha (ICPh) is visible in an intercellular space within the mesophyll (ms).
The cytoplasm of an adjacent mesophyll cell has become completely disorganised (arrow), even though it has not been penetrated by the fungus.

(ii) This micrograph illustrates the penetration neck (N), where the intraepidermal penetration hypha (IEPh) has exited the epidermal cell (E), into the intercellular space of the mesophyll. The epidermal cell wall (CW) has been slightly distorted around the penetration site, with the bending of fibrils in the direction of penetration (arrows). The single wall layer of the penetration neck is continuous with the inner wall layer of the intraepidermal penetration hypha and the intercellular penetration hypha (ICPh). Numerous multivesicular bodies (mvb) are associated with microtubules (mt) passing through the neck area. An elaborate, membranous, lomasome-like structure (L) can be seen along the wall of the intercellular penetration hypha, near the neck. It appears to be closely associated with invaginations of the plasma membrane, as well as with elements of endoplasmic reticulum (ER). Glycogen-like particles (GP) are abundant in the intraepidermal penetration hypha, but are scarce in the intercellular penetration hypha.

(iii) An extracellular, adhesive matrix (AM) is present around the intercellular penetration hypha (ICPh). A proliferation of the endomembrane system is apparent in the intercellular penetration hypha. Elements of ER are abundant, forming parallel arrays in some cases (arrows), and some of these elements appear to be budding vesicles (b). Many elements of ER are in association with mitochondria (M). A number of lomasome-type bodies (L) can be seen along the wall of the intercellular penetration hypha.

4.4 DISCUSSION

Urediospore germlings of *P. apoda* appear to be very similar to those of *P. pachyrhizi*, not only at a morphological level, but also at an ultrastructural level. A few differences became apparent during this TEM investigation, but these two fungi are remarkably alike, especially considering they are different species infecting very different host plants, one being a monocotyledonous, and the other a dicotyledonous plant.

An unusual feature of the appressorium of *P. apoda*, is the thinned, very electron-dense area of the basal wall around the appressorial pore (Figs 2 and 4). This would only seem to have been observed in germlings of *P. apoda* and *P. pachyrhizi*. In a paper by Koch *et al.* (1983), the thinned, electron-dense region is clearly visible in electron micrographs of the appressoria of *P. pachyrhizi*. However, thinning of the basal wall of the appressorium, usually resulting in the development of a wall-less region over the penetration site, has been observed in many direct-penetrating fungi (Littlefield and Heath, 1979; Bourett, Hoch and Staples, 1987; Mims and Richardson, 1989; Swann and Mims, 1991), and has been implicated in the mechanisms responsible for directional peg emergence from the base of the appressorium (Wynn and Staples, 1981).

Certainly the most striking ultrastructural feature of the appressorium of *P. apoda*, is the elaborate cone structure which surrounds the appressorial pore. Appressorial cones are probably involved specifically with the direct penetration process, as they have only been found in appressoria which produce infection structures that penetrate through the cuticle and epidermis of the host leaf (Mims and Richardson, 1989). In the rust fungi, cones have been found in several direct-penetrating species: in the appressoria of basidiospore (Metzler, 1982; Mims and Richardson, 1989), aeciospore (Pady, 1935; Swann and Mims, 1991) and urediospore (Hunt, 1968; Koch *et al.*, 1983) germlings.

The two species of rust fungus with urediospore germlings known to produce a cone-like structure in the base of the appressorium, are *R. humphreyana* (Hunt, 1968) and

P. pachyrhizi (Koch *et al.*, 1983). Even though Hunt (1968) does not comment on their presence, cones in appressoria of *R. humphreyana* have been drawn clearly into several of his illustrations of germlings penetrating the host leaf. In the case of *P. pachyrhizi*, a more detailed study of the cone, with the aid of transmission electron microscopy, has been conducted. Koch *et al.* (1983) described a cone very similar to that which was observed in the present study of *P. apoda*.

The cone of *P. apoda* would appear to have a complex structure, not only in terms of its convoluted morphology, but also in terms of its wall composition.

A very distinct collar-like structure would appear to constitute the outermost part of the cone apparatus. The electron-dense wall layer in the immediate vicinity of the penetration site, made it difficult to determine whether the collar is continuous with the basal appressorial wall, or whether it is a separate, distinct structure that terminates at the pore (Fig. 4).

If the collar represents basal wall material that has been invaginated or extended, then the structure of the cone would be very similar to that described for some species of *Colletotrichum*. In several species of *Colletotrichum* (e.g. Mercer, Wood and Greenwood, 1970; Brown, 1977; Landes and Hoffmann, 1979; Mould, Boland and Robb, 1991), a collar-like structure is formed, apparently via extension and invagination of one of the basal appressorial wall layers. In these anthracnose fungi, the cone, consisting of distinct wall material, is formed upon the collar and extends into the appressorium, beyond the collar, and to the edge of the pore where it is continuous with the penetration peg.

A collar-like structure, associated with the cone, was not described for *P. pachyrhizi* (Koch *et al.*, 1983), even though an electron-dense, outer cone layer very similar to the collar seen in *P. apoda* was present. However, this structure was not as distinct and well defined as in *P. apoda*, and Koch *et al.* (1983) did not consider the possibility that it may be an extension of the electron-dense basal appressorium wall. In fact, according to Koch *et al.* (1983), there were obvious morphological differences between the cone-like structures found in *Colletotrichum* spp., and those found in *P. pachyrhizi*, precisely because of the absence of a structure equivalent to a collar in *P. pachyrhizi*. In *P. apoda*, the wall material of the collar is the same thickness and electron density as the surrounding, thinner, electron-dense, basal wall, whereas in *P. pachyrhizi*, it appears to be slightly more diffuse and of a greater thickness than the electron-dense basal wall.

In order to understand how the cone develops in *P. apoda*, particularly with regard to collar formation, further specimens need to be examined between 5 and 6 hpi.

Attached to the collar of *P. apoda*, is the bulk of the appressorial cone (Fig. 3). The convoluted nature of the membrane-bound cone, and its association with the endomembrane system, would seem to indicate that its structure is orientated towards increasing the surface area available for biological activity in the vicinity of the penetration site. The numerous mitochondria associated with the infoldings of the cone, could also be seen as indicative of biochemical activity in the region. In *P. pachyrhizi*, the cone is also elaborately branched and convoluted, with numerous, associated mitochondria (Koch *et al.*, 1983). Cone-like structures of this elaborate nature have not, to the present author's knowledge, been reported to occur in any other genus of rust fungus.

In the appressoria of aeciospore germlings of *A. peckianus*, numerous membranous elaborations were found in association with the cone, and although there was no branching of the cone wall, numerous invaginations were observed in the inner cone wall (Swann and Mims, 1991). Swann and Mims (1991) commented on the similarity of these elaborations with the irregular outgrowths of wall material found in transfer cells of higher plants. In plants, these outgrowths are involved in short-distance transfer of solutes (Gunning and Pate, 1969), and Swann and Mims (1991) suggested that the large membranous surface area in the cones of *A. peckianus* may somehow be involved in the transfer of material to or from the host surface. It is possible that, in a similar way, the branched cones in *P. apoda* and *P. pachyrhizi* are involved in the production and/or transfer of material to the penetration site and to the infection structures within the host.

The numerous electron-dense particles that are associated with the cone surface in *P. apoda*, are similar to what have been described in the literature as glycogen granules (Revel, 1964). Harder (1977) demonstrated the presence of particles of a polysaccharide in teliospores of *Puccinia coronata* Cda. f. sp. *avenae* Eriks., which he characterised as 'glycogen-like', since the particles were arranged in rosettes which is typical of the α-form of glycogen (Revel, 1964). The granules in *P. apoda* also appear to have this rosette arrangement, but to determine whether they are in fact composed of a polysaccharide, cytochemical tests such as those used by Harder (1977) will have to be performed. De Bruijn's (1973) fixation method could also be used to test for the presence of glycogen in the particles.

Littlefield and Heath (1979) reported that clearly recognizable glycogen granules had not been seen either in rust fungus infection structures formed on artificial membranes or in those developed in the host, apart from in *Uromyces phaseoli* (syn. *U. appendiculatus*) var. *typica* (Mendgen, 1973), where they were found in the substomatal vesicles formed in the host. These particles are very similar to those observed in *P. apoda*. Both are about 0.03 µm in diameter, and have a granular texture. In a subsequent study, glycogen particles were observed by Swann and Mims (1991) in the appressoria of aeciospore germlings of *A. peckianus*. Their appearance was similar to the particles observed in *P. apoda*, and also seemed, in some cases, to be associated with, or clustered around, vesicles in the appressorium. They also appeared to be more densely clustered at the base of the appressorium.

Glycogen particles have been found in the appressoria of other direct-penetrating fungi, e.g. *Spilocaea pomi* Fr. (Corlett and Chong, 1977) and *Magnaporthe grisea* (Herbert) Barr (Bourett and Howard, 1990).

The present author observed the presence of electron-dense particles in transmission electron micrographs of *P. pachyrhizi* appressoria published by Koch *et al.* (1983). These particles appeared to be far scarcer in the infection structures of *P. pachyrhizi* than in those of *P.apoda*.

Since glycogen probably represents a source of stored energy (Muller and Hohl, 1975; Harder, 1976), it is not surprising that these particles would be so abundant around the penetration sites in direct-penetrating appressoria. Direct penetration is no doubt an energy-intensive process. Bourett and Howard (1990) implicated glycogen particles in another role in the process of direct penetration from appressoria of *M. grisea*. They suggested that glycogen metabolism may raise the appressorial turgor, thereby creating a mechanical component to the penetration process.

The penetration peg wall of *P. apoda* is clearly continuous with the innermost layer of the cone (Fig. 4). This layer extends up into the cone for a short distance, before merging with the bulk of the cone material. This phenomenon, of the penetration peg wall being continuous with an inner layer of the cone, has been reported in all the fungi known to form cone-like structures in their appressoria, and in the rust fungi, this has been observed in basidiospore germlings of *Gymnosporangium juniperi-virginianae* Schw. (Mims and Richardson, 1989) and *Uromyces appendiculatus* (Pers.) Unger (Gold and Mendgen, 1984), and in urediospore germlings of *P. pachyrhizi* (Koch *et al.*, 1983).

Littlefield and Heath (1979) suggested that extension of developing structures from an inner wall layer of the parent structure, is a common method of effecting a change in fungal wall structure from one morphological form to the next, e.g. as occurs during germ tube development from the urediospore, and also in infection peg development from the appressoria of many indirect-penetrating urediospore germlings. In the case of *P. apoda* and *P. pachyrhizi*, there is potential for an even more dramatic change in wall structure, as the penetration peg does not arise from an inner wall layer of the appressorium, but from the cone. Kunoh (Bonde *et. al.*, 1976) suggested that the appressorial cone of *P. pachyrhizi* could be considered as being part of the penetration peg, and that synthesis of the cone probably represents one of the initial stages of peg formation.

In *P. pachyrhizi*, the continuity of the peg wall and an inner cone layer has been confirmed through the use of gold-labelled WGL, which labels chitin (Ebrahim-Nesbat *et al.*, 1985).

The wall of the intra-epidermal penetration hypha has two clearly defined layers, with a region of greater electron density at the interface between these two layers. The nature of this electron-dense material, and whether it constitutes a definite wall layer has yet to be determined. As demonstrated by Chong, Harder and Rohringer (1985), various chemical treatments can be performed to enhance the resolution of the wall structure in rust fungi.

