APPRESSORIUM INDUCTION IN THE CEREAL RUSTS

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Declaration

This thesis has been composed by myself and the work of which it is a record has been carried out by myself. All sources of information have been specifically acknowledged by means of reference.

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Abstract

The aims of the project were to define the signals provided by the cereal host which induce cereal rusts to form appressoria over stomata, and to analyse the mechanism by which these signals are transduced.

Using polystyrene replicas of microfabricated silicon wafers to provide precisely defined topographies, appressoria were induced in all the cereal rusts analysed (*Puccinia graminis* f. sp. *tritici, P. graminis* f. sp. *avenae, P. graminis* f. sp. *secalis, P. recondita* f. sp. *tritici, P. coronata* f. sp. *avenae* and *P. hordei*) by topographies with closely spaced (1.5 μm) ridges. Ridges of different heights (between 0.116 μm and 2.4 μm) were tested. The patterns of response to different ridge heights was not correlated to host nor rust species. Only two rusts, *P. graminis* f. sp. *secalis* and *P. recondita* f. sp. *tritici*, formed significant numbers of appressoria (up to 62% and 9% respectively) on ridges spaced 50 μm apart (= single ridges).

The formation of appressoria on host leaves was found to be very efficient, 92–95% of germlings encountering a stoma formed an appressorium over the stoma. On polystyrene host leaf replicas, however, the efficiency was much less, and varied significantly between rusts (4%–63%). Again, the variation was not correlated to host or rust species. The implication was that while topographical signals alone are sufficient to induce appressoria *in vitro*, topography of the host alone was not sufficient to explain the high efficiency of appressorium induction seen *in vivo*.

It was shown for *P. graminis* f. sp. *tritici* that the volatile compounds *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol, produced by the host plant as part of the lipoxygenase pathway, induced appressoria in the absence of topographical stimuli. Induction was dose dependent and optimal at 1 mM when applied to the fungus as a solution. Appressoria were also induced when the germlings were exposed to vapour from solutions of *trans*-2-hexen-1-ol. Induction was over a very narrow range of concentrations (1– 2 mM).

When the *trans*-2-hexen-1-ol and optimal topography (2.0 μm high ridges spaced 1.5 μm

apart) treatments were combined, there was a much more rapid induction of appressoria. After 4 h, 85% of germlings were induced with combined treatment, whilst only 7% of the germlings were induced by either individual treatments alone. There appeared to be a synergy between the two different signals.

Evidence for other potential stomatal recognition signals was also obtained. Nile red staining indicated that exposed cutin was only associated with the guard cell lips, and 1 mM ammonia hydroxide treatment revealed UV autofluorescence of the guard cell cuticle indicative of phenolic acids. However, the phenolic acids: ferulic, diferulic and *p*-coumaric acids, were found to have no biological activity, either on flat surfaces nor by increasing the response to topography.

Differences in the transduction mechanism of the topographical and *trans*-2-hexen-1-ol signals were found. Induction by topography, optimal at pH 7.2, was inhibited by low (6.2) external pH whilst *trans*-2-hexen-1-ol induction was not. Measurement of cytosolic pH by confocal ratio imaging of the ester loaded pH sensitive ratio dye SNARF-1, showed that the external pH did not affect cytosolic pH. Topographical induction was also inhibited by the calmodulin antagonists, calmidazolium, W-7, W-5 and TFP, whilst induction by *trans*-2-hexen-1-ol was not affected at the IC_{50} of each of these inhibitors for topographical induction. Topographical induction was also inhibited by RGD, an integrin binding antagonist, and D_2O, a microtubule stabilising agent. Again, *trans*-2-hexen-1-ol induction was unaffected at the IC_{50} of RGD or D_2O for topographical induction. Interpretation of results obtained with inhibitors with respect to topographical induction was difficult due to their effects on tip growth. Induction by *trans*-2-hexen-1-ol was specifically inhibited by silver ions (IC_{50} = 0.2 μM $AgNO_3$) and the protein kinase C inhibitor H-7, suggesting that the signal transduction of *trans*-2-hexen-1-ol may have similarities with the ethylene receptor found in plants and *Colletotrichum*.

Abbreviations

Standard SI (*International System of Units*) were used throughout this thesis. Non-SI units are as follows:

AM	acetoxymethyl ester
BHK	baby hamster kidney
CaM	calmodulin
cAMP	adenosine-3'.5'-cyclic monophosphate
cGMP	guanosine-3'.5'-cyclic monophosphate
d	days
DAG	diacyl glycerol
DAPI	4, 6-diamidino -2-phenylindole
dH_2O	distilled water
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dr	differentiation related
DS	differentiation specific
ECM	extracellular matrix
f. sp.	*forma specialis*
h	hour or hours
H-7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HEPES	n-[2-hydroxyethyl]piperazine -n'-[2-ethanesulphonic acid]
HMC	haustorial mother cell
HMDS	hexamethyldisilazene
IC_{100}	concentration producing 100% inhibition
IC_{50}	concentration producing 50% inhibition
IH	infection hypha
LOX	lipoxygenase
LSCM	laser scanning confocal microscope
LTSEM	low-temperature scanning electron microscopy
MAPKKK	mitogen activated kinase kinase kinase
MES	2-[n-morpholino] ethanesulphonic acid
min	minute or minutes

MS	mechanosensitive
nd	not determined
PIP_2	phosphotidylinosityl 4, 5-bisphosphate
Plan Apo	plan-apochromatic
RGD	arginine-glycine-aspartic acid
RGE	arginine-glycine-glutamic acid
RH	relative humidity
sec	second or seconds
SEM	scanning electron microscopy
sem	standard error of mean
SNARF–1-AM	5(6)-carboxyseminaphthorhodafluor-1
SSV	substomatal vesicle
TFP	trifluoperazine
UV	ultra-violet
v.	version
W-5	N-(6-aminohexyl)-1-naphthalenesulfonamide
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide
w/v	weight per volume

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1. REVIEW OF THE LITERATURE

1.1 General introduction

There are an estimated 4,000 species of rust fungi (Laundon, 1973) constituting the three families (Alexopoulus & Mims, 1979) in the order Uredinales; all are biotrophic parasites. As with many obligate biotrophs, adaptation to a single host species means that some rust species are classified sub-specifically. In the case of the cereal rusts the sub-specific taxon is termed *forma specialis* (f. sp.) and usually refers to the primary host of the rust (see Section 1.1.1). For example the primary host of *P. graminis* f. sp. *tritici* is wheat (*Triticum aestivum*) and for *P. graminis* f. sp. *avenae* it is oat (*Avena sativum*).

Most cultivated plants can suffer a rust disease, with the exceptions of the Cruciferae, Curcurbitaceae and Solanaceae. Due to the biotrophic nature of rusts, crop losses are mainly indirect and seldom significant. Only cereal crops, forest trees and some vegetable and flower crops suffer serious rust disease (Gjaerum *et al.*, 1988).

1.1.1 The rust life cycle

The life cycle of rust fungi is complex, involving up to 5 different spore types (in the macrocylic rusts) and two hosts (in heteroecious rusts) (Figure 1.1). Amongst the large number of species within the order Uredinales, there are several variations on the basic life cycle. Some species have only one host (autoecious) and others have fewer spore types (microcyclic).

The rust fungi derive their common name from the pustules of uredospores, which are for most species are a rusty-brown colour and are the main symptom of the disease.

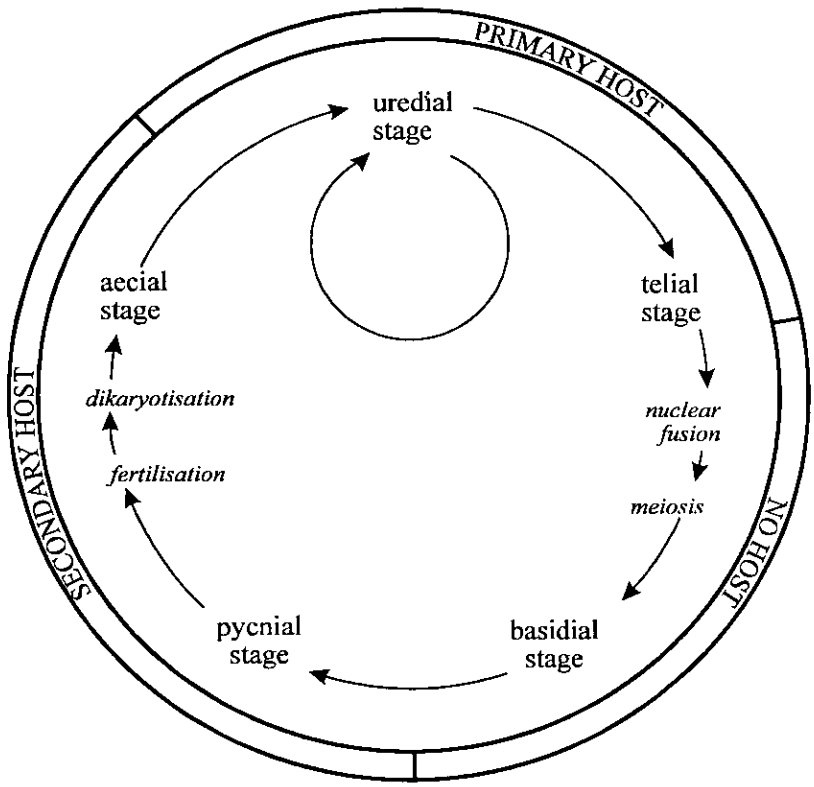

Figure 1.1 . Typical life cycle for a macrocyclic, heteroecious rust fungus

The uredospore is dikaryotic and the main propagative and dispersal spore of rust fungi. There is evidence that *P. graminis* uredospores have, at least three times this century, spread from East Africa to Australia (8,000 km) (Watson & de Sousa, 1983). These distances are exceptional though, more typical distances of dispersal are 100 km and occasionally up to 2000 km (Watson & de Sousa, 1983). The uredospore germinates, producing a germ tube which penetrates the primary host leaf through a stoma. If the infection is successful a uredospore producing pustule, a uredium, will develop. Uredia are comprised of uredospores on stalk-like projections which burst through the host epidermis. As the host matures the uredia stop producing uredospores and start to produce teliospores, which are also dikaryotic. When the pustule contains teliospores only it is termed a telium. Telia may also be produced directly by uredospore germlings infecting a mature host.