The constriction of the penetration hypha at the penetration peg and the penetration neck conforms to a common pattern among fungi, with penetration of a host wall usually being associated with a narrowing in diameter of the fungal hypha (e.g. Allen, 1930; Pady, 1935; Gold and Mendgen, 1984). This has been observed in a wide range of different pathogenic fungi, and is obviously a result of the fungus economising on the production of enzymes and or other energy-consuming aspects of penetration. It is interesting that both the penetration peg (Fig. 4) and the penetration neck (Fig. 5 (ii)) of this fungus consistently contains conspicuous, parallel arrays of microtubules, and numerous multivesicular bodies, which appear to be contiguous with the microtubules.

Microtubules have been associated with cytoplasmic migration in previous studies of rust fungus germlings (eg. Littlefield and Heath, 1979; Hoch, Tucker and Staples, 1987; Mims and Richardson, 1989), and have been observed to lie parallel to the longitudinal axis of rust germling infection structures (Kwon, Hoch and Aist, 1991). Microtubules were clearly illustrated in the appressorium and penetration hypha of *S. pomi*. They were orientated parallel to the penetration peg, passing through the appressorial pore in exactly the same fashion as in *P. apoda*.

It is generally accepted that the migration of nuclei is mediated by microtubules (Littlefield and Heath, 1979; Kwon *et al.*, 1991). It is conceivable that the microtubules remain at the penetration sites in preparation for nuclear migration, which would appear to take place at a stage later than 12 hpi.

Multivesicular bodies (mvbs) were rarely observed in the appressorium and within the penetration hypha, and yet they are abundant in the peg and neck regions (Figs 4 and 5 (ii)). Could the mvbs in some way support the activity of the microtubules, or are the microtubules simply aiding in their transfer through the narrow hyphal regions? Interestingly, mvbs have been associated with astral microtubules at the poles of mitotic nuclei in aeciospore appressoria of *A. peckianus* (Swann and Mims, 1991).

The question remains as to whether the same mvbs remain associated with the microtubules, or whether they are created specifically for the transfer of materials across the peg and neck areas, with their subsequent degeneration on the other side. This may tie in with the apparent absence of glycogen-like particles in the penetration peg and neck. Perhaps glycogen has to be converted into another form to be moved across the narrow penetration points. The mvbs may contain the products of the converted glycogen. Lipid droplets have been known not to associate with microtubules during cytoplasmic migration (Littlefield and Heath, 1979). Perhaps the same applies for the glycogen-like particles, and where the fungus is of a very narrow diameter, and contains numerous microtubules for the movement of mitochondria and other organelles, this conversion may be required.

Mvbs would also appear to be present in the penetration pegs of *P. pachyrhizi*, although Koch *et al.* (1983) did not comment on their presence. They were not observed in the penetration neck, and microtubules were not visible in either the peg or neck regions. However, the micrographs concerned were not very clear, and the presence of microtubules cannot be precluded altogether. Interestingly, glycogen-like particles did appear to be present within the penetration peg of *P. pachyrhizi*.

The most conspicuous ultrastructural feature of the intraepidermal penetration hypha, is the long elements of smooth ER, which are arranged predominantly parallel to the long axis of the hypha, and which often appear to be associated with the glycogen-like particles which are scattered throughout the cytoplasm (Fig. 4). Once the penetration hypha becomes intercellular, it becomes very different ultrastructurally (Fig. 5). Conspicuously few glycogen-like particles are present, but the endomembrane system becomes very elaborate. It is possible that the glycogen-like particles, which are associated with the endomembrane system in the intraepidermal region of the penetration hypha, provide the energy required for the production of these membrane systems.

Within the intercellular penetration hypha, parallel arrays of smooth ER are present. These arrays are reminiscent of golgi-equivalents, as some of the elements appear to be involved in the production of vesicles, with budding occurring at the tips of some of the strands of ER.

The unusual, elaborate membrane structures observed in association with the plasmamembrane, particularly near the penetration neck (Fig. 5 (ii)), are similar in appearance to lomasomes.

None of the elaborate membranous structures seen in penetration hyphae of *P. apoda* were visible in micrographs of *P. pachyrhizi*, although, as already mentioned, the ultrastructural detail provided in these micrographs was not very clear. However, the apparent proliferation of the fungal endomembrane system during the infection process is apparently not unusual. According to Mendgen and Deising (1993), the endoplasmic reticulum within pathogenic fungi is often more differentiated during growth of the fungus within the host plant. In *U. appendiculatus*, relatively simple strands of ER were found in the urediospore germtubes (Hoch and Staples, 1983), but in intercellular hyphae and haustoria, the ER formed tubular vesicular complexes (Welter, Müller and Mendgen, 1988).

The septum delimiting the penetration hypha from the primary hypha, was previously observed in germlings of *P. apoda* on artificial membranes (Adendorff and Rijkenberg, 1996) and possibly within the host (24 hpi) (Adendorff and Rijkenberg, 1995b). However, it was not observed by 12 hpi, during the course of this study with transmission electron microscopy (e.g. Fig. 6). It is likely that septum formation occurs at a slightly later stage. In *P. pachyrhizi*, an apparently equivalent septum was observed 24 hpi (Koch *et al.*, 1983).

The nuclei of *P. apoda* were still in the appressorium 12 hpi, even though the penetration hypha had already exited the epidermal cell. Perhaps migration only occurs immediately prior to the formation of the septum delimiting the primary hypha from the penetration hypha. It is not uncommon in direct penetrators for the nuclei to 'lag behind' during the infection process, i.e. the nuclei do not always migrate into infection structures as soon as they are formed. In basidiospore germlings of *G. juniperi-virginianae*, the nuclei also remain in the basidiospore whilst penetration takes place (Mims and Richardson, 1989), and in *Uromyces vignae*, the two nuclei in the basidiospore migrate into the intraepidermal vesicle, and undergo mitosis, when the primary hypha is about 40 µm long (Heath, 1989).

Two obvious reactions of the host to infection by *P. apoda* occur. Firstly, cytoplasmic aggregates form along the walls of mesophyll cells adjacent to the intercellular penetration hypha (or even adjacent to infected host cells), and associated with these aggregates, are electron-dense, papilla-like structures, which are deposited between the host plasmalemma and the cell wall (Fig. 7). The formation of cytoplasmic aggregates against the wall in contact with a fungus, is a common plant response to fungal invasion (Kunoh, Aist and Hayashimoto, 1985). This is often followed by the deposition of a papilla.

The second host reaction that occurs, is the complete disorganisation of the cytoplasm of some mesophyll cells adjacent to an infection site, resulting in the dispersion of the chloroplast lamellae throughout the cell (Fig. 5 (i)). According to Shaw and Manocha

(1965), the first signs of an adverse reaction to a fungal pathogen are often apparent in the chloroplasts. Individual chloroplasts first increase in size, and then the chloroplast ruptures, and the thylakoids become dispersed in the surrounding hyaloplasm.

A question that is always raised in the study of direct penetrating fungi, is whether the penetration process is effected by mechanical or enzymatic means. Often the answer to this question is based on the appearance of the penetration hole - smooth edges with no distortion imply enzymatic activity, but inward distortion of the host wall fibrils, and a more ragged edge, imply that a mechanical process is involved. If these assumptions are in fact valid, then germlings of *P. apoda* would appear to penetrate the host wall predominantly by enzymatic means. The host wall exhibits very little deformation around the penetration peg (Fig. 4). A thin layer of electron-dense material was observed lining the penetration hole produced by *P. apoda*, and this could be seen as evidence of chemical alteration of the host wall through enzymatic activity.

Some researchers have advocated the theory that penetration of the cuticle is effected mechanically, but that enzymes are used to penetrate the wall itself (Landes and Hoffman, 1979). Others, have implicated enzymatic activity in both these processes (see Kolattukudy, 1985). The cuticle of *P. clandestinum* was either very thin, or was removed during preparation of the specimens, and consequently, no conclusions could be drawn as to how the penetration process altered the cuticle.

Germlings of *P. apoda* would be ideal candidates for freeze-substitution, particularly if urediospores were germinated on artificial membranes, rather than on the host leaf, thereby avoiding the difficulties experienced in processing material thicker than 10 μm (Howard and O'Donnell, 1987). Freeze-substitution is known to greatly improve the fixation of the endomembrane system in particular (e.g. Swann and Mims, 1991), and since this system is so spectacular in the infection structures of *P. apoda* germlings, exploitation of this technique would seem to be a logical progression to make in future studies.

Issues which would need to be addressed in future studies, would include the early development of the cone (between 4 and 6 hpi) and the structure of the basal appressorium wall, whether the electron-dense particles observed in association with the cone are in fact glycogen particles, whether penetration of the cuticle and cell wall is effected enzymatically, and how the nuclei behave during later stages of development.

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CHAPTER 5 GENERAL DISCUSSION

5.1 METHODOLOGY

5.1.1 Inoculum and inoculation techniques

As mentioned in Chapter 1, attempts to establish a source of *Phakopsora apoda* in the glasshouse, proved to be unsuccessful. Conditions were probably too hot and dry to maintain rust infections on the kikuyu grass, and insufficient control of the glasshouse environment could be achieved to make the conditions more conducive to infection by the rust fungus. As a result, fresh material was harvested from the field as required. Infected leaf material was used within 2 days of harvesting, as host leaf curling[1] and the growth of saprophytic fungi excluded the use of older material. Hall (1991) also experienced great difficulty in maintaining infections of *P. apoda* in the glasshouse, and also had to resort to harvesting fresh material from the field.

A further complication in the process of obtaining inoculum, was the very low yields of spores produced by the fungal uredia. This factor led to difficulties both in the collection of large numbers of spores for storage, and for the efficient inoculation of leaf and plastic materials for study. Collection of spores by means of a spore collector proved to be very time consuming, and resulted in small spore masses with high levels of contamination by debris from the leaf surfaces. Levels of germination of the collected spores were very low when compared with spores scraped directly from freshly collected uredia, and the viability of the spores decreased dramatically within a week of storage in a refrigerated environment.

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1 'Leaf curling' may not sound like much of a problem, but when dealing with narrow kikuyu grass leaves, old specimens proved to be very difficult to handle, especially bearing in mind that the uredia of *P. apoda* are so small (about 0.2 x 0.3 µm) and awkward to work with in the first place.

Hall (1991) also reported difficulties associated with the low yield of spores per uredium of *P. apoda*, and he found that few infections were obtained with inoculum procured with a spore collector. The only inoculation method which Hall (1991) found to be successful, was the 'shaking method', i.e. rubbing or shaking infected material over the leaves to be inoculated. The present author found that the most successful results, in terms of the ease of inoculation and spore germination rates, were produced by gently pressing freshly collected, infected material onto the leaf or plastic surfaces to be inoculated, or by scraping spores directly from sporulating material onto these surfaces with a scalpel. It did become apparent, during the course of this study, that if spores were scraped from deep within the uredium, instead of only from the surface, the rates of germination were greatly reduced, presumably because of the increased percentage of immature spores in the inoculum.

These relatively crude inoculation techniques, which prevented even estimates of spore concentrations to be made, deprived this study of a certain degree of precision and consistency. It is possible that the high concentrations of spores inoculated onto the host surface could have created abnormal germling development and responses, as well as unusual host reactions. This is certainly an area that needs to be refined in future studies. Hunt (1968) was also concerned about the validity of results obtained in studies using the very artificial method of heavily inoculating detached leaves. Upon examining naturally infected leaves however, he concluded that the results from the artificial inoculations were identical to those occurring in the natural situation. In the current study, the short incubation times (under 24 hours) probably reduced the effects of using detached leaf pieces, as there appeared to be insufficient time for significant changes such as chlorosis to occur in the leaf tissue.

An important factor contributing to the success of spore germination during incubation was water, both in terms of humidity levels and the availability of free water. This became especially apparent when spores were incubated on artificial membrane surfaces. To prevent drying out of the inoculated substrates, and hence the termination of the infection processes, both the lids of the petri dishes, as well as the black plastic bags into which the petri dishes were placed, were sprayed with distilled water. To prevent drying of membrane surfaces during incubation, as well as to create a uniformly moist environment for sporeling development, a number of researchers (e.g. Allen, Hoch, Stavely and Steadman, 1991;Kwon and Hoch, 1991) have advocated the practice of floating the membranes spore-side down in distilled water. The present author however, decided not to adopt this practice, to approximate natural conditions more closely. The main concern with total submersion of the germlings, is the potential effect this could have on oxygen levels available to the germlings during their development.

5.1.2 Microscopy

Three types of microscopy were utilised to investigate the processes involved in the penetration of kikuyu grass leaves by urediospore germlings of *Phakopsora apoda*. Each of these techniques revealed different types of information regarding the development of the fungus and its interactions with the host. This allowed for the creation of a multifaceted depiction of the infection process, with sufficient overlap to provide a degree of continuity.

Light microscopy, combined with the use of artificial membranes allowed for detailed observations to be made of the morphological development of early infection structures in the absence of host material. Two major advantages were that large numbers of germlings could be viewed at relatively low magnifications, and that specimens could be viewed immediately following incubation, without further processing. An important aspect of germling development that could be observed very easily, was nuclear behaviour. Nuclear observations made with the use of TEM, required the painstaking, time-consuming practice of making and comparing serial sections of the infection structures. Fluorescence microscopy, using UV light and DAPI stain, facilitated the immediate examination of live, intact infection structures.

Scanning electron microscopy provided a fascinating three-dimensional view of the infection structures on the host leaf. The unique perspective provided by SEM transforms the distant world of the ultra-small into something easy to relate to and understand. In terms of creating spacial awareness, this technique is unparalleled, and this was particularly evident when viewing torn leaf material, where the fungus could be seen within the host tissue. SEM was the only form of microscopy which revealed textural effects, such as the formation of waxless areas on the leaf surface where appressoria had been removed (Chapter 2, Fig 4), and the formation of smooth appressorial rings in the bases of appressoria (Fig 10). SEM also allowed for the rapid viewing of large numbers of germlings, as opposed to the meticulous sectioning of individual specimens for TEM. This aspect of SEM proved to be particularly useful in determining the exact time, following inoculation, of host leaf penetration.

Stripping the appressoria from the leaf allowed for examination of both the undersides of the appressoria, as well as the infected leaf surface, providing direct evidence of penetration hole formation at precise times post inoculation.

TEM provided the most intensely detailed and fascinating information produced during this study. The beauty of transmission electron micrographs, is that the information they provide can be interpreted at so many levels. The most basic interpretation relates to the observation of purely morphological aspects, for example where a septum is formed, where the hyphae become narrower in diameter, where the fungus penetrates and exits cells. This represents an area of overlap with SEM and light microscopy. At a finer level, TEM provides a unique view of the activities within the bounds of the host and fungal cell walls. Interpretation of these activities is far more complex and is often based on deduction, but the picture provided gives insight into the chemical activities occurring at the subcellular level.

Of course, in addition to their own, unique advantages, each type of microscopy has its technical challenges. Some of these are discussed below.

5.1.2.1 Light microscopy

The major disadvantages of light microscopy, when studying the development of fungal germlings on the leaf surface, mainly relate to poor resolution and reduced visibility due to interference from underlying tissues. The use of plastic membranes, however, dramatically improves the visibility of fungal germlings. This is especially apparent when making nuclear observations using UV fluorescent dye, as there are no host leaf nuclei obscuring the activities of the fungal nuclei. Unfortunately, when studying obligately parasitic fungi, membranes can usually only be used in the examination of the early stages of fungal infection structure development, as the germlings seldom develop beyond the primary and secondary hyphae in the absence of a host.

5.1.2.2 Scanning electron microscopy (SEM)

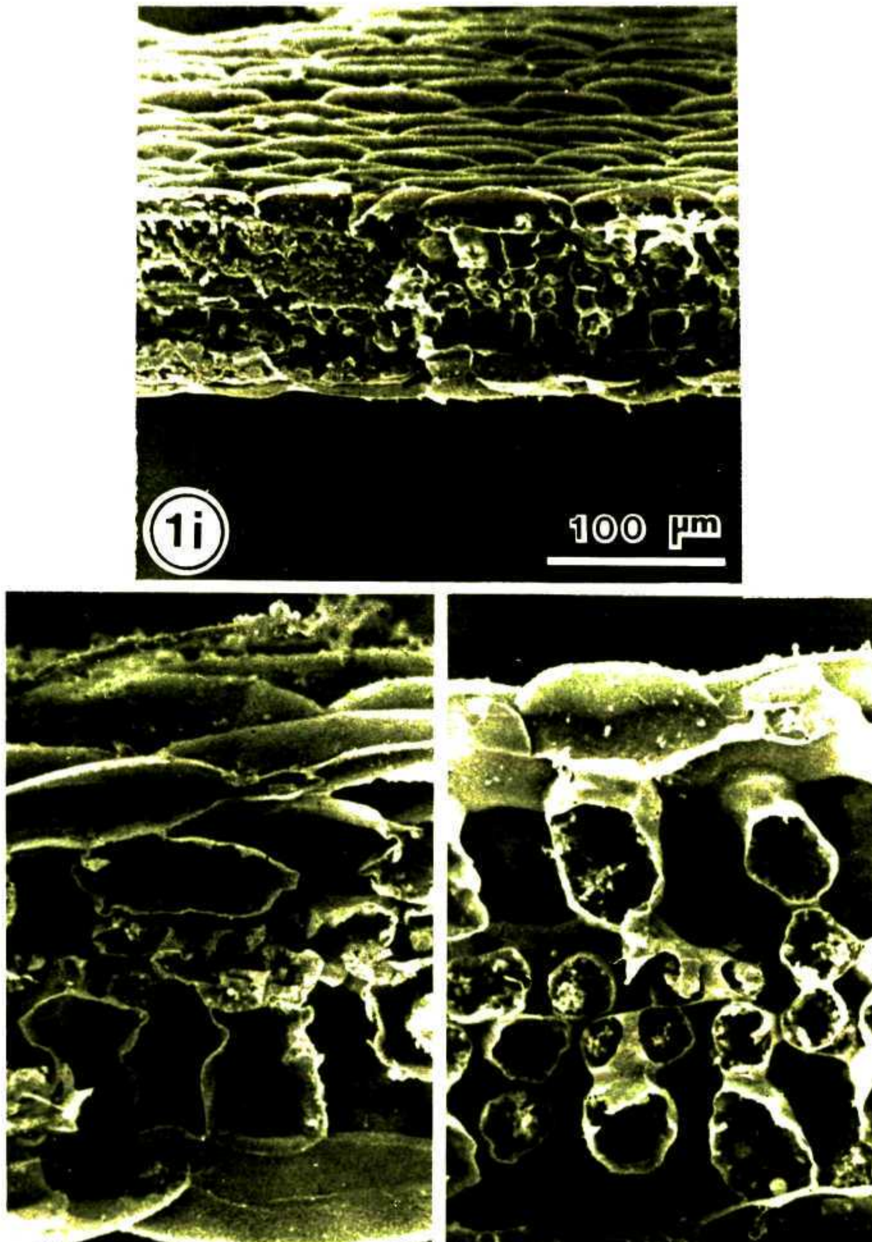
The stripping technique described by Hughes and Rijkenberg (1985), whereby appressoria are removed from the leaf surface with adhesive tape, proved to be very useful in the examination of the appressorial bases and also the leaf surface (eg. Chapter 3, Figs 10 and 11). Stripping of the entire epidermal layer, which provides valuable information about the entry of stomatal penetrators into the leaf, would not have been as useful for a direct penetrator like *P. apoda*, although it may have given some insight as to how and where the fungus emerges from the epidermal cell. Unfortunately, this technique proved to be very difficult to perform on kikuyu grass. The leaves seem to have a very brittle epidermal cell layer which is resistant to separation from the underlying tissues.

Cutting critical-point dried material with a razor blade, to expose infected leaf tissue in cross section, resulted in some crushing and deformation of the cells. Tearing of the leaf material, however, did not result in any perceptible deformation of the host or fungal structures, as a lateral, rather than a vertical, force was applied to the material (see and compare Figs 1(ii) and (iii)). In addition, the amount of debris obscuring the exposed surface was greatly reduced in torn material. Although cytoplasmic contents

Figure 1: (i) A piece of kikuyu grass leaf, viewed edge-on. The left half of the leaf segment was cut with a razor blade following critical point drying, and the right half was torn.

(ii) An enlarged portion of the kikuyu leaf in (i), that was cut with a razor blade following preparation for SEM. Note the deformation of cells, and the jagged appearance of the cell walls along the cut edge.

(iii) An enlarged section of leaf tissue in (i) that was exposed through tearing of the leaf tissue. Note the crisp fracture surface, and the absence of cell deformation.


1i
100 µm
ii
40 µm
iii
40 µm

sometimes obscured the finer details of infection structures within the host cells, this technique of leaf tearing, rather than cutting, was very successful (e.g. Chapter 3, Fig. 15).

A serious disadvantage of SEM, relates to the dehydration of the specimens during processing. Dehydration inevitably causes some contraction of the tissues being processed. This became apparent when comparing the sizes of unstained appressoria viewed with light microscopy, and those that were viewed with the scanning electron microscope. The average diameter of the latter, was 2 µm smaller than that of the unprocessed appressoria. Apparently, it is not unusual for specimens processed for SEM, to shrink by up to 20% of their original size[2].

In addition to the conventional specimen preparation, culminating in critical-point drying of the material to be viewed, fresh, inoculated leaf material was cryogenically preserved prior to viewing with the scanner. This technique had the advantage of reducing the number of spores and appressoria that were removed during processing, but resolution was reduced, and there was a higher incidence of 'charging'. In addition, although the specimens could be fractured after freezing, to reveal the leaf in cross section, the degree of control afforded by the available equipment was inadequate. Being able to locate the most densely infected areas on critical-point dried material under the dissecting microscope when tearing the leaf, was a more efficient and more accurate procedure.

5.1.2.3 Transmission electron microscopy (TEM)

Specimen preparation for TEM is a time-consuming and sometimes tedious procedure. Often, slight modifications have to be made in the protocol of specimen preparation, to accommodate the particular characteristics of the tissue being studied.

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Much of the early work conducted during this study, was aimed at investigating the structure of the uredia of *P. apoda*. This proved to be an unfortunate choice in terms of an introduction to the techniques involved in transmission electron microscopy, as the leaf material bearing uredia generally displayed a high degree of cellular disruption, and most of the cells contained numerous, large, apparently amorphous, electron-dense inclusions. These inclusions, presumably the product of a host response to the fungal infection, proved to be very difficult to section. Glass knives could not be used with any success, and even ultrathin sections cut with a diamond knife were holey and tended to have 'chatter' marks.

This material was also not embedded with great success, the inner tissues often remaining insufficiently polymerised. Problems were also experienced with freshly infected grass material, in terms of inadequate resin infiltration and polymerisation. This may have been related to the hard, siliceous nature of kikuyu grass leaves, which are not nearly as soft and pliable as many of other crop plants that have been studied in a similar context, e.g. soybean, maize and wheat. However, these infiltration problems were largely overcome by cutting the leaf pieces very small (under 1 mm x 1 mm) prior to processing for resin embedding. The disadvantage of cutting the specimens so small, was that the tissues along the edge, that were inevitably damaged due to a certain amount of crushing, constituted a substantial volume of each specimen.