Teliospores remain in the straw over winter and the nuclei undergo karyogamy (nuclear fusion) to become diploid. In the spring teliospores, having been vernalised

(freeze/thawed) or stratified (heated/cooled), will germinate, undergo meiosis and form a four-celled basidium. Each cell in the basidium produces a single haploid basidiospore. Basidiospores may be considered the first spore in the sexual part of the rust life cycle. Basidiospores infect the secondary host (in heteroecious rusts) or the only host (in autoecious rusts) by directly penetrating the leaf cuticle. The basidiospore infection occurs only once in a season, unlike uredospore infection which can have many infection cycles in a single season.

A successful basidiospore infection will result in the formation of a pycnium, the site of sexual reproduction. This flask-shaped structure is haploid and produces minute, uninucleate pycniospores in a nectar like syrup. The syrup attracts insects which then disperse the pycniospores. The pycnium also produces branched, thin-walled hyphae termed flexuous hyphae. If a pycniospore is brought within close proximity to a flexuous hypha of opposite mating type, it will germinate producing a short germ tube. The germ tube anastomoses with the flexuous hypha and the nucleus of the pycniospore migrates into the flexuous hypha. The nucleus then multiplies and migrates throughout the fungal tissue, resulting in the dikaryotisation of the pycnium. The now binucleate hyphae of the pycnium grow through the host tissue and give rise to another spore-producing structure, an aecium, generally on the under side of the leaf. If the pycnia are not fertilised they do not develop further.

The aecium consists of a peridial wall surrounding chains of aeciospores. The aeciospores are forcibly released by rounding-off of the flattened interface between adjacent cells. Once the aeciospore is released it infects the primary host producing a uredium. The aeciospores, like uredospores, infect through stomata, and represents the last spore stage in the sexual cycle. The whole sexual cycle (basidium-pycnium-aecium) is fairly localised, usually not spreading more than several hundred metres or so.

For microcyclic rusts which do not have the pycnial stage, such as *P. striiformis*, there is evidence that exchange of genetic material can occur during the uredial stage.

Uredial hyphae of different genetic makeup can anastomose within the host leaf and exchange genetic material (Stubbs *et al.*, 1986). Such somatic hybridisation is also present in macrocyclic rusts and also occurs between *formae speciales* (Watson & Luig, 1959). The exchange of genes between *formae speciales* is of particular concern to plant breeders that have incorporated resistance genes from one cereal species to another.

The uredial stage can be considered the most important in the rust life-cycle. Uredospores are the primary means of propagation, dispersal and yield loss occurs as a direct result of the uredial stage. In many of the cereal rusts uredospores are also a major means of over wintering and there is some evidence that they may also be involved in gene transfer between races, which seems to be the single role of the sexual stage of the rust life cycle.

1.1.2 Cereal rust diseases

1.1.2.1 *Puccinia graminis* Pers. f. sp. *tritici*: Wheat stem rust

P. graminis f. sp. *tritici* has a global distribution. The fungus attacks all the above ground parts of the plant, except the seeds. The net result is loss of foliage, poor root development, reduced tillering, reduced number of seeds per head and the kernels that are produced are small and of poor food quality. More than 1 million tonnes of wheat are lost annually in North America because of this disease. In epidemic years the losses can be tens or hundreds of millions of tonnes. In other parts of the wheat growing world losses are at least as severe, and generally more severe, especially in developing countries (Agrios, 1988). In some parts of Europe and European USSR losses average out to be 10% of the annual crop (Gjaerum *et al.*, 1988; Parry, 1990). In the UK though, the cooler climate makes this disease less significant, except for the occasional epidemic in the south east.

The fungus is a heteroecious, macrocyclic rust (Figure 1.1) infecting leaves, leaf sheaths and stems and predominately infects *Triticum aestivum* (bread wheat) and *T.*

turgidum (durum wheat). Other primary hosts include Triticale and barley. The main alternate host is *Berberis vulgare* (barberry) which is an thorny bush. The wood of barberry makes good tool handles, extract from the bark is a dye and the fruit can be used to make jams. As a result of its versatility, barberry was introduced to much of the US wheat growing areas as hedging. This unfortunate situation, where the primary and secondary hosts were planted together, resulted in the creation of new combinations of genes for virulence and aggression. Since then, eradication of barberry has led to a reduction in stem rust epidemics in North America and Europe (Roelfs *et al.*, 1992).

The yellowish-brown uredia (2 × 5 mm) are produced 14 days after successful infection and are often surrounded by split epidermis. The uredia of *P. graminis* f. sp. *tritici* produce approximately 10,000 uredospores per day; *P. recondita* f. sp. *tritici* (wheat leaf rust, see Section 1.1.2.4) uredia only produce approximately 3,000 uredospores per day. Uredospores of *P. graminis* f. sp. *tritici* also survive longer than those of *P. recondita* f. sp. *tritici* because they are better protected on the sheaths and stems than on the leaves. However, the infection rate is much lower for *P. graminis* f. sp. *tritici* than for *P. recondita* f. sp. *tritici* (10% compared to 33%) (Roelfs *et al.*, 1992). Uredospores of *P. graminis* f. sp. *tritici*. are echinulate, ovoid (21–42 μm x 16–22 μm) and have 4 germ pores arranged equatorially. Over-wintering in the uredial stage is possible but the fungus is relatively thermophilic; inhibition of the pathogen occurs below 15°C (Parry, 1990) and asexual over-wintering has not been recorded north of the Alps (Gjaerum *et al.*, 1988). The high temperature requirements of the fungus means that the disease occurs late in the season.

As the host matures, teliospores are formed. The telia are black producing chestnut brown teliospores (35–60 μm x 12-22 μm). Teliospores are the more common spore type to over-winter. Once germinated in early spring, the teliospore produces four hyaline basidiospores which infect the young *Berberis* leaves. The aecia and aeciospores are bright orange on the abaxial face of the *Berberis* leaf, but have never been isolated in Britain. The disease in the UK arises through uredospores spreading

from Western Europe; the pattern of races in the UK follows closely that in Europe (Wilson & Henderson, 1966).

1.1.2.2 *Puccinia graminis* f. sp. *secalis*: Rye stem rust

Globally rye stem rust is not an important disease, although in 1982 there was a severe epidemic in Brazil (Roelfs, 1985a). The main reasons given for its lack of importance world-wide are that rye is cross pollinated ensuring a diversity for resistance and rye is grown in only limited areas (Roelfs, 1985a). Also, rye leaves normally senesce at an earlier growth stage than other cereals, so infection needs to be severe early in the season to have much effect on yield (Roelfs, 1985b).

The main interest in rye stem rust is due to its close taxonomic relationship with wheat stem rust and their putative hybrids. Parasexual recombination occurs between wheat and rye stem rusts (Watson & Luig, 1959) and transfer of rye genes into wheat and the use of Triticale as a crop has renewed interest in rye stem rust (Roelfs, 1985a).

1.1.2.3 *Puccinia graminis* f. sp. *avenae*: Oat stem rust

Oat stem rust occurs almost everywhere oats are cultivated and has periodically caused severe crop losses (Martens, 1985). In 1977, 35% of the oat crop (385,00 tonnes) in Manitoba was lost (Martens, 1978).

Late onset of the disease is probably the reason why there are few epidemics of oat stem rust (Roelfs *et al.*, 1993) since most cultivars are susceptible to most races of *P. graminis* f. sp. *avenae* (Roelfs, 1993).

1.1.2.4 *Puccinia recondita* Rob. ex Desm f. sp. *tritici*: Wheat leaf rust

P. recondita f. sp. *tritici* is found throughout the wheat growing world and may be considered the most widespread of the wheat rust diseases and arguably the most significant rust of wheat in the world (Gjaerum *et al.*, 1988). The disease is confined

to the leaf; the stem is rarely infected. Under certain conditions pustules may appear on awns, glumes and leaf sheaths. The effect on the plant is to reduce kernel number and size (Stubbs *et al.*, 1986) reducing yield by 5–10%, although loss can be as high as 40% in epidemic years (Gjaerum *et al.*, 1988). The fungus has a temperature optimum of 15–25°C, and disease occurs from middle to late in the season.

Puccinia recondita f. sp. *tritici* is a heteroecious, macro-cyclic rust with five spore stages (Figure 1.1). The main primary host is *Triticum aestivum*, although *T. turgidum* can be more important where it is grown more extensively (e.g. Mediterranean, Middle East and India). Leaf rust is not important on *T. monococcum* and *T. dicoccum*, but is considered a threat to Triticale. Secondary hosts, on which sexual reproduction occurs, include *Clematis* spp. (including *C. vitalba*, or old man's beard) and *Thalictrum* spp. (the meadow rues).