Epon-Araldite resins proved to be more successful as an embedding medium than Spurrs. Placing the specimens under a moderate vacuum in the resin solutions, did not appear to greatly assist in improving infiltration, and increased the risk of tissue damage.

The quality of both fixation and resin infiltration, varied substantially between batches of material prepared at different times. Exactly what factors contributed to the varying degrees of success achieved was not established, but two potentially beneficial practices were identified. The first involves the exclusion of all traces of water in the later stages of the embedding process. When the specimens are placed in 100 %

alcohol for the first time, all the vial lids are washed in a beaker containing 100 % alcohol. This removes the liquid that inevitably splashes onto the lids during the earlier stages of processing. It is also important to use alcohol which is stored in a vessel containing a molecular sieve, to further ensure the exclusion of water. The second, apparently beneficial, practice that was adopted during the embedding procedure, was to leave the specimens in fresh, 100 % resin for an additional day.

When studying the interactions between a host or membrane and a single spore and its germling, it is important that the specimens are easy to find once they have been fixed and embedded, and that they are easy to orientate for sectioning. Ideally, all the components of the fungus should be included in the sections, with the spore, germ tube and appressorium all visible in one plane. Some beautiful examples of such precision sectioning have been published by Grey, Amerson, and van Dyke (1983), Gold and Mendgen (1984) and Mims and Richardson (1989). These authors made use of the thin-embedding techniques described by Mims, Richardson and Taylor (1988). Random sectioning of samples is only possible when germlings are present in reasonably large numbers, and even then, this method is not suitable for efficiently studying large numbers of samples (Mims *et al.*, 1988). During this study, visibility of the germlings on the host leaf was, on the whole, not adequate to allow for accurate orientation of the germlings. Even when thin-embedding techniques were employed, the density of the leaf tissue precluded accurate identification of specific spores and their appressoria.

The density of appressoria and spores on the inoculated leaf pieces was always greatly reduced by the time the specimens were polymerised in the resin blocks, presumably as a result of germlings being dislodged each time the specimens were rinsed during processing. Furthermore, nearly all the appressoria that were observed with the TEM, had lifted from the surface of the leaf. One would have expected the appressoria to have been completely removed if they had been dislodged at any time during the embedding process, unless they were loosened from the leaf surface during the actual polymerisation phase. It is conceivable that exposure to the oven temperatures of 60°C for 24 hours may have caused the cuticle to lift away from the leaf or to partially dissolve, causing the appressoria to separate from the leaf surface, as has happened in all the figures in Chapter 4. Very few spores and appressoria remained on specimens incubated for 6 hours and less. Bearing in mind that penetration of the leaf occurs between 6 and 8 hpi, this may be an indication that the intrafoliar penetration structures help to anchor the appressorium to the leaf.

Once the specimens had been polymerised, each piece of leaf tissue was examined under the light microscope to locate appressoria for sectioning. A number of measures were taken to improve the visibility of the spores on the resin-embedded leaf pieces:

- Leaf pieces were cut into a trapezoid shape (see Fig. 2);

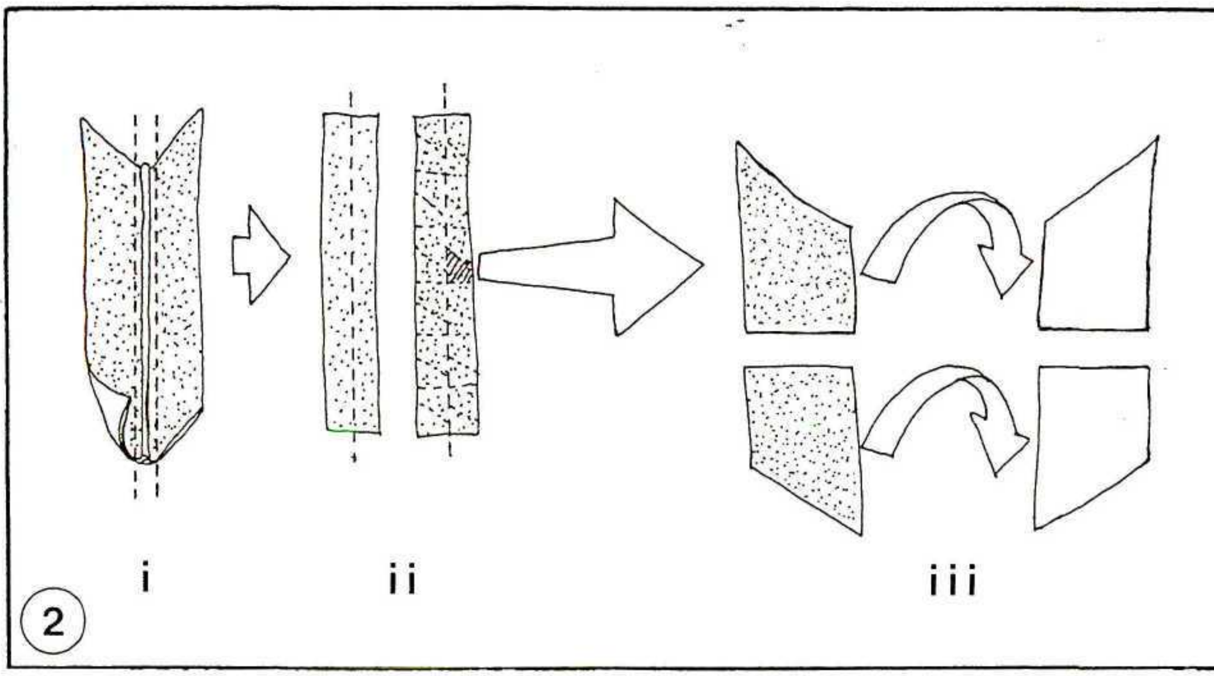
  This allowed for identification of the inoculated surface following embedding, and also allowed most pieces to be orientated spore-side down in the embedding dish. This ensured that time was not wasted searching for germlings on the wrong side of the leaf. Furthermore, the specimens inevitably sink to the bottom of the dish prior to polymerisation, leaving only a thin layer of resin between the leaf and the bottom of the block. Having the leaf pieces spore-side down, means that when viewing the polymerised block, there is less resin between the microscope lens and the germlings, and hence there is improved visibility.

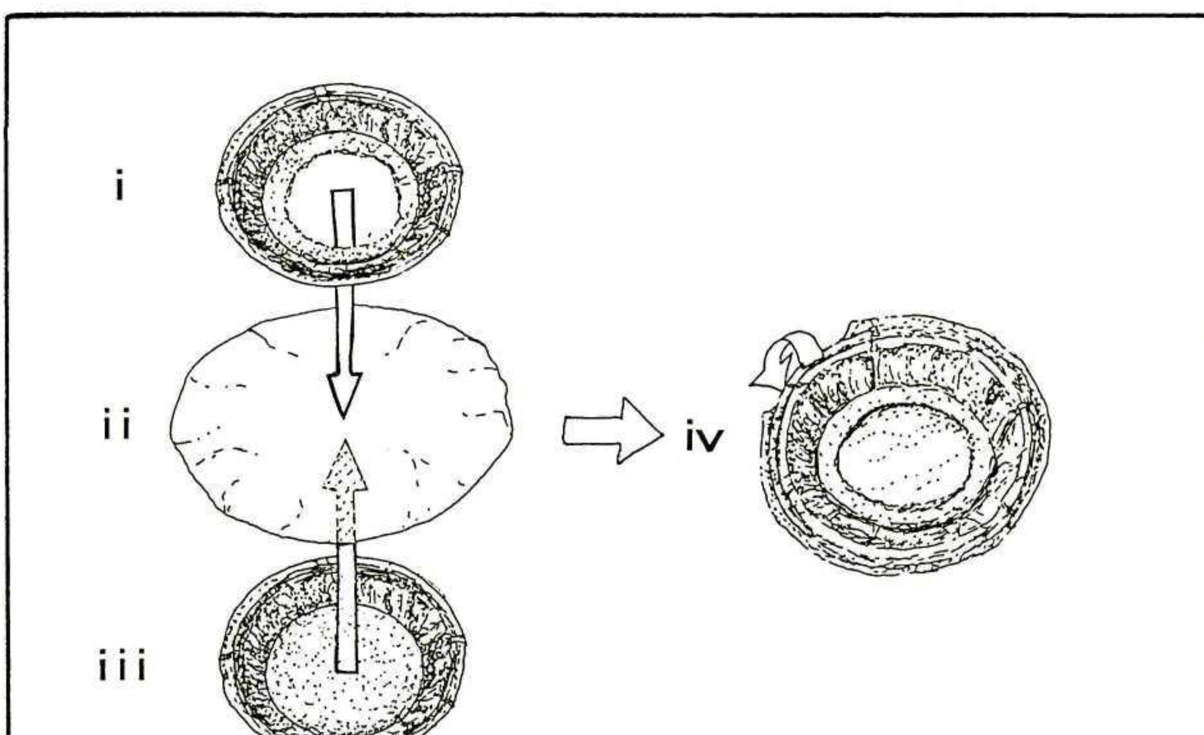
- Embedding dishes were lined with polypropylene (see Fig. 3);

  The polypropylene base meant that the undersurface of the polymerised resin block was very smooth, greatly enhancing germling visibility. Simply placing a piece of polypropylene in the base of a foil embedding dish was not successful, as the plastic floated to the surface of the warm resin during polymerisation. When the plastic was glued to the base of the dish, it tended to buckle in the oven, resulting in an irregular block base.

Figure 2: Only one side of each leaf sample was inoculated (as indicated by the stippled areas). Following incubation, the leaf segments were cut in half (along the midrib) **(i)**, and then into quarters, lengthways **(ii)**. Finally, areas most densely covered in spores were identified under the dissecting microscope, and were cut into a characteristic trapezoid shape, which allowed the inoculated surfaces to be identified during processing and examination **(iii)**. This made the location of spores and appressoria in embedded material easier.

Figure 3: A circular piece of polypropylene film **(ii)** is sandwiched between two foil dishes **(i & iii)** (drawn to scale), the upper dish having had its centre removed. The edges of the dishes are turned over, clamping them together **(iv)**.


i
ii
iii
2


i
ii
iii
iv
3

The best method of attaching the plastic, was to clamp it between two foil dishes of the same size, a circle of foil having been excised from the base of the upper dish. The two dishes are held together by folding over their rims. Any thin, plastic film could be used instead of polypropylene, provided that it is heat resistant, and will not dissolve in the resins used.

- During fixation, specimens were placed in osmium tetroxide for less than 2 hours;

Specimens left in osmium tetroxide for more than 2 hours became very dark. This greatly reduced the visibility of the germlings on the leaf pieces. If left in osmium tetroxide for only half an hour to an hour, the leaf pieces were less opaque. Fortunately, the small size of the specimens allowed for reduced exposure to the osmium.

Upon examination under the light microscope, each leaf piece was drawn, and the positions of observed spores/appressoria were indicated. This information was particularly useful when orienting the excised blocks on stubs for sectioning, as well as during the sectioning process itself. Once suitable specimens had been identified in the resin block, they were excised and hand-trimmed with a razor blade for mounting onto perspex stubs.

As mentioned earlier, sectioning specimens of infected kikuyu grass proved to be quite a challenge. Glass knives were not very successful. The knife edge developed score marks very rapidly, and sections were often 'chattered', i.e. there were narrowly spaced, regular, variations in section thickness, giving the sections a 'rippled' appearance. The use of a diamond knife virtually eliminated these problems, provided that the resin block face was kept very small at all times (well under 0.5 mm x 0.5 mm).