1.1.2.5 *Puccinia coronata* Cda. f. sp. *avenae* F. et L.: Oat crown rust

This rust derives its common and specific name from the wall protuberances from the apex of the teliospores, giving a crown-like appearance to the spore. *Puccinia coronata* f. sp. *avenae* is a heteroecious macrocyclic rust (Figure 1.1).

The primary hosts for *P. coronata* f. sp. *avenae* are oats and several other grass species. The secondary hosts include *Rhamnus cartharticus* (buckthorn) and *Frangula alnus* (alder buckthorn).

Crown rust has a world-wide distribution and is probably the most significant pathogen of oats. Losses in the US can be up to 20%, although in the UK the disease is limited to occasional outbreaks in the south-west (Parry, 1990). The disease is generally seen on adult plants late in the season with uredia primarily on leaf blades but also on leaf sheaths, floral parts and occasionally on culms. Yield loss is through reduction of grain number and weight.

1.1.2.6 *Puccinia hordei* Otth.: Barley brown rust

P. hordei is found wherever barley is grown and is considered the most important disease of barley (Gjaerum *et al.*, 1988). The significance of the disease in the last 10–15 years in North West Europe has increased considerably due to the intensification of barley cultivation (Gjaerum *et al.*, 1988). In 1970 and 1971 there were epidemics of barley brown rust in the UK on spring barley (Clifford, 1985). In 1989 and 1990 severe infections of winter barley occurred in England and Wales (Polley & Slough, 1992).

Yield loss is primarily through reduction in grain size and number and can be as much as 20% (Gjaerum *et al.*, 1988). The sexual stage on the secondary hosts (*Ornithogalum*, *Leopolia* and *Dipcadi* species) is rare in the UK and considered epidemiologically insignificant in Europe, but essential for survival in Israel (Clifford, 1985). Over-wintering occurs on volunteer plants or autumn-sown crops.

1.2 The infection process

The infection processes of rust fungi have been most closely studied for the uredial stage of the disease. Infection during this stage of the life cycle typically occurs through stomata and requires differentiation of the germ tube into a series of specialised infection structures. This section shall describe the separate stages of the infection process prior to penetration of the host mesophyll cells, starting with adhesion of the uredospore to the leaf and concluding with the formation of the haustorial mother cell.

1.2.1 Germination and pre-penetration growth

Most rust fungi are spread via airborne uredospores which, once deposited by either sedimentation, impaction or rain scrubbing (Roelfs, 1985b), adhere to the surface initially through hydrophobic interactions (Clement *et al.*, 1993a) and secondly, via a

localised accumulation of water. This localised accumulation of water is thought to be a passive effect brought about by capillary action (Clement *et al.*, 1994). Thirdly, extracellular matrix (ECM) accumulates between spore and substratum. Similar accumulation of ECM has also been observed by conidia of *Erisyphe graminis* (Kunoh *et al.*, 1988). In *Uromyces viciae-fabae* the ECM forms a pad ('adhesion pad') below the uredospore which has been shown to contain esterases and a cutinase (Deising *et al.*, 1992). It has been proposed that these enzymes embedded in the ECM modify the properties of the surface and facilitate adhesion (Nicholson & Epstein, 1991).

Prior to germ tube emergence, low molecular weight carbohydrates and some high molecular weight peptides are released, probably as a result of lysis of the germ pore plug (Clement *et al.*, 1993b). Germination is influenced by temperature, moisture and light. For *P. graminis*, high intensity blue and far-red light inhibits uredospore germination. Light inhibition is thought to synchronise infection with environmentally optimum conditions (Knights & Lucas, 1980). It has been shown that nonanol, a volatile present in uredospores, promotes germination (French, 1992). Nonanol is also found in the volatile compounds associated with wheat leaves (Hamilton-Kemp & Anderson, 1984) and may act in host recognition.

Upon germination the germ tube usually emerges from the uredospore via the germ pore closest to the surface. It has been suggested that this is a contact-mediated response (Read *et al.*, 1992). Emergence of the germ tube correlates with a release into the extracellular medium of high molecular weight polymers including proteins, uronic acid and carbohydrate and a drop in pH of the medium (Clement *et al.*, 1993b). Polypeptides released show phosphatase, esterase, β-glucosidase and protease activity (Clement *et al.*, 1993b). The pH optima for these enzymes was predominately neutral to slightly alkaline; leaf leachates tend to be alkaline (Tukey, 1971).

Once emerged from the spore, the germ tube adheres tightly to the surface with

localised secretions of ECM. The ECM is essential for the induction of appressoria and contains proteins and glycoproteins (Moloshok *et al.*, 1994). Treatment of germlings with pronase-E disrupts normal adhesion (Epstein *et al.*, 1987). It has been observed that the cell wall thins where there are secretions of the ECM (Littlefield & Heath, 1979) although this observation has been disputed (Kwon *et al.*, 1991b). If the hyphal wall does thin at the points of contact with the substratum, it may have significance in the mechanism of contact sensing because a thinner cell wall may be more easily deformed (see Section 1.3.1). The ECM preferentially adheres to hydrophobic surfaces such as epicuticular wax; germ tubes on hydrophilic surfaces (e.g. agar) tend to be aerial (Wynn & Staples, 1981).

Johnson (1934) was the first to report that rust germlings show directional growth. In 1969, Dickinson recognised the directional growth of cereal rusts as a contact-mediated cell response. It has also been suggested pH gradients in the host epidermal cell wall may be important in orientating the growth of *U. viciae-fabae* germ tubes towards stomata (Edwards & Bowling, 1986). For *P. graminis* f. sp. *tritici* it has further been suggested that the wax crystal microstructure may induce directional growth of germ tubes (Lewis & Day, 1972).

On artificial substrata, rust directional growth has been shown to be a response to ridges in the substratum; the ridges are thought to be mimicking epidermal cell junctions. It was found that the wider the ridge spacings were, the greater the 'wandering' of the germ tube (Hoch *et al.*, 1987b). When a germ tube encounters a ridge or groove, it will reorientate its direction of growth to subsequently grow at 90° to the ridge or groove. Epidermal cells of dicotyledonous plants are irregular in shapes so that even if a germling leaves a cell junction at 90°, there is no way of predicting the orientation of the next cell junction to be encountered. This means that because of the directional response to the irregular orientation of the epidermal cell junctions, the germlings effectively wander randomly across the dicotyledonous epidermal cells. However, the cell junctions of the companion cells associated with the guard cells encircle the stomatal aperture (Figure 1.2), and once a cell junction is

encountered, the germling will be directed to the centre of the circle (i.e. the stomatal aperture). The directional growth increases the chance of locating a stoma by effectively increasing the size of the target from the stoma to the companion cells.

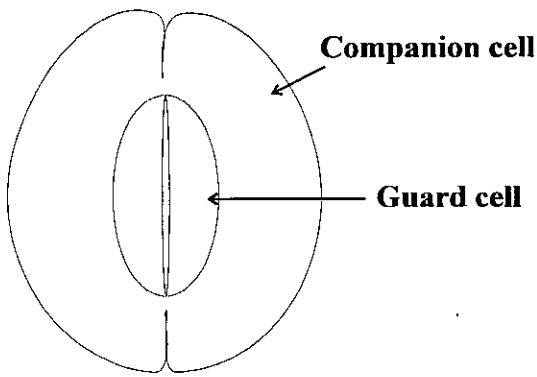

Figure 1.2. Diagram of the stomatal complex of *Phaseolus vulgaris* (French bean), illustrating how the companion cells encircle the stomatal aperture.

For cereal rusts the benefit of directional growth is less obvious. It has been suggested that the arrangement of the stomata on a cereal leaf in staggered parallel rows means that this mode of growth increases the probability of any one germ tube locating a stoma. It may also reduce the distance which a germ tube has to grow prior to locating a stoma. For *P. hordei* it has been shown that the distance of germ tube growth on the leaf surface is negatively correlated to the final fungal colony size (Niks, 1990). The interpretation was that the more energy the fungus uses to find a stoma, the less it had to produce an aggressive infection.

The ability of cells to sense and respond to topographical features of the substratum on which they are growing (thigmotropism) is not limited to rust fungi. Observations have been made of growth along grooves in the substrata for *Candida albicans* (Sherwood *et al.*, 1992; Gow, 1994), *Cochliobolus sativus* (Clay *et al.*, 1994) and the clover pathogen *Cymadothea trifolii* (Roderick, 1993). The migration of animal cells during embryogenesis is guided by the orientation of fibrils in the extracellular matrix (Dunn, 1991). Animal BHK cells are able to distinguish single grooves greater than 1 μm or 0.1 μm deep multiple grooves spaced 260 nm apart (Clark *et al.*, 1987). Pollen tubes of *Lilium longiflorum* have been shown to orientate their growth when growing across a woven mesh (Hirouchi & Souda, 1975). In many of these cases however, it is unclear whether the observations are of an active response to a physical stimulus (i.e. genuine thigmotropism) or merely passive growth along the

path of least physical resistance.

1.2.2 Stomatal penetration

For most rusts, uredospore germlings penetrate the host through stomata. Once the stoma is encountered the fungus differentiates a series of infection structures. These follow the sequence: appressorium, penetration peg, sub-stomatal vesicle, infection hyphae, haustorial mother cell and finally the haustorium (Figure 1.3).