A worthwhile technique, which saved much time by greatly increasing the accuracy of 'picking up' ultrathin sections onto copper grids, was the flaming of the grids immediately prior to use. Usually, the grids disperse the water when brought immediately beneath the sections to be lifted, resulting in the sections being deposited on the grid surface slightly off centre, or even resulting in the sections skating off the grid altogether. Flaming the grids, by passing each through the flame of a bunsen burner immediately prior to submersion into the water in the boat of the knife, greatly reduces this effect, allowing the grid to closely approach the sections without disturbing their positions. Accurate positioning of the sections in the centre of the grid helps to reduce the number of sections partially or wholly obscured by the specimen holder in the microscope. Generally, the longer the grids are exposed to the flame, the less hydrophobic they became, although the copper grids combust readily if heated for too long. The best results achieved by the present author, were obtained by passing the grids through the flame at such a rate that they became rainbow-coloured. Naturally, formvar-coated grids would have to be flamed for a much shorter period of time.

5.1.3 Artificial membranes

Artificial membranes were used in two contexts during the course of this study (Chapter 2). The first, exploited the transparent nature of plastic membranes such as polyethylene, which provided an unobstructed view of the early stages of development of fungal germlings both morphologically and in terms of nuclear behaviour. The second, involved the manufacture of plastic films with microfabricated surfaces, which were used to investigate the effects of topography on appressorium induction.

5.1.3.1 Membranes and transmission electron microscopy (TEM)

The value of using transparent plastic membranes when viewing fungal germlings under the light microscope has already been discussed in Chapter 2. These membranes, which vastly improve the visibility of spores and germlings, can be put to effective use in transmission electron microscopy. As mentioned previously, the precise location and orientation of germlings for sectioning is very important in TEM studies, and the improved visibility afforded by artificial membranes allows for a greater degree of accuracy.

Preliminary attempts were made to embed and section membranes bearing germinated spores. Polyethylene membranes were inoculated with spores, incubated, and processed for TEM in the same manner as the host leaf material.

Along with variable spore germination rates, many difficulties were experienced in processing and sectioning the membranes. Only plastics resistant to dissolution in the solvents used during processing could be utilised. This precluded the use of polystyrene. Teflon, polypropylene and polyethylene membranes were investigated for their suitability in this application.

One of the most serious problems experienced during processing of inoculated membranes, was the tendency of the germlings to become dislodged, particularly in the more viscous resin stages. Very few spores remained on the membranes by the time the resin was polymerised. Processing the membranes in covered petri dishes, which were kept stationary, greatly increased the numbers of germlings remaining on the membranes.

Extracting specimens from the block often resulted in the separation of the plastic from the resin, leaving the germlings immediately adjacent to the edge of the block. Sections cut from this material often curled along the edges, obscuring the specimens. Even when the plastic did not separate during specimen extraction from the block, it usually separated during sectioning, curling away from the resin component of the section.

Teflon proved to be particularly prone to separation from the resin following polymerisation, with polypropylene and polyethylene performing marginally better in this respect.

Certain artificial membranes, such as dialysis tubing and polycarbonate, have the advantage of allowing fungal material to be processed by means of freeze-substitution. This technique has been shown to greatly improve the fixation of material to be viewed with transmission electron microscopy (eg. Hoch and Howard, 1980; Hoch and Staples, 1983; Swann and Mims, 1991). Freeze-substitution cannot be used with much success on material more than 10 μm thick (Howard and O'Donnell, 1987), without the use of high pressure, rapid freezing techniques. This requires the use of expensive equipment to process host leaf material. By using artificial membranes, the host tissue can be excluded, allowing for the processing of the fungal germlings with conventional freeze-substitution techniques. Although freeze-substitution was not used during the course of this study, this technique could be seen as the next step that needs to be taken in this line of research.

5.1.3.2 Microfabricated membranes

In order to study the thigmotropic responses of *P. apoda* germlings to changes in surface topography, it was necessary to manufacture membranes with superficial ridges or grooves. Initially, attempts were made to scratch membranes such as polyethylene with various implements, including copper brushes, fine needles, various grades of sandpaper and steel wool. The result was an assortment of scratch marks, with an unsatisfactory degree of resolution and regularity. The edges of these scratches tended to be jagged, and the dimensions of the features varied considerably. Even when testing the well defined thigmotropic responses of a stomatal penetrator such as *Puccinia sorghi* (Mr M. Savary[3], personal communication), these membranes yielded inconclusive results. In a direct penetrator, such as *P. apoda*, which is capable of appressorium formation in the absence of a well defined thigmotropic stimulus, one would expect any thigmotropic response indicative of preferential site selection, to be fairly difficult to detect. Since a preference, rather than a requirement of the fungus, is involved, any testing process would need to be sensitive.

- Making microfabricated membranes

As described in Chapter 2, ridged polystyrene membranes were cast from a laser-

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etched silicon wafer using liquid solutions of polystyrene in methylene chloride, and polycarbonate in chloroform, as described by Hoch, Staples, Whitehead, Comeau and Wolf (1987), Allen *et al.* (1991a) and Kwon and Hoch (1991). These precisely fabricated membranes provided the level of sensitivity required for this investigation.

The silicon wafer was obtained from Prof. H.C. Hoch[4]. The laser-etched grooves in the wafer were 2.0 μm wide and 0.9 - 1.1 μm deep, and were spaced 60 μm apart, in a grid pattern. The wafer itself appeared to be very fragile, and scratched easily, so care had to be taken when making membranes and when cleaning the surface of the wafer. The wafer was glued to a glass disc (5mm thick) to strengthen it and to make handling easier.

Casting ridged membranes involved pouring the dissolved plastic medium over the wafer, spreading it evenly over the surface, and then floating the dried membrane off the wafer in warm water.

The concentration of the polystyrene solution used for casting membranes in this study, was determined on the basis of texture rather than exact, volumetric measurements. As a starting point, Hoch's recommendation of 20% [w/v] was used, and then adjusted accordingly. Thicker solutions resulted in the production of thicker membranes. Very thick membranes tended to result in reduced transparency and increased optical distortion, and took much longer to dry. On the other hand, membranes that were too thin were more difficult to lift from the template, and tended to tear easily and to curl. Generally, solutions with the consistency of hot syrup gave the best results.

Spreading the dissolved polystyrene evenly over the surface of the wafer proved to be more difficult than anticipated. The rapid evaporation rate of the solvent, coupled with the danger of scratching the wafer, made spreading the polystyrene thinly and evenly enough, a difficult task. Various 'spreaders' were tested with limited success, including microscope slides wrapped in thin polypropylene sheeting. By accident, one of the

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pieces of polypropylene fell onto the pool of polystyrene, with a surprising result. The polystyrene spread under the piece of polypropylene, which appeared to react with the solvent, causing it to curl away from the surface of the wafer. When the curling polypropylene was gently slid off the edge of the wafer, a thin, even layer of polystyrene remained. The best results were obtained when only a third or a half of the wafer's surface was used at a time. All casting of membranes was conducted under a fume hood.

Lifting the membranes from the wafer was relatively easy, although the edges usually needed to be gently teased with a scalpel. Very thin pieces of membrane often did not lift at all. The wafer was cleaned by rinsing its surface with methylene chloride or acetone.

Once the polystyrene membranes had been lifted from the silicon template, it was still possible to check, with the naked eye, which side of the membrane bore the ridges. The ridged side glowed with a rosy colour when angled under a bright light. Although the smooth side also had a pinky hue when viewed at an angle, the effect was not as marked. In addition, when the membranes were tilted at a sharp angle under the dissecting microscope, a grid pattern could be seen in the most brilliantly illuminated areas of the ridged surface whereas, on the reverse side, these areas were smooth.

- Interpretation of results

In the past, authors have cited figures representing percentages of appressoria formed on ridges as opposed to smooth areas on microfabricated membranes, in support of the inducive properties of the ridges for appressorium formation (e.g. Allen *et al.*, 1991a). This work has mainly centred on the indirect or stoma-penetrating germlings that usually only differentiate appressoria when presented with a specific thigmotropic stimulus. By comparing the number of germ tubes that grew over a ridge, with the number that formed appressoria immediately following contact with the ridge, it is possible to accurately assess the induciveness of the ridge to appressorium formation.

When dealing with direct-penetrators that do not form long germ tubes prior to appressorium formation, measuring a thigmotropic response is not as simple as for indirect-penetrators. The lack of exploratory growth does not allow for a fair assessment of 'growth prior to ridge contact' versus 'growth after ridge contact'. Indirect penetrators generally do not form an appressorium if they do not encounter a suitable stimulus - you know when they have not recognised a potential signal (in the form of a ridge), because they grow over it. With the direct penetrators, they may form an appressorium close to the spore, irrespective of whether there is a ridge there or not. Simply counting the number of appressoria that are in contact with a ridge, may therefore produce results that are biased by the proportion of ridge-associated area to smooth area on the membrane.

Presumably, an appressorium has potentially formed in association with a ridge when there is some overlap with the ridge. Hence, we can assume that any appressorium within a distance less than the average diameter of an appressorium from a ridge, may have formed in association with that ridge. If this area of ridge-associated membrane, within a single block on a microfabricated membrane, was equal to the area of smooth membrane enclosed by the ridges, then we could simply count all the appressoria formed in each zone and make a direct comparison. In this study, the area of ridge-associated membrane was 44.5%, but the smaller the average appressorial diameter of the fungus being studied, the smaller the ridge-associated area on the membrane. Conversely, the larger the appressorium, the larger the ridge-associated area. To take this to a ridiculous extreme, imagine an appressorium that has a diameter equivalent to over half the distance between two ridges. This means that every appressorium formed on the membrane will be in contact with a ridge. Does this automatically imply that ridge-associated induction of the appressoria took place? The temptation is to interpret a high percentage of appressoria formed in apparent association with ridges as being illustrative of an inducive response, whereas, in a totally random arrangement of appressoria on the membrane, one would expect a certain proportion to have formed in the ridge-associated areas anyway.

This method of relating areas on a membrane to proportions of appressoria that are expected to form in these areas, is not entirely correct. There is some overlap of the smooth and the ridge-associated areas, and this was not taken into account when performing the statistical analysis of the results obtained in this study. Bearing this in mind, it is possible that the apparent preference on the part of the fungal germlings for ridge-associated differentiation, which was significant at the 5% level of significance, is an artifact of an imperfect analytical technique.

5.1.4. Statistical validity of results

For a number of reasons, this study did not have the benefit of statistical corroboration of the results obtained. This was particularly apparent in the TEM study. The time-consuming nature of this work, and the difficulties experienced in finding specimens, meant that too few individuals were examined to constitute a population size large enough to allow statistically sound conclusions to be drawn. Scanning electron microscopy was more accommodating, in so far as observations of infection structures on the leaf surface were concerned, as large numbers of infection structures could be examined over a relatively short period of time. However, the rarity of intraepidermal discoveries, precluded any hopes of frequency-based analyses.

Studies involving the germination of spores on membrane surfaces, were the most conducive to statistical analysis, as the location and observation of germlings was unhindered in the absence of host tissues. Ironically, it is also these circumstances that provided the greatest variability, and which inspired the least confidence in terms of reflecting 'natural' processes.

5.2 THE INFECTION PROCESS

The lifecycle of *P. apoda* appears to involve only one spore stage on one host. As discussed in Chapter 1, teliospores of this fungus have been described in the literature, but have not been found in Hawaii, New Zealand, or South Africa. Although Hall's

(1991) attempts to induce teliospore formation were unsuccessful, it would be of great interest and potential value to pursue this line of research and establish what conditions are required for teliospore formation, and whether these conditions fall within common climatic parameters experienced in South Africa. If kikuyu grass rust should ever become a problem in South African pastures, it would be worth knowing the potential genetic diversity of the pathogen. Dealing with a fungus limited to asexual reproduction, would imply a reduced potential for rapid development of resistance to chemical agents.