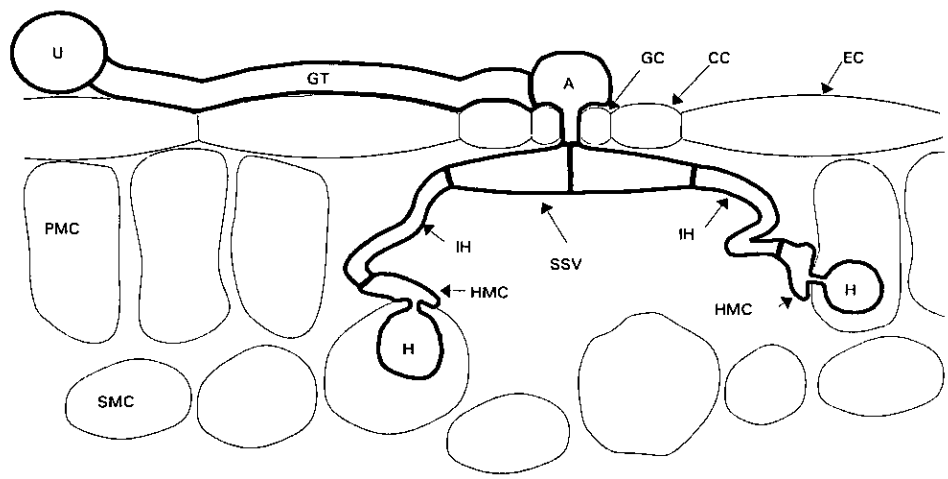

Figure 1.3. Diagram of typical infection structures of a cereal rust. **A**, appressorium; **CC**, companion cell; **EC**, epidermal cell; **GC**, guard cell; **GT**, germ tube; **H**, haustorium; **HMC**, haustorial mother cell; **IH**, infection hypha; **PMC**, palisade mesophyll cell; **SMC**, spongy mesophyll cell; **SSV**, sub-stomatal vesicle; **U**, uredospore.

It was first observed in 1949 that certain rusts form infection structures in response to irregular substrata (Dickinson, 1949b). Several other fungal pathogens are thought to form appressoria in response to mechanical stimuli: *Magnaporthe grisea* (Bourett & Howard, 1990); *Colletotrichum* spp. (Staples & Macko, 1980; Lapp & Skoropad, 1978) and *Metarhizium anisopliae* (St Leger *et al.*, 1989). However these pathogens rely on non-topographic signals (Read *et al.* 1992); rusts rely on both topographic and non-topographic signals from the host.

Early work on topographical sensing in rust fungi involved germinating uredospores on various substrata with uneven topographies. Substrata used included oil-collodion membranes (Heath, 1989), stretched nitro-cellulose, rubber and gelatine membranes (Dickinson, 1969), polystyrene leaf replicas (Wynn, 1976), and scratched surfaces such as glass, polystyrene and polyethylene (Staples *et al.*, 1983b). Although these substrata have provided much information on topographical sensing, there is no way to control the precise dimensions of the topographic features. Precisely defined topographies can be obtained using techniques employed in the electronics industry, in the form of microfabricated silicon wafers. Polystyrene replicas of these wafers provide an inert substratum with a precisely defined, transparent topography on which rusts can be grown and studied. Most work addressing appressorium formation in the rust fungi has been done on the bean rust, *Uromyces appendiculatus*.

Using polystyrene leaf replicas of the host, it has been shown for *U. appendiculatus* that *in vitro*, leaf topography alone is sufficient to induce the percentage appressorium formation observed *in vivo* (Wynn, 1976). The precise dimensions of inductive topographic signals for *U. appendiculatus* were calculated by growing the fungus on polystyrene replicas of microfabricated silicon wafers. It was shown that *U. appendiculatus* optimally responded to ridges (or grooves) 0.5 μm high. The differentiation was reduced from 75% on 0.5 μm ridges to 5% on 1.0 or 0.25 μm high single ridges. Furthermore, it appeared that only two changes in angle were required, i.e. a 0.5 μm step-up or a 0.5 μm step-down (Hoch *et al.*, 1987b). These dimensions correspond to those of the bean guard cell lip (Hoch *et al.*, 1987b) and ledge (Terhune *et al.*, 1991); either of which may be the inductive signal *in vivo*. Glass with 0.5 μm high ridges was silanised using various organic silanes, producing substrata with a range of hydrophobicities and it was found that perception of the inductive topographic feature was dependent on the hydrophobicity of the substratum. It was found that the more hydrophobic the substratum, the more appressoria were formed (Terhune & Hoch, 1993).

The time course of events for appressorium formation in *U. appendiculatus* were

studied in detail by Kwon and Hoch (1991a) (Table 1.1). The initial response was the cessation of polarised growth of the germ tube. This occurred within 4–6 min of the apex reaching the leading edge of the inductive ridge. Ten min later the hyphal tip began to swell and the two nuclei started to migrate towards the apex. 15 min after induction, 15 differentiation-related (dr) proteins were synthesised. These proteins were not present in germlings until after they had been induced to differentiate. One such dr-protein is cellulase (Heiler *et al.*, 1993). There was also a downshift in the synthesis of other proteins after induction. Northern blot analysis has shown that there are at least 5 genes, the differentiation specific (DS) genes, dedicated to the differentiation process (Staples & Hoch, 1988). The functions of these genes and proteins have not been elucidated.

Table 1.1, Summary of cellular events initiated when *U. appendiculatus* germling tip encounters an inductive ridge (adapted from Kwon & Hoch, 1991a).

Time after encountering inductive ridge (min)	Cell event
0	contact with ridge
4	tip growth stops
10	apex swells; nuclei migrate toward tip
30	both nuclei enter tip
45	nuclear division and septation initiated
55	mitosis
60	septation complete; DNA synthesis initiated
120	DNA synthesis complete; penetration peg initiated

Approximately 50 min after reaching the ridge, the nuclei entered the terminal swelling and mitosis and septation were initiated. One hour after encountering theinductive ridge, the appressorium was complete, containing four nuclei and separated from the rest of the germ tube by a septum. After two hours DNA replication had occurred and a penetration peg was initiated (Kwon & Hoch, 1991a).

Employing the same technique used to define the dimensions of the inductive topographic signal for *U. appendiculatus*, Allen *et al.* (1991a) analysed appressorium topographic signals inductive for appressorium formation in 27 rust fungi. The responses to the single ridges of different heights were grouped into four categories (Figure 1.4).

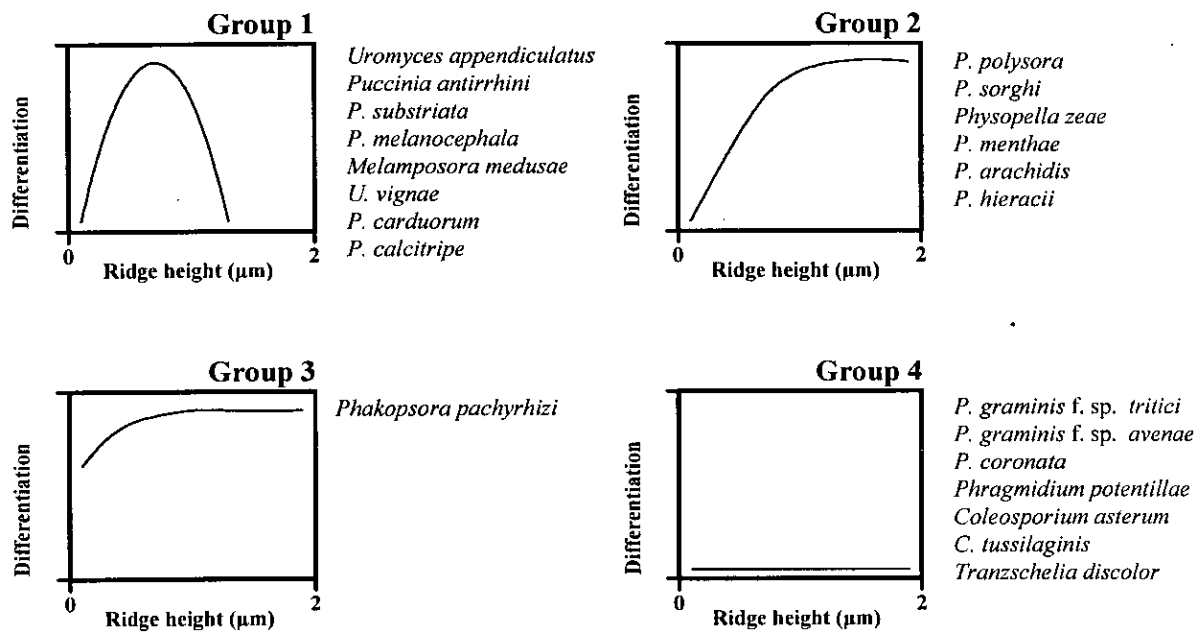

Figure 1.4. Patterns of appressorium differentiation by rusts in response to microfabricated substrata with single ridges of varying height (adapted from Allen *et al.*, 1991a).

Most of the rusts fell into group 1 and optimal differentiation was on topographies with single ridges within a specific height range, topographies with ridges higher or lower than these heights were less inductive (Figure 1.4). *Uromyces appendiculatus* was in this group. Group 2 contained rusts which differentiated on topographies with ridges over a minimum height and had high differentiation even on the highest ridges tested (2.24 µm). The soybean rust *Phakopsora pachyrhizi* (Figure 1.4, Group 3) formed a high number of appressoria on flat surfaces but preferentially in association with ridges. *Phakopsora pachyrhizi* and *Physopella zeae* (Figure 1.4, Group 2) are rusts which penetrate the cuticle directly, not via stomata. Their contact-sensing ability is reflected in the fact that *in vivo* their appressoria are formed predominately

over anticlinal walls of epidermal cells (Allen *et al.*, 1991a). Rusts in Group 4 showed no differentiation on any topography with single ridges tested. Three cereal rusts were in this group: *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *avenae* and *P. coronata.* A fourth cereal rust, *P. recondita*, formed a low percentage of appressoria on single ridges, optimally 0.5 μm high. This low induction suggests that a single ridge may not be the optimum inductive signal. Subsequently, it has been shown that *P. hordei* (not tested by Allen *et al*, 1991c) and *P. graminis* f. sp. *tritici* are induced to form appressoria by multiple closely spaced ridges, optimally 1.5 μm spaced (Read *et al.,* 1996). As well as the specific spacing there is also an optimum height (2.0 μm) for the multiple ridges to be maximally inductive (Read *et al.,* 1996).

There is evidence that for *P. graminis* f. sp. *tritici* the signal for appressorium induction is, at least in part, provided by the chemistry of the wheat leaf. A number of volatile products of the lipoxygenase pathway of plants, particularly *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol, have been shown to induce appressoria in *P. graminis* f. sp. *tritici* in the presence of a crude leaf extract, sucrose, casein and a number of salts (Grambow, 1977; Grambow & Reidel, 1977). These volatile alcohols are, in part, responsible for the 'green odour' of cut grass and are derivatives of the lipoxygenase pathway in plants.

Induction of appressoria by *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol in *P. graminis* f. sp. *tritici* was dependent on the presence of leaf extract (Grambow, 1977; Grambow & Reidel, 1977). Components of leaf extract found to be inductive in conjunction with *trans*-2-hexen-1-ol were a number of unidentified phenolic compounds which co-localised with ferulic and *p*-coumaric acid on 2-D thin layer chromatography (Grambow & Grambow, 1978). Ferulic acid and *p*-coumaric acid had no biological activity though (Grambow & Grambow, 1978). The lignan nordihydroguaretic acid was found to be highly inductive even in the absence of the volatile compounds. However, it is not present in the wheat leaf (Grambow, 1978).

The role of host chemical signals in induction of infection structures has been studied

in a number of other host-pathogen systems. The genus *Colletotrichum* contains a large number of epiphytic plant pathogens which form infection structures in response to a wide variety of host chemical signals including sucrose (Grover, 1971), chlorogenic acid (Swinburne, 1976; Harper & Swinburne, 1979), phenolics (Parberry & Blakeman, 1978) and wax fatty acids (Podila *et al.*, 1993). *Colletotrichum gloeosporioides* synchronises infection with host ripening by utilising ethylene, the gaseous ripening hormone of the host, as a trigger for appressorium induction (Flaishman & Kolattukudy, 1994).

In the rice blast fungus *Magnaporthe grisea*, polar wax fractions (i.e. wax esters, aldehydes and alcohols) promoted appressorium differentiation, while non-polar fractions (i.e. alkanes) did not (Uchiyama & Okuyama, 1990). Since the inductive fractions are also very hydrophobic, it is not clear as to whether the fraction is acting as a physical signal and/or chemical signal.

Colletotrichum and *Magnaporthe* penetrate the host directly through the epidermis and so do not require the signal which initiates infection to be unique to a particular part of the leaf. Rusts penetrate through the stomatal aperture and therefore require the signal which induces appressorium formation to be unique to stomata. Therefore, the inductive chemical must only be present or at an active concentration over the stomatal aperture.

1.2.3 Post-appressorial infection structures

After the penetration peg has penetrated the leaf, a sub-stomatal vesicle (SSV) forms in the sub-stomatal cavity. Prior to the onset of SSV development, synthesis of two new proteins has been demonstrated in *P. graminis* f. sp. *tritici* (Wanner *et al.*, 1985). There is also a round of mitosis in the vesicle (Heath & Heath, 1978). Stomatal penetration in *P. graminis* f. sp. *tritici*, but not *P. recondita* f. sp. *tritici*, is inhibited by light and also by high CO_2 levels independent of light (Yirgou and Caldwell,

1968).

Infection hyphae develop from the SSV. In some species (e.g. *P. coronata* and *P. hordei*) there are two infection hyphae while other species (e.g. *U. appendiculatus* and *P. recondita*) produce a single infection hypha. When the tip of an infection hypha comes into contact with a host cell, it stops elongating and differentiates a haustorial mother cell (HMC) (Wynn & Staples, 1981). The HMC adheres to the host cell wall prior to penetration and haustorium formation. Formation of a HMC seems to require a second signal from the host since HMC differentiation is only observed *in vitro* in a few rust species (Deising *et al.*, 1991; Heath & Perumalla, 1988).

There is some evidence that the adherence of HMC is specific to the appropriate host tissues and may be a factor in determining host-specificity (Mendgen & Deising, 1993).

HMCs can be induced to form haustoria *in vitro* in *U. vignae* by addition of a complex mixture of carbohydrates (Heath, 1990) or an inactivated, fungal enzyme extract (Heath, 1989).

In the cereal rusts, the compatibility of the fungus/host interaction is determined after the haustorium has entered the host cells. There is a gene-for-gene relationship between resistance and avirulence, with avirulence genes being dominant in the fungus and resistance genes being dominant in the host (Rohringer *et al.*, 1979). An incompatible reaction results in early cessation of hyphal growth associated with the rapid collapse of the infected cell and accumulation of lignin (or lignin-like compounds) and callose (Ogle & Brown, 1971).

1.3 Transduction of the appressorium inductive signal

The mechanism(s) by which the rust germ tube senses changes in the surface topography of the substratum is (are) not fully understood. In the following section the current views on topographical sensing mechanisms are analysed. In animal cells, mechanical signals are transduced via the cytoskeleton (Ingber, 1991) and/or Ca^{2+} mechanosensitive channels in the plasma membrane (Sachs, 1989). The evidence for the involvement of these two mechanisms in induction of rust appressoria is discussed. What is known about the transduction of ethylene signals in plants and fungi will be described as it may provide a model for transduction of the volatile signals, *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol which induce appressoria in *P. graminis* f. sp. *tritici* (Grambow, 1977; Grambow & Reidel, 1977). Finally, other intracellular signals involved in signal transduction are described and their possible roles in appressorium induction discussed.

1.3.1 The cytoskeleton

The fungal cytoskeleton has a well established role in: vesicle transport; mitosis and meiosis; organelle and cytoplasmic motility; and also cytokinesis and septation (for review see Heath, 1995). With the exception of meiosis, appressorium formation involves nearly all of these processes. The apolar arrangement of the cytoskeleton towards the fungus-substratum interface (Staples & Hoch, 1988), and the observation that only the underside of the germling is sensitive to mechanical perturbation (Correa & Hoch, 1995) suggests that the cytoskeleton may be involved in topographical sensing because its components are concentrated at the site of topographic sensing. It has been proposed that a transient depolymerisation of the cytoskeleton is required for appressorium differentiation (Hoch & Staples, 1985).

Using laser scanning confocal microscopy (LSCM) a detailed study of the cytoskeleton in germ tube apices of *U. appendiculatus* during appressorium formation has been made (Kwon *et al.,* 1991; Kwon & Hoch, 1991b). It was observed that the cytoskeleton abruptly ceased where the plasma membrane was

indented by a ridge. However, it was also pointed out that the apparent termination of the cytoskeleton may have resulted from the microtubules bending over the ridge and out of the plane of focus. When grown on mylar (Bourett *et al.*, 1987) or polycarbonate (Kwon *et al.*, 1991, Kwon & Hoch, 1991b), distinct invaginations in the cell wall are seen as the germ tube grows over ridges in the substratum. However, recent work has not shown these distinct invaginations of the plasma membrane of germlings which had differentiated over stomata, suggesting that either the invaginations are transient or the cytoskeleton is not disrupted by the *in vivo* topography (Terhune *et al.*, 1993). It is also possible that the mechanical signal is transduced via stress and/or strain on the intact cytoskeleton (Wang *et al*, 1993; Forgacs, 1995), rather than disruption of the cytoskeleton.

There is evidence for the involvement of the cytoskeleton in topographical induction of appressoria from experiments using drugs which interfere with normal cytoskeletal functioning. It was demonstrated that cytochalasin E, an F-actin depolymerising drug, could induce germling nuclear division, one stage in appressorium differentiation (Staples & Hoch, 1982). However, induction of nuclear division only occurred after prolonged exposure to the inhibitor (Tucker *et al.*, 1986). Transient disruption of F-actin resulted in a swelling of the germ tube apex, but nuclear division or septation was not initiated (Tucker *et al.*, 1986).

Appressoria can be induced on non-inductive topographies by certain ions and metabolites added exogenously (Kaminskyj & Day, 1984a; Kaminskyj & Day, 1984b) and also by 30°C heat treatment (Wanner *et al.* 1985). Stabilisation of the microtubule cytoskeleton by drugs such as D_2O and taxol inhibits formation of appressoria in response to K^+ and heat shock suggesting that disruption of microtubules is required for appressorium formation. However, induction by a topographical stimulus cannot be prevented by microtubule stabilising drugs (Hoch *et al.*, 1986). It is possible that the stabilisation of the microtubules was not adequate to resist the force exerted by a relatively immovable ridge and so disruption of the microtubules was still possible. However, appressorium formation on inductive

substrata in this rust is reduced by treatment with microtubule destabilising drugs (griseofulvin, nocodazole and vincristine sulphate). The published evidence suggests that appressorium formation induced by topography requires an intact microtubule cytoskeleton, whilst induction by heat-shock and K^+ requires the microtubule to be disrupted.