Another area of research that may help with future control of kikuyu grass rust, as well as disease prediction, involves the determination of the effects of environmental factors on disease development. This would include determining the conditions most suitable for 1) development and dispersal of the fungal urediospores, 2) development of fungal infection structures on/in the host, and 3) host predisposition to infection. Such a study would have to take both climatic and microclimatic effects into account. Of particular interest to the present author, is the effect of sunlight on disease development. Does shaded kikuyu grass represent a host more predisposed to infection due to reduced vigour, or are the moister, cooler conditions more favourable to proliferation of the pathogen? Perhaps both these factors have a role to play.

A great deal of time and effort has been expended during the course of this study in determining whether or not the germlings of *P. apoda* respond to topographical features during their selection of suitable sites for host leaf penetration. If this organism had not been a rust fungus, the present author would probably not have gone further than stating that most appressoria appear to form at epidermal cell junctions. But, because of the long history of investigation into the thigmotropic responses of the rust fungi, it seemed quite logical that this rust should also be scrutinised with the aid of scratches, ridges and suchlike. Unfortunately, the outcome was a set of fairly inconclusive observations. The germlings of *P. apoda* may or may not associate abrupt changes in elevation of the substrate with suitable penetration sites. Quantification of thigmotropisms in urediospore germlings of *P. apoda*, with the aid of microfabricated membranes, needs to be refined considerably to produce more convincing results. At this stage however, it is quite clear that topographical signals do not play a huge role in the infection process, as would be expected in a direct-penetrator. It would be interesting to quantify the thigmotropic responses of each direct penetrator that has been identified, and to compare the results with an assessment of the respective host leaf topographies. Does a more pronounced response to topographical features on an artificial membrane correspond to the presence of deeper 'valleys' between epidermal cells on the host leaf?

In Chapter 1, a number of theories were described as to why direct penetrators might target epidermal cell junctions as sites of host wall penetration. One obvious possibility that was not mentioned, is that the host cell wall might become thinner towards the junction with an adjacent cell. The occurrence of thinner anticlinal and inner epidermal cell walls was observed in kikuyu grass leaves (see Chapter 4, Fig. 6 (i) and (ii)), and this could help to explain why *P. apoda* tends to select penetration sites near epidermal cell junctions. As described in Chapter 3, approximately 81 % of appressoria of *P. apoda* on the host leaf surface, formed at epidermal cell junctions. At first glance, this appears to provide convincing testimony in favour of preferential penetration-site selection by the fungus. However, if one takes into account that the average epidermal cell is only approximately three times the width of an appressorium, then the chances of an appressorium randomly forming in contact with an epidermal cell junction is high, and it becomes difficult to prove the existence of selectivity on the part of the fungus. This could possibly be achieved with computer-aided image analysis, whereby the area available for apparently 'junction associated' appressorium formation could be compared with the area accommodating appressorium formation in the absence of an associated junction.

Such calculations have been made regarding the inducive effects of microfabricated membranes, as discussed in Chapter 2 and the Methodology section of this thesis. In the case of microfabricated membranes, the potential thigmotropic stimuli are well defined and regular, and associated areas can be measured exactly and with ease.

However, in the case of the epidermis, the cells are not perfect rectangles, and exact area measurements become more complicated.

Following penetration site selection, the fungal germling forms an appressorium, and it is this structure that has been the focus of the present study. Under the light microscope the appressorium of *P. apoda* was interesting, providing the first glimpse of a cone-like structure in the base of the appressorium, and confirming a pattern of nuclear behaviour commonly observed in the appressoria of many rust fungi in the uredial stage. As seen with the aid of the electron microscopes however, the appressorium was fascinating. Using three different microscopes to view the appressorium, provided an intriguing composite picture of the structures and processes involved in the penetration process.

One of the earliest stages of the penetration process, is the creation of a bond between the fungus and the surface of the host leaf. Scanning electron microscopy (SEM) revealed the presence of apparently wax-less areas on the leaf surface, which represented sites of appressorial attachment. Although many theories have been put forward, the significance of this close bond between the host surface and the appressorial base has never been conclusively demonstrated (refer to Chapter 1).

It is presumed that enzymatic activity is responsible for the alteration and apparent dissolution of the wax layer on the leaf surface. Unfortunately, this aspect could not be examined with TEM, as the wax and cuticle layers on the kikuyu grass leaves appeared to have been removed during processing (probably also accounting for the lifting of the appressorial bases away from the leaf surface prior to polymerization).

Within the region beneath the appressorium, the fungus appears to be able to exercise further selectivity as to exactly where within the infection court penetration is to take place. This allows the fungus to avoid penetration at a cell junction or through a stomatal pore, should the appressorium extend across these features, and suggests that a very specific and sensitive contact or chemical stimulus is involved.

The first indication of penetration activity occurring in the appressorium, involves the alteration of the basal appressorial wall, culminating in the development of a pore surrounded by a ring of wall material with a different texture, optical properties and electron density to the rest of the wall in the basal region. How this pore develops is unknown, as only the later stages of the penetration process were observed with TEM, but with SEM it became clear that the formation of the ring and the pore occurred in concert.

The benefits of using several techniques in any one study, was particularly evident in the elucidation of the structure of the thinned basal appressorial wall. With light microscopy, the pore was visible in the base of the appressorium. In a ring immediately surrounding the pore, the basal wall appeared to have slightly different optical properties to the rest of the wall. This was attributed to the presence of a cone which appeared to surround the pore. With scanning electron microscopy, it was clear that a pore did develop in the base of the appressorium, and that the wall surrounding the pore was altered in some way. This ring was composed of wall with a very smooth texture, and was approximately 1.5 µm in width. Transmission electron micrographs revealed that the area up to 1.5 µm from the appressorial pore, corresponded exactly with the thinned, electron-dense area that is so conspicuous in the basal wall when viewed in cross section (see Fig. 4).

Thinning of the basal wall, and the development of a 'wall-less' region as a precursor to penetration peg development has been observed in many direct penetrating fungi. It seems that the thinning is usually the result of one of several wall layers in the appressorium being dissolved in a localised region around the pore. In the appressorium of *Spilocaea pomi*, it is the inner wall layer that tapers away around the infection site, with the outer wall remaining in contact with the substrate right up to the actual penetration pore (Corlett and Chong, 1977), and in some *Colletotrichum* spp., it is the outer appressorium wall layer that tapers away, with the inner wall layer remaining in contact with the substrate in the vicinity of the penetration pore (Landes and Hoffman, 1979). Gold-labelling has revealed that in *P. pachyrhizi*, it is the outer

Figure 4: Diagram of the infection site created by *P. apoda* on the kikuyu grass host leaf.

E - host epidermal cell;

CW - host cell wall;

A - appressorium;

AM - extra-appressorial matrix;

Pp - penetration peg;

pw - penetration peg wall;

IEPh - intraepidermal penetration hypha;

clr - collar;

ap - appressorial pore;

1 - appressorium wall;

2 - thinned, electron-dense region of basal appressorial wall;

3 - thin, moderately electron-dense outer layer in cone wall;

4 - point at which the penetration peg layer merges with the bulk of the cone wall;

5 - 2-dimensional fit of the collar into the appressorial pore;

6 - electron-dense layer of material lining the penetration pore.

Figure 5: Diagram of the infection process from the appressorium to the formation of the extracellular penetration hypha of *P. apoda* on kikuyu grass leaves.

E - host epidermal cell;

ms - host mesophyll cell;

ics - intercellular space in the mesophyll;

A - appressorium;

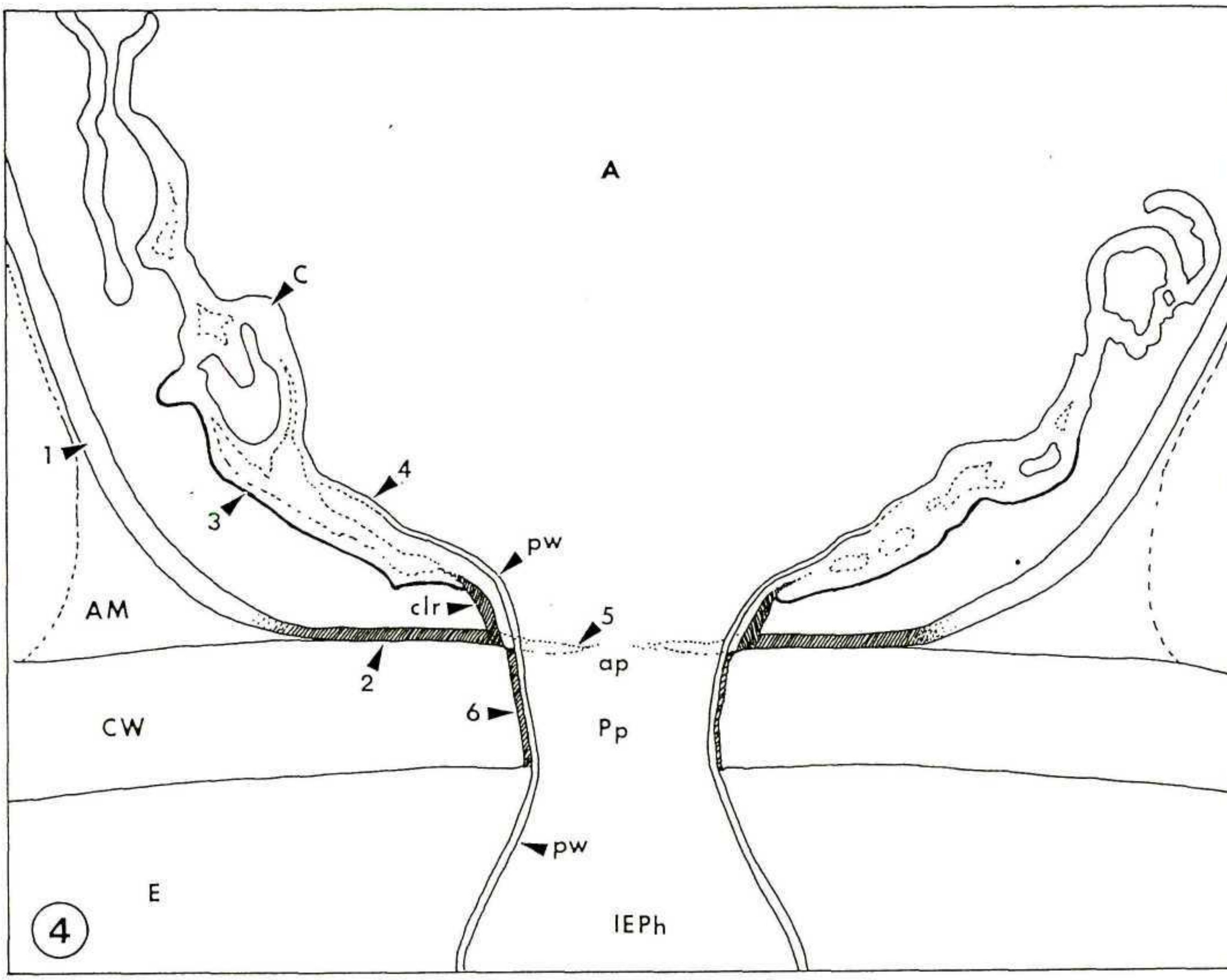
C - cone;

Pp - penetration peg;

IEPh - intraepidermal penetration hypha;

N - penetration neck;

ICPh - intercellular penetration hypha.


A
C
1
3
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pw
AM
clr
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2
ap
CW
6
Pp
pw
E
IEPh
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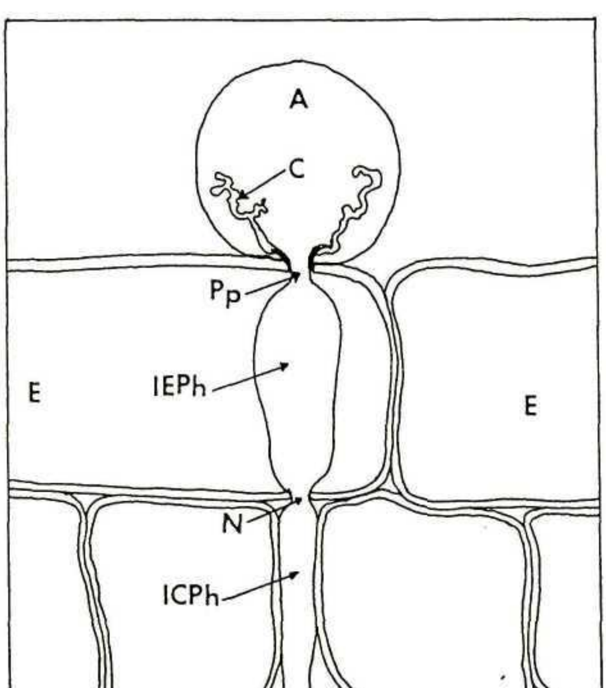
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appressorial wall layer that tapers away towards the pore, exposing the inner wall layer in the vicinity of the pore (Ebrahim-Nesbat, Hoppe and Rohringer, 1985). This exposed inner wall layer is very electron-dense, as is the case with *P. apoda*. It seems reasonable to infer that the structure of the altered basal wall in *P. apoda* is similar to that of *P. pachyrhizi*. It would be interesting to observe, with SEM, the basal walls of critical-point dried appressoria of *P. pachyrhizi* to see if there is also a ring of smooth wall material around the appressorial pore in this fungus.

The increased electron-density of the inner wall layer that is in contact with the host surface, is puzzling. It could relate to the dissolution of the outer wall in this area. How do the enzymes required for wall dissolution reach the outer wall layer, and how are the products of outer wall layer dissolution reassimilated? The most elegant option is that the inner wall layer is only deposited following the dissolution of the outer wall layer and the formation of a wall-less region in the appressorial base, but the appressorium wall prior to pore development appears to be the same thickness as that following the formation of the pore. If the inner wall layer is already present when dissolution of the outer wall layer occurs, then either i) both layers are removed and the inner layer re-deposited, or ii) the chemical activities involved in outer wall layer dissolution occur via apoplastic transport through the inner wall. In either case, the inner wall layer would probably be chemically altered in some way.

This chemical alteration may have some significance during the penetration process. It is possible that the alteration is specifically orchestrated - perhaps the altered wall is particularly resistant to the enzymes used during penetration, and acts as a type of shield, preventing the apoplastic movement of degrading enzymes away from the penetration site.

The most striking feature of appressorial ultrastructure in *P. apoda*, as well as in *P. pachyrhizi*, is the cone. As discussed in earlier chapters, cones are not unusual features in the appressoria of direct-penetrating fungi. They probably represent a means of increasing the surface area available for the biochemical processes that occur during penetration. They may also served to 'funnel' the various products of these processes towards the penetration site.

Unfortunately, because the transmission electron microscope (TEM) observations described in this study focused on specimens processed 12 hpi, the potentially fascinating early stages of cone development, that occur at approximately 6 hpi, can only be speculated upon at present.

If we imagine a model of basal wall alteration similar to that described for *P. pachyrhizi* (Ebrahim-Nesbat *et al.*, 1985), then there is wall material that is being 'dissolved' as the outer wall layer tapers away towards the appressorial pore, as well as inner wall layer material that is removed with the formation of the pore itself. It is possible that the products of wall dissolution are used in the construction of the cone, unless of course, the development of the cone preceeds the development of the pore. The size of the collar is a piece of evidence which makes it very tempting to conclude that the cone wall material has originated from the basal wall. Fig. 4 illustrates how the collar could easily fit into the diameter of the appressorial pore. However, one has to bear in mind that the collar is conical in shape, and a 2-dimensional cross-section does not give an accurate reflection of the situation.

As was discussed in Chapter 4, the terminology that has been used in describing and differentiating between the various penetration structures has probably not been entirely accurate. The penetration peg should perhaps refer to the transitional peg structure which is seen in the host cell wall during its penetration, and could be applied to the penetration structures arising from the appressorium and also the haustorial mother cell. This fleeting stage was not observed during this investigation, only the constricted region of hypha that remained within the outer epidermal cell wall was seen. However, in most studies of direct penetrators the narrow region of hypha within the host cell wall which links the appressorium to the intracellular infection structures, is referred to as the penetration peg.

The penetration peg wall appears to be continuous with an inner wall layer of the cone. As discussed in Chapter 1, this phenomenon is very common amongst direct-penetrating fungi, and even in those that do not produce cones, the penetration peg wall is continuous with an inner appressorial wall layer. The net effect of this is a discontinuity in wall structure from the appressorium to the intra-foliar infection structures. This may represent a mechanism whereby the fungus is able to effect a radical change in the structure and composition of the walls of the infection structures. In *P. pachyrhizi*, studies using gold-labelling techniques, have demonstrated that the penetration peg and subsequent infection structures of this fungus contain chitin, whereas the outer appressorium wall does not contain detectable amounts of this substance (Ebrahim-Nesbat *et al.*, 1985).

As to the mode of penetration employed by *P. apoda*: it would seem to be predominantly enzymatic. There is strong evidence in the literature in support of mechanical mechanisms of penetration in some direct-penetrating species of fungus, but these fungi are mostly anthracnose fungi with melanised appressoria and tiny penetration pegs (<0.5 µm as opposed to 1.5 µm in *P. apoda*), and the mechanical component to the infection process is thought to be directed more at cuticular penetration, with enzymatic mechanisms operating on the host cell wall itself (Brown, 1977; Mould, Boland and Robb, 1991). There is little evidence in support of mechanical penetration mechanisms amongst the rust fungi, and the 'neatness' of the penetration hole in leaf tissue infected by *P. apoda* would seem to support a predominantly enzymatic mode of penetration. However, the depressed areas often observed immediately surrounding the penetration hole on the leaf surface, could imply that some mechanical force is applied during infection.

The spiralling or corkscrew growth of the intraepidermal penetration hypha that was observed to occur in germlings of *P. apoda* on artificial membranes and occasionally with SEM in the host leaf, was not observed with TEM. Of course, detection of this pattern of growth is more difficult with TEM, which only allows for three dimensional visualisation through serial sections. Why the intraepidermal penetration hypha sometimes adopts this spiral growth pattern, is not clear, especially as it has not been consistently observed. There must be some sort of directional growth involved in the development of the intraepidermal penetration hypha to prevent random growth within the epidermal cell and failure to reach the opposite side of the cell. Generally, the penetration hypha traversed the epidermal cell perpendicularly to the leaf surface or at a slight angle, and sometimes the hypha grew a short distance along the opposing epidermal cell wall before exiting.

None of the penetration hyphae observed, exited the epidermal cell directly into an underlying mesophyll cell, as has been reported to occur in *P. pachyrhizi*. In some cases, it appears that the fungus has a means of detecting suitable sites for exiting the epidermal cell. See in Chapter 3, Fig. 5(i) how the penetration hypha exits immediately adjacent to an underlying mesophyll cell. In Fig. 6 the penetration hypha neatly exits between two mesophyll cells.

The penetration neck, where the intraepidermal penetration hypha exits the epidermal cell and becomes the intercellular penetration hypha, was very similar in appearance to the penetration peg, with the exception that the wall was continuous from the intraepidermal hypha through to the intercellular penetration hypha. It seems that the fungus does not require a dramatic change in wall structure when commencing intercellular growth.

With regard to the nuclear behaviour observed in the infection structures of *P. apoda*, the results obtained from germlings grown on artificial membranes were not conclusive. During the early stages of penetration structure development, the pattern of behaviour was consistent, with the two nuclei present in the urediospore moving into the appressorium, where they each underwent mitosis, resulting in four nuclei in the mature appressorium. However, following the formation of the penetration hypha, the results became varied and contradictory. This may well be related to the fact that the nuclei remain behind in the appressorium in the host leaf, and are not observed to move into the penetration hypha prior to 12 hpi. On membranes, the nuclei moved into the penetration peg shortly following its development. This seems to suggest that either the penetration process itself, or some interaction with the host, ensures a delay in the movement of the nuclei out of the appressorium.

Why do the nuclei remain for so long in the appressorium? Why are there so many glycogen-like particles, mitochondria and elements of ER associated with the cone, long after penetration of the outer epidermal cell wall has occurred, and how is the intraepidermal penetration hypha able to penetrate the inner epidermal cell wall with such apparent ease? Perhaps the appressorium represents the 'power-house' of the infection process, and is not simply there to penetrate the outer epidermal cell wall. The entire process (see Fig. 5), possibly right up until septum formation in the penetration hypha, may be orchestrated from the appressorium. This would help to explain why the nuclei remain in the appressorium for so long. Perhaps the enzymes and materials required for later infection processes are, at least in part, produced within the appressorium and then transported along the penetration hypha, via what appears to be a well developed transport system with an elaborate endomembrane network and strategically placed microtubules.

Infection structures produced by rust fungi on artificial substrates, usually have an identical morphology to those produced on/in the leaf, and nuclear behaviour also appears to correlate in most cases (Mendgen and Deising, 1993). Hunt (1968) found that the behaviour of germinated urediospores of *Puccinia psidii* and *Ravenelia humphreyana* on membranes was similar to that observed on the leaf surfaces but germ tubes were, on average, twice as long as those on leaves. In the case of *P. apoda*, the development of infection structures on membranes appeared to be very similar to that of germlings on the host, especially in the early stages. However, as mentioned above, nuclear behaviour appeared to become aberrant during the development of the penetration and primary hyphae, and the rate of infection structure development appeared to be retarded on the membranes.

The rate of germling development on artificial membranes is often different to that on/in the host, with the development of infection structures usually taking longer on membranes. For example, it took basidiospore germlings of *Uromyces* spp. days to develop primary hyphae on artificial membranes, while only 4 hours were required in host tissue (Freytag, Bruscaglioni, Gold and Mendgen, 1988).

As can be seen from the table below, development of infection structures by *P. apoda* on artificial membranes was considerably slower than that in the host, although the structures appeared to be morphologically identical.

Table 1: Times of development of early infection structures of *P. apoda* in the host and on artificial membranes (hpi = hours post inoculation).

INFECTION STRUCTURES OBSERVED	*IN VIVO* (SEM and TEM)	*IN VITRO* (Light microscopy)
Appressorium formation	5 hpi	6 hpi
Penetration pore in the base of the appressorium	6 hpi	8 hpi
Penetration peg	6 hpi	12 hpi
Penetration hypha	8 hpi	16 hpi

Perhaps one of the most interesting results of this study, is the fact that penetration from urediospores of *Phakopsora apoda* on a monocotyledonous host, appears to be very similar to that observed for *Phakopsora pachyrhizi* on a dicotyledonous host. These similarities have become apparent at both a morphological and an ultrastructural level.

Tables 2 and 3 represent comparisons between the development of infection structures from urediospores of each fungus on artificial membranes and on their respective hosts. Unfortunately, most of the information on *P. pachyrhizi* that is given in Table 3 resulted from observations made 24 hpi, as opposed to 12 hpi in the case of *P. apoda*.