More circumstantial evidence put forward for the involvement of microtubules in topographical sensing is that heat-shocked germlings (30°C for 40 min) will differentiate (Wanner *et al.*; 1985); heat shock is known to depolymerise microtubules in animal cells (Turi *et al.*, 1981).

Since the cytoskeleton has a pivotal role in orchestrating the differentiation process itself, it is possible that many of the results from pharmacological experiments are due to inhibition of appressorium formation rather than initiation. It is possible that in these experiments the topographic signal is detected but the fungus cannot effect a response because the cytoskeleton has been disrupted.

1.3.2 Integrins

Work with animal and plant cells suggest that their ECM attachments are the sites at which the forces are transmitted (Wang *et al.*, 1993). In animal cells the cytoskeleton is linked to the ECM via trans-membrane ECM receptors, such as members of the integrin family of proteins (Wang *et al.*, 1993). There is evidence that integrins are involved in the transduction of mechanical signals across the plasma membrane (Ingber, 1991).

Intracellular signalling via integrin-like proteins has been widely studied in animal systems (Hynes, 1992) and has been observed in the alga *Chara* (Staves & Wayne, 1993) and the higher plant *Glycine max* (Shindler *et al.*, 1989). Integrins are a family of heterodimer, transmembrane proteins which bind to fibronectin, vitronectin and other related extracellular matrix proteins, typically containing the arginine-glycine-

aspartic acid (RGD) motif. Oligopeptides containing the RGD sequence of amino acids are used experimentally to inhibit integrin-mediated signalling. The cytoplasmic domain of integrins are generally bound to actin microfilaments via a range of proteins including vinculin, talin, paxillin and tensin (Schwartz, 1992). There is evidence that integrins are involved in the modulation of tyrosine kinase activity; integrin activation can result in an increase in cytosolic free Ca^{2+} and cytosolic pH; and it has been proposed that integrin can stimulate phosphatidylinositol bisphosphate (PIP_2) synthesis (Hynes, 1992; Schwartz, 1992).

Integrin proteins have been antigenically identified in the yeast *Candida albicans* (Marcantonio & Hynes, 1988; Santoni *et al.*, 1994) and oomycete *Saprolegnia ferax* (Kaminskyj & Heath, 1995). In *Candida*, integrins are thought to bind to fibronectin molecules in the host basal membrane and thus aid in pathogenesis (Klotz & Smith, 1991). In *Saprolegnia*, integrins are located in a tip-high gradient and are thought to tether the plasma membrane to the cell wall (Kaminskyj & Heath, 1995). It may be significant that the receptor for the topographical signal in rusts has been shown to be located in the tip-most 6 μm of the germ tube (Kwon *et al.*, 1991b) and topographical sensing ability is lost from parts of the germ tube 40 μm from of the apex (Correa & Hoch, 1995).

1.3.3 Calcium mechanosensitive channels

Calcium has been implicated in the mediation of a large number of differentiation events in fungi (see review by Gadd, 1995). In *Uromyces* appressoria can be induced by exogenous Ca^{2+} (Hoch *et al.*, 1987a; Read, N. D. & Taylor, K. unpubl.). In *P. graminis* f. sp. *tritici* topographically induced appressoria were inhibited by the inorganic Ca^{2+} channel blockers neodymium, lanthanum and gadolinium (C. Bauch, S. Horne, B. Meyer, S. Ruffert, T. J. Collins, B. Moerschbacher, N. D. Read, unpubl.). Gadolinium ions also inhibit appressorium formation in response to a contact stimulus in the entomopathogenic fungus *Metarhizium anisopliae* (St Leger *et al.*, 1991). Gadolinium has been shown to inhibit MS channel activity in a number

of organisms (Naruse & Sokabe, 1993; Quasthoff, 1994; Cui *et al.*, 1995). Mechanosensitive channels which pass Ca^{2+} have been shown to be present in *Uromyces* protoplasts by patch clamp analysis and the activity of the channels is inhibited by gadolinium (Zhou *et al.*, 1991). There is some controversy over where the channels are located, in the plasma membrane of hyphal tip or in the tonoplast of the vacuole (Garrill *et al.*, 1992b). Ca^{2+}-MS channels have also been found in the non-topographic sensing yeast (Gustin *et al.*, 1988; Garrill *et al.*, 1992a) and oomycete *Saprolegnia ferax* (Levina *et al.* 1994; Garrill *et al.*, 1993) and have been implicated in polarised hyphal tip growth (Wessels, 1990). It may be argued though, that this provides a basis for the evolution of the sensing mechanism by modification of the existing tip growth mechanism.

Read *et al.* (1992) suggest that adhesion of the germ tube to the substratum, essential for the detection of the signal (Epstein *et al.*, 1987), allows localised stress at the cell-substratum interface which may activate Ca^{2+} MS channels in the plasma membrane. The activated channels will allow an influx of Ca^{2+} resulting in a localised increase in free cytosolic Ca^{2+} which, once reaching a threshold concentration, might trigger appressorium formation.

As a second messenger, Ca^{2+} mediates its effect either by binding to calmodulin (CaM) (the primary receptor for Ca^{2+} in eukaryote cells) or by directly regulating protein and lipid kinases, cytoskeletal elements or ion channels. On binding Ca^{2+}, CaM undergoes a conformational change which increases its affinity for its target proteins which include protein kinases, phosphodiesterases, adenylate cyclases and Ca^{2+}-ATPases (Gadd, 1995). Calmodulin has been implicated in the transduction of a wide range of signals resulting in a wide range of cell response including cell proliferation, cell cycle control, nuclear division (Gadd, 1995) and appressorium formation in *Zoophthora radicans* (Magalhães *et al.*, 1991). Calmodulin has been identified in *U. appendiculatus* (Laccetti *et al.*, 1987).

1.3.4 Transduction of volatile signals

Nothing is known about the transduction of the volatile chemical signals *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol. The best studied signal transduction pathway for a gaseous chemical signal is that for ethylene in plants. Early work led to the concept that the 'ethylene receptor' was a metallo protein. Unsaturated aliphatic compounds, such as ethylene, differ from other aliphatic and most aromatic compounds in their ability to form complexes with metals. Also, the ability of CO_2 to competitively inhibit ethylene action and the fact that CO_2 typically affects metalloenzymes lent weight to the concept of a metal-based receptor for ethylene (Beyer, 1976). The observation that Zn^{2+} deficient plants were ethylene insensitive suggested that the metal in the receptor was Zn^{2+} (Burg & Burg, 1967). However, Cu^{2+} is also proposed as a candidate for the metal ion in the ethylene receptor because of its ability to form complexes with ethylene (Beyer, 1976). It is thought that the well documented inhibition of ethylene responses by Ag^{3+} is due its to substitution with the metal in the ethylene receptor (Beyer, 1976). Ag^{3+} inhibition is not due to ethylene scavenging or irreversible binding because Hg^{3+} is more efficient than Ag^{3+} at these processes, yet does not inhibit the ethylene response of plants (Beyer, 1976). Despite the evidence for a metal based receptor for ethylene, attempts to purify it have failed (Hall *et al.*, 1990).

Several *Arabidopsis thaliana* mutants which are insensitive to ethylene have been found. The mutant most strongly insensitive, *etr1* is thought to contain a mutation in the gene encoding a protein very early on in the signal transduction pathway (Ecker, 1995). ETR1 has three putative membrane spanning domains and has much similarity with the two-component histidine kinases (Chang & Meyerowitz, 1995). Whether ethylene is perceived at the membrane or in the cytosol is not known since ethylene has a higher solubility in lipid than water and may not require a trans-membrane receptor. A second mutant, *ers,* shows much similarity with *etr1* and is thought to act sequentially with *etr1* or the two are subunits of an ethylene sensing complex (Hua *et al*, 1995). Another mutant, *ctr1*, is further downstream of *etr1* and encodes a protein resembling a serine-threonine kinase (Kieber *et al.* 1993). CTR1

appears to be a negative regulator of ethylene signal transduction and shows greatest amino acid homology with the Raf family of protein kinases, most similar to mitogen-activated protein kinase kinase kinase (MAPKKK) (Kieber *et al.*, 1993). MAPKKK and related proteins are involved in a variety of stress response in eukaryotes (Herskowitz, 1995; Marshall, 1995). The transduction of ethylene signal in plants shows similarities with the yeast *Saccharomyces cerevisiae* osmolarity signalling pathway and prokaryotic two-component pathway (Hua *et al*, 1995).

In *Colletotrichum gloeosporioides*, ethylene produced by the host as a ripening signal, is used by the fungus as a signal for appressorium formation and initiation of infection (Kolattukudy *et al.*, 1995). The transduction of the ethylene signal in *C. gloeosporioides* has similarities to the transduction of the ethylene signal in plants, namely it is inhibited by Ag^{3+} ions (Flaishman & Kolattukudy, 1984) and the protein kinase inhibitor H-7 (Kolattukudy *et al.*, 1995).

cis-3-Hexen-1-ol and *trans*-2-hexen-1-ol also have an unsaturated carbon bond and it is possible that transduction of these signals may have similarities with the ethylene signal transduction pathway.