Table 2: A comparative summary of infection structure development from urediospores of *Phakopsora apoda* and *Phakopsora pachyrhizi* on artificial membranes.

	Phakopsora apoda	*Phakopsora pachyrhizi*
Germ tubes	Usually short, or appressorium sessile to the urediospore.	Usually short, or appressorium sessile to the urediospore.
Thigmotropic induction of appressoria on ridged membranes	No clear response to ridges; appressoria form freely between and on ridges.	Appressoria form in 'loose association' with ridges; appressoria form freely between and on ridges.
Appressoria	• clear region (appressorial pore) develops in base of appressorium; • cone-like structure develops in base of appressorium; • four nuclei present in fully developed appressorium.	• no appressorial pore described; • cone-like structure develops in base of appressorium; • four nuclei present in fully developed appressorium.
Penetration hyphae	• very low rates of formation, only on a few types of membrane; • develops from the base of the appressorium, apparently originating from the cone; • corkscrew growth habit frequently observed.	• very low rates of formation on non-host surfaces; • develops from the base of the appressorium, apparently originating from the cone; • corkscrew growth habit observed in some cases.
Primary hypha	• a septum delimits the primary hypha from the intercellular penetration hypha; • primary hypha bifurcates to form two secondary hyphae.	• a septum delimits the primary hypha from the intercellular penetration hypha; • primary hypha bifurcates to form two secondary hyphae.

Table 3: A comparative summary of infection structure development from urediospores of *Phakopsora apoda* and *Phakopsora pachyrhizi* on their respective host leaves.

	Phakopsora apoda	**Phakopsora pachyrhizi**
Germ tubes	Usually short, appressoria often sessile to the urediospore.	Usually short, appressoria often sessile to the urediospore.
Appressorium formation at epidermal cell junctions	81 %	85 %
Penetration sites	Always directly into a host epidermal cell; penetration not observed through stomata or between epidermal cells.	Always directly into a host epidermal cell; penetration not observed through stomata or between epidermal cells.
Appressoria	• basal wall thinned, and electron-dense around a basal pore; • cone-like structure present in the base of the appressorium.	• basal wall thinned and electron-dense around a basal pore; • cone-like structure present in base of the appressorium.
Appressorial cones	• elaborately branched cone wall; • well-defined, electron-dense collar around base of cone; • numerous mitochondria associated with the cone elaborations; • numerous electron-dense glycogen-like granules associated with the cone elaborations.	• elaborately branched cone wall; • poorly defined, electron-dense collar around base of cone; • numerous mitochondria associated with cone elaborations; • Electron-dense glycogen-like particles present in low numbers in the appressorium.

Penetration peg	• represented by narrowing in diameter of the penetration hypha, within the host wall; • peg wall continuous with wall layer in the cone; • numerous microtubules and associated multivesicular bodies present; • no glycogen-like particles present.	• represented by narrowing in diameter of the penetration hypha, within the host wall; • peg wall continuous with wall layer in the cone; • no microtubules observed, but multivesicular bodies present; • very few glycogen-like particles present.
Intracellular penetration hypha	• numerous strands of ER; • evenly distributed glycogen-like particles; • only observed to exit directly from a epidermal cell into an intercellular space in the mesophyll.	• no well defined elements of ER visible; • glycogen-like particles not visible; • occasionally observed to exit the epidermal cell and immediately enter and traverse a palisade cell, prior to exiting into an intercellular space in the mesophyll.
Intercellular penetration hypha	• numerous elements and arrays of budding ER; • very few glycogen-like particles.	• no proliferation of the endomembrane system observed; • glycogen-like particles not observed.
Inner-wall penetration neck	• microtubules passing through neck area; • multivesicular bodies present in association with microtubules.	• no microtubules observed; • no multivesicular bodies observed.
Primary hypha	No septum observed delimiting a primary hypha from the intercellular penetration hypha (12 hpi).	Septum observed delimiting a primary hypha from the intercellular penetration hypha (24 hpi).

Host response to infection	Obvious signs of disorganisation in host cells in the vicinity of infection site, within 12 hpi.	Obvious signs of host cell disorganisation well within 24 hpi.
Host range	Infects at least 7 species of grass.	Infects many leguminous species.

Because *P. pachyrhizi* is closely related to *P. apoda*, it was anticipated that there would be certain similarities between the two fungi. However, the similarities that have been observed, exceeded all expectations. It was really only at the ultrastructural level that some differences between the two species of *Phakopsora* became apparent, and some of these differences could perhaps be accounted for in terms of differences in specimen preparation, and of course incubation times.

Amongst the indirect-penetrating rust fungi, significant differences have been noted in infection structure morphology between members of the same genus. This is very likely to be related to the adaptation of the indirect penetrators to the morphology of their respective hosts, e.g. the size and orientation of the substomatal chambers. In the direct penetrators, the fungi are perhaps less influenced by the structure of the host in terms of their morphology in the early stages of penetration, as they do not have to navigate intercellular features of the host tissue until later in the infection process.
It is possible that the mode of penetration employed by rust fungi from the urediospore, is a genetically conserved feature. Following their investigations of direct penetration from urediospores of *P. pachyrhizi*, Bonde, Bromfield and Melching (1982) decided to investigate the mode of penetration effected from urediospores of *Physopella zeae*. This was decided on the basis of the close taxonomic relationship between the genera *Phakopsora* and *Physopella*, which Bonde *et al.* (1982) felt may have a bearing on the penetration method employed by the fungus. They found that *Physopella zeae* was indeed a direct penetrator from its urediospores. It is possible that additional direct penetrators may be discovered by investigating species closely related to those fungi that have already been observed to penetrate directly into the host leaf.

Bearing in mind that many rust fungi can effect both direct and indirect penetration, depending on the spore type involved, it is not very surprising that there are variations in the methods of penetration that we have come to expect in the rust fungi. If a fungus can penetrate directly from its basidiospores and indirectly from its urediospores, then why not have a fungus that can penetrate directly from its urediospores? Staples and Hoch (1987) described the differentiation response of the direct-penetrating rust fungi as representing 'a phylogenetic degeneration' from the more sophisticated stoma-recognition response exhibited by the indirect-penetrators. The present author prefers to see both mechanisms as being superb adaptations to the respective host plants, with the germlings using different approaches to the same problem. Indirect-penetrating rusts require longer germ tubes to find specialised points of entry on the leaf surface, i.e. stomata. Although a large amount of energy is spent on finding a point of entry, energy is probably saved on the enzymes and other processes required for penetrating the cuticle. In direct-penetrating rusts, where the requirements for site selection are less specific, e.g. any of the abundantly occurring shallow depressions between cells, the fungus can economise on germ tube growth and focus its energy on the actual penetration process. There is no evidence to suggest that direct-penetrators are less successful than indirect-penetrating species, and each mode of penetration appears to have its advantages and disadvantages.

The reaction of the kikuyu grass host to infection by *P. apoda* appears to be more rapid and extreme than one would expect when considering that a rust fungus is involved. Even by 12 hpi, the cytoplasm of mesophyll cells adjacent to infection sites had become totally disorganised. Presumably, this represents one of the disadvantages of penetrating a host cell so early in the infection process.

It makes sense that the epidermal and palisade cells of a plant would have more sophisticated enemy detection and defense mechanisms than the mesophyll. It is unfortunate, therefore that the direct penetrators have to invade the epidermal cells first. Perhaps indirect penetration should not only be seen as a means of avoiding epidermal cell penetration, but also as a means of avoiding the plant's first line of defense.

The work of Deverall, Keogh and McLeod (1977) demonstrated this interesting concept in their observations of the reactions of a susceptible soybean host to *P. pachyrhizi*. To summarize, the first cells in contact with the penetration hypha following penetration through the epidermal cell were palisade cells, and these showed some sort of incompatibility reaction, as evidenced by a change in the staining properties of the cells. However, once the penetration hypha reached the mesophyll, no incompatibility reaction was observed until much later in the infection process. Deverall *et al.* (1977) suggested that the cells of the lower mesophyll were therefore more compatible with the intercellular hyphae and haustoria for a longer period than the palisade cells. They also proposed that this evidence may point to some mechanism whereby haustorium formation prevents the onset of incompatibility reactions in the host. In the case of *P. apoda*, only the early stages of infection were observed, but this phenomenon would be worth investigating in future studies.

Future investigations into the infection process from urediospore germlings of *P. apoda*, would have to address issues including how and why the basal appressorial wall becomes thinned and electron- dense; how the cone develops and what its composition and function is; whether penetration is effected enzymatically and if so, where these enzymes are produced; the exact composition and function of the glycogen-like particles found in the appressorium and intraepidermal penetration hypha; the mechanisms of resistance in the host; the formation and structure of haustorial mother cells, and how they compare to the appressoria. Techniques such as freeze-substitution, low-temperature embedding and immunocytochemistry could all play a role in resolving some of these questions.

One of the above questions that particularly intrigues the present author, is how the appressorium of a direct penetrator such as *P. apoda* differs from the haustorial mother cells (HMC's) formed later on in the infection process. In the indirect-penetrating rust fungi, the first host wall penetration occurs from the HMC, whereas in direct penetrators, the first penetration of a host cell wall occurs from the appressorium.

The essential differences between the appressorium and the HMC of a direct-penetrator are:

(i) the appressorium is formed outside the host leaf, and the HMC within the host tissue;
(ii) the appressorium has to initiate penetration through the cuticle and a host wall, whereas the HMC only has to initiate penetration of a host wall;
(iii) the HMC has to mediate two-way transport of materials (initially into, and later out of, the penetrated host cell), whereas the primary function of the appressorium appears to be the initiation of host infection.

An investigation into how these differences relate to the morphological and biochemical aspects of each structure may contribute to an understanding of the penetration process as a whole. Several similarities become apparent when making even a superficial comparison between the appressorium of *P. apoda* and the HMC's of some rust fungi. These include the occurrence of membrane elaborations associated with the penetration process and wall modifications around the penetration pore.

Heath and Heath (1975) were the first to describe convoluted membrane protrusions at the septum delimiting the D-haustorium of a rust fungus from the intercellular hypha. These protrusions have since been found in several rust fungi (Chong, Harder and Rohringer, 1981; Hu and Rijkenberg, 1998). The membrane protrusions originate from the fungal plasmamembrane of the intercellular hypha at the HMC septum and contain an electron-dense matrix (Chong *et al.*, 1981). Presumably, the function of these protrusions and the cone elaborations of *P. apoda* is to increase the surface area of the fungal plasmamembrane in the vicinity of host wall penetration. Numerous mitochondria have been found in association with the HMC-associated protrusions (Chong *et al.*, 1981; Hu and Rijkenberg, 1998) and with the cone elaborations of *P. apoda*.
In several species of rust fungus, development of a wall thickening or torus occurs in the base of the HMC at the site of host cell penetration (Chong *et al.*, 1985; Hu and Rijkenberg, 1998). Could this serve a similar function to the cone or at least the collar in the appressoria of *P. apoda*? The intrawall penetration torus described by Chong *et*

al. (1985) in the HMC's of *Puccinia graminis* Pers. f.sp. *tritici* Eriks and Hen., clearly demonstrates the similarities between these structures, including an increased electron density of the wall material in the torus.

The present author feels fortunate to have worked with such an unusual and interesting rust fungus as *Phakopsora apoda*. It seems that almost every aspect of this fungus is extraordinary, including its lifecycle, uredial morphology and mode of host infection. Even at the ultrastructural level, this fungus continues to reveal surprising and fascinating details.

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