1.3.5 Other intracellular signals

There are a number of other molecules known to have key roles in transduction of signals in fungi including cAMP, inositol 1, 4, 5-trisphosphate (IP_3), diacyl glycerol (DAG) and protein kinases (Gadd, 1995).

A number of these intracellular signalling molecules have been implicated in the process of appressorium formation. To date, the chemicals that have been observed to induce differentiation when added exogenously include: phosphatidic acid (part of inositol lipid metabolism) and DAG (Staples & Hoch, 1988); cAMP and cGMP (Hoch & Staples, 1984); K^+ and Mg^{2+} (Kaminskyj & Day, 1984b); acrolein (Staples *et al.*, 1983b); and simple sugars (Kaminskyj & Day, 1984a; Hoch *et al.*, 1987a). As

mentioned above, some of these chemicals are known to be involved in intracellular signalling in other organisms and may have a role in signal transduction in rusts.

1.3.6 pH

There is evidence that changes in cytosolic pH can be involved in signal transduction in animal cells. For example, many protein growth factors stimulate cell proliferation and also activate a Na^+/H^+ exchanger which results in an increase in cytosolic pH. If the exchanger is inhibited with amyloride, then proliferation does not occur, suggesting that the pH increase is critical for the cellular response to the growth factor. An increase in cytosolic pH activates DNA and protein synthesis in sea urchin eggs upon fertilisation (Alberts *et al.*, 1984). Changes in cytosolic pH have been implicated in a wide range of cellular processes (see review by Busa & Nuccitelli, 1984) including the dimorphic transition of *Candida albicans* between yeast and mycelial forms (Stewart *et al.*, 1988). There is evidence that external pH influences appressorium induction in rust fungi. For *U. appendiculatus*, the optimum external pH for topographical induction of appressoria was pH 5.5–6.5 and at pH 7.0 and pH 4.5 there was 50% inhibition of appressorium induction (Stumpf *et al.*, 1991). It is possible that external pH may be influencing cytosolic pH, ECM-cytoskeleton interactions, plasma membrane ion channel activity or simply affecting tip growth.

The influence of pH on appressorium induction may be significant *in vivo.* Measurements of the pH of epidermal cell walls in *Commelina communis* with pH sensitive microelectrodes showed that the guard cell wall ranged from pH 7.0 when closed to pH 6.3 when open (Edwards & Bowling, 1986). The epidermal cells had a pH of 7.5–8.0 when closed and 6.0 when open. The closely related *Tradescantia virginiana* showed a similar pattern of cuticle pH. These differences in wall pH when the stomata are open arise due to the uptake of K^+ by guard cells by a K^+/H^+ antiport (Salisbury & Ross, 1984), thereby acidifying the apoplast. It was suggested that the rust might degrade the cuticle and perceive these differences in pH which underlie the cuticular waxes (Edwards & Bowling, 1986). If, however, the cuticular waxes are

not degraded, the phylloplane pH will be determined by the leaf leachates which are characteristically alkaline (Tukey, 1971).

1.4 Introduction to the thesis

The first aim in this study was to determine how widespread induction of appressoria by multiple ridged microfabricated topographies was in the cereal rusts. The significance *in vivo* of appressorium induction by topography was determined by comparing appressorium formation on polystyrene replicas of host leaves with appressorium formation on host leaves themselves.

It was found that all of the six cereal rusts studied were induced to form appressoria *in vitro* by topographical features alone. However, formation of appressoria over stomata on leaf replicas was significantly lower on polystyrene replicas than on host leaves. This suggests that although the cereal rusts are capable of forming appressoria in response to host physical features alone, *in vivo* there are other, non-topographic features of the host which induce appressorium formation.

The focus of the research was then switched to *P. graminis* f. sp. *tritici*, in which appressoria had been previously induced by *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol (Grambow, 1977; Grambow & Reidel, 1977). The aim here was to investigate the possible role of these wheat leaf volatile compounds in appressorium induction. It was confirmed that *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol will induce appressoria in the absence of topographical features. Furthermore, in the presence of an inductive topography, it was found for the first time that 1 mM *trans*-2-hexen-1-ol increased the speed with which appressoria were induced indicating cross talk between the signalling pathways induced by topography and *trans*-2-hexen-1-ol.

The final aim of the research was to characterise the two signal transduction pathways in more detail using various pharmacological agents. It was found that

topographical induction could be inhibited by a number of calmodulin antagonists, whilst *trans*-2-hexen-1-ol induction was not, indicating that only the former was Ca^{2+}-mediated. It was also found that topographical induction of appressoria was sensitive to external pH (optimum pH 6.7–7.5; 50% inhibition: pH 6.2), whilst *trans*-2-hexen-1-ol induction was not. Topographical induction could also be inhibited by the integrin-binding antagonist RGD. It was suggested that inhibition of topographical induction of appressoria by low pH (< pH 6.5) may be through disruption of the binding between putative integrin-like proteins in the rust plasma membrane and putative extracellular ligands. Induction by *trans*-2-hexen-1-ol was not inhibited by RGD, suggesting that integrins are not involved in *trans*-2-hexen-1-ol signalling.

Induction of appressoria by *trans*-2-hexen-1-ol could be inhibited by $AgNO_3$ and the protein kinase antagonist, H-7. These patterns of inhibition are characteristic of ethylene signal transduction in *Colletotrichum* and *Nicotiana.*

2. MATERIALS AND METHODS

2.1 Plant and fungal material

The six cereal rusts surveyed, along with the hosts used for spore propagation, are detailed in Table 2.1. *Puccinia striiformis* was not studied because it penetrates the host without producing an appressorium (Kellock, 1994). The hosts were grown at 22°C with a 16 h day for 3 weeks prior to inoculation. Hosts were inoculated with a soft bristle brush or spore settling tower (see Section 2.2) then placed under misted domes with a relative humidity (RH) of 100% and kept at 22°C for 3 h in the dark before being returned to the 16 h day cycle. The misted domes were removed after 36 h. Uredia started to produce spores after 12–14 d and continued to for a further 14 d or so.

Table 2.1. Details of the rusts and cereal hosts used.

Rust	Disease	Isolate/race	Host cultivar
Puccinia graminis f. sp. *tritici* [1]	Wheat Stem Rust	race 32	Armada
Puccinia graminis f. sp. *avenae* [2]	Oat Stem Rust	isolate IVP	Pendragon
Puccinia graminis f. sp. *secalis* [2]	Rye Stem Rust	isolate IVP1	Rogo
Puccinia hordei [2]	Barley Brown Rust	121	Golden Promise
Puccinia coronata f. sp. *avenae* [3]	Oat Crown Rust	CRO 251	Pendragon
Puccinia recondita f. sp. *tritici* [3]	Wheat Leaf Rust	BRW-NIAB 93/1	Armada

Supplied by: [1] B. M. Moerschbacher, Institut für Biologie III (Phlanzenphysiologie) der RWTH Aachen, Germany; [2] R. Niks, Plant Breeding Department (IvP), Agricultural University, Wageningen, The Netherlands; [3] National Institute of Agricultural Botany, Cambridge, UK.

2.2 Incubation times and methods

Approximately 5 mg of uredospores were collected fresh from host plants and inoculated onto the substrata (wafer replicas, leaf replicas, flat surfaces or leaves) with a spore settling tower giving a spore density of approximately 100–150 spores

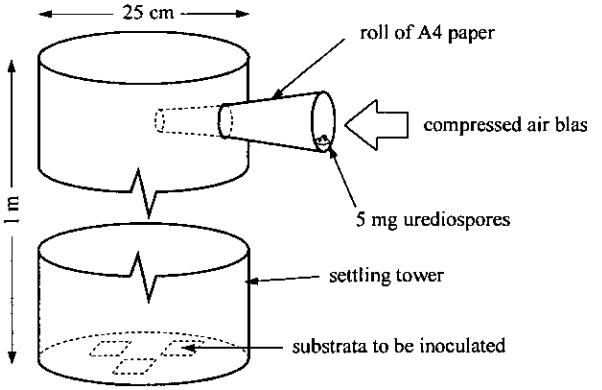

Figure 2.1. Diagram of the spore settling tower used to inoculate substrata with uredospores.

cm^{-2}. The settling tower consisted of a covered aluminium cylinder 1 m high, 25 cm diameter with a 5 cm diameter hole in the side 20 cm from the top. Spores were placed inside a rolled out sheet of A4 paper and blown in the side hole of the settling tower with two blasts from a compressed air canister (Kenro, H.A. West, Edinburgh, UK). After having been blown into the tower, the spores were left for 20 min to settle onto the substrata at its base (Figure 2.1). Prior to inoculation the interior of the settling tower was swabbed down with 70% ethanol.

For experiments in 100% RH (relative humidity), six pieces of the substratum were placed spore side up on a circle of wet filter paper (Whatman no. 1) in the base of a 8.5 cm diameter Petri dish (Disposables Media, Philip Harris Ltd., Clydebank, UK). The lid of the Petri dish was sprayed with distilled H_2O and sealed with Parafilm.

For experiments in liquid, each substratum was placed spore side down in 3 ml of liquid in a 5 cm diameter Petri dish (Fisons, Loughborough, UK) and sealed with Parafilm.

For experiments in vapour, each substratum was placed spore side up in a 8.5 cm diameter Petri dish. The lid of a 5 cm diameter Petri dish (Fisons, Loughborough, UK) containing 3 ml of the test liquid was placed next to the substratum in the large Petri dish which was then sealed with Parafilm.

The substrata were incubated, in the dark in a cooled incubator at 22°C, unless stated otherwise. The experiments were stopped by adding ethanol to the germlings after

24h unless otherwise stated.

2.3 Manufacture of wafers

Precisely defined repeated microtopographies were etched into 7.6 cm diameter Silicon (100) wafers by a process of reduction projection photolithography and reactive ion beam etching.

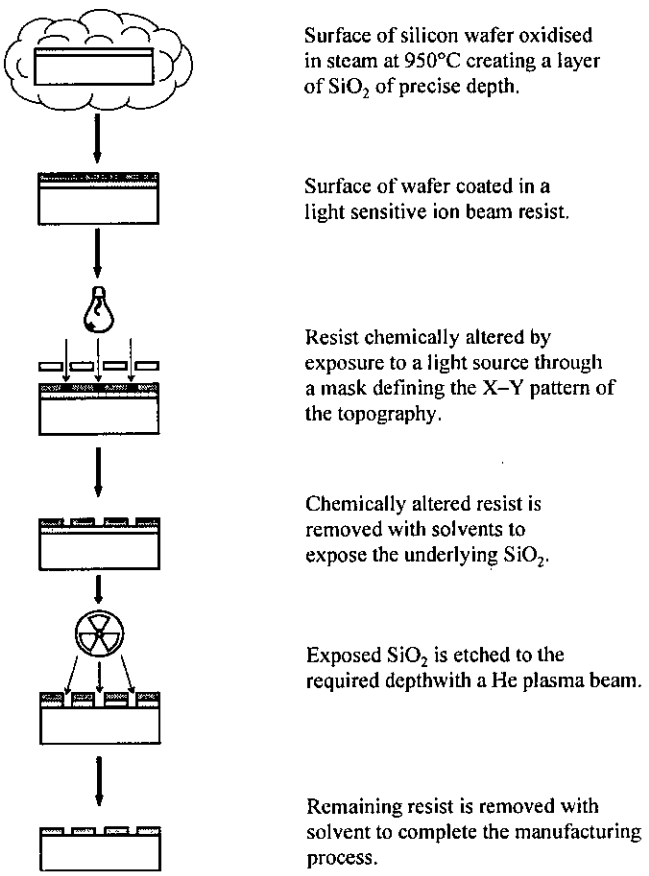

Figure 2.2. Summary of the main steps in microfabrication of silicon wafers.

2.3.1 Preparation of the silicon wafers

The Z dimension of the microtopography was defined by a layer of SiO_2 in the surface of the Si wafer, created by thermal oxidisation in steam (burning H_2 in O_2) at 950°C. The duration of oxidisation controls the depth of the oxidised layer, and thus

the Z-dimension of the topography. Wafers with topographies 0.116 and 2.4 μm deep had oxidisation times of 30 min and 48 h respectively.

Preparation of the wafers for definition of the X-Y dimensions of the topographical pattern involved spin coating the oxidised wafers with HIPR6512 photoresist (Olin Ciba Geigy, Livingstone, UK). Prior to coating, the adhesion of the resist to the wafer was improved by vacuum-baking the wafer in hexamethyldisilazene (HMDS). The solvent in which the resist was dissolved was then removed by baking at 90°C then at 125°C.

2.3.2 Definition of wafer dimensions

The X–Y dimensions of the microtopography in the Si was defined by a polished chromium 'mask'. The pattern of the masks were designed using CADENCE CAD software. These data was used by an electron beam pattern generator to print the pattern onto the top layer of an anti-reflective, chromium-coated, 11 cm square glass plate which was coated with an electron beam positive resist (Compugraphics International, Glenrothes). The exposed resist was removed with standard solvents, then the exposed chromium layer was removed with ferric chloride. The remains of the electron resist was removed with fuming HNO_3. Using a Reduction Direct Step On wafer machine (Eaton Optimetrix), a 10× reduced image of the mask was printed onto the resist with 436 nm light from a short arc Hg lamp. The pattern was projected onto the wafer 13 times resulting in thirteen 5 mm × 5 mm areas of each topography per wafer. The exposed resist is removed by developing in dilute methyl ammonium hydroxide which reacts with the carboxylic acid created when the photoresist is exposed to UV. The X–Y dimensions result from etching the exposed SiO_2 only.

The definition of the Z-dimension of the topography relies on the fact that SiO_2 is preferentially etched over the Si (at a SiO_2 : Si etch ratio of >12:1). Precise control over the depth of the topography was achieved by etching the exposed SiO_2 completely. The SiO_2 was etched at a rate of approximately 1 μm h^{-1} and the surface

fluorinated with a mixture of CHF_3 and He plasma in a Plasmatherm PK2440. Once all the exposed SiO_2 was etched, the remains of the photoresist was removed by low pressure O_2 plasma and then immersion in fuming HNO_3.

The microfabricated silicon wafers used had 2.0 μm wide ridges of heights 0.116, 0.223, 0.55, 0.72, 1.20, 1.50, 2.00 and 2.40 μm.

2.4 Polystyrene replicas of silicon wafers

Wafer replicas were made by heat-pressing polystyrene onto the silicon wafer. 12 mm x 12 mm pieces of Petri dish (Disposables Media, Philip Harris Ltd., Clydebank, UK) were cut with a Stanley knife on a nylon chopping board. Two wafers were placed 'ridge-side' up on a glass microscope slide (BlueStar, Chance Proper, Warley, UK) and each wafer had a piece of Petri dish placed on top of it followed by a second microscope slide. Five microscope slides were placed on a ceramic kitchen tile which was 'glazed-side' down on a stainless steel baking tray allowing manufacture of 10 wafer replicas per ceramic tile. A second ceramic tile was placed on top of the microscope slides 'glazed-side' up, and a 250 ml conical flask containing sand was placed on top of the tile. The amount of sand in the conical flask was adjusted so that the total mass on the ten wafers was 450 g (Figure 2.3).

This was placed in an oven at 200°C for 60 min before the wafer/replicas were plunged into dH_2O and left for several hours or occasionally overnight. The wafer and replicas were then carefully separated and kept face down on Whatman no. 1 filter paper. Many types of disposable glove have talcum powder associated with them, and so to prevent effects of this on the fungus, polystyrene pieces, silicon wafers and replicas were handled with un-powdered polythene gloves (A. J. Beveridge, Edinburgh, UK). Replicas were handled with watchmakers forceps with the tips bent inwards to ensure minimum damage to the polystyrene topographies.

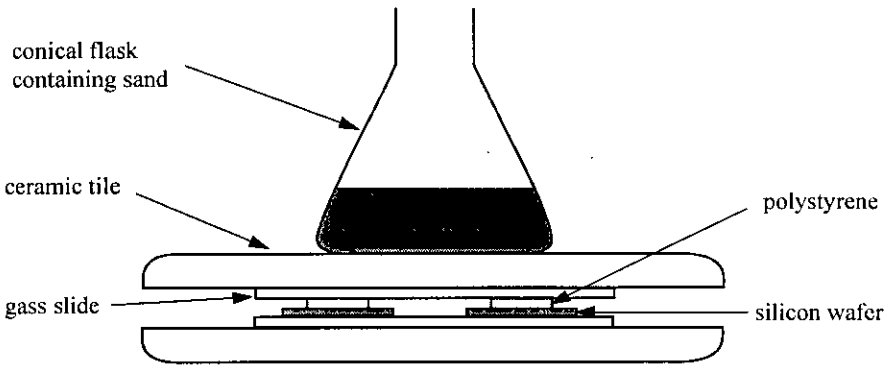

Figure 2.3. Apparatus for production of polystyrene replicas of silicon wafers

Control substrata (flat surfaces) were prepared in the same way, except the silicon wafers were not included in the sandwich.

2.4.1 Cleaning wafers

Wafers were cleaned by first soaking them in chloroform for 4–5 h to remove pieces of polystyrene then washed briefly in clean chloroform and allowed to dry on filter paper 'ridge side-up'. The wafers were then placed in a beaker and 50 ml concentrated H_2SO_4 and 25 ml of 30% H_2O_2 were added and left in a fume cupboard overnight. The mixture was poured off and the wafers washed in copious running water for 10 min then washed in a large volume of dH_2O before being dried 'ridge side down' on filter paper.

2.5 Polystyrene replicas of host leaves

The low viscosity, two part polysiloxane silicone polymer Extrude Wash (Kerr, (UK) Ltd., Peterborough, UK) was used to make negative moulds of 4 week old leaves. The blue base and white catalyst were mixed in equal amounts (2 ml) on a microscope slide with a micro-spatula. The mixture was then dribbled along 3 cm of the abaxial side of a leaf, which was resting on a microscope slide and still attached to the plant. A second microscope slide was then gently placed on the wet polymer

mixture. After the polymer had set (30 min) the slides and leaf were removed.

Positive leaf replicas were made by heat pressing polystyrene into the negative leaf moulds. Leaf moulds were each put on a microscope slide and five of these slides were placed on a ceramic wall tile on a stainless steel baking sheet. Polystyrene Petri dishes (Disposables Media, Philip Harris Ltd., Clydebank, UK) were cut with a Stanley knife into 2.5 cm × 0.5 cm rectangular pieces and a piece placed on each leaf mould. A second slide was the placed on the polystyrene and a second wall tile placed on top of it. Finally, a conical flask containing sand was placed on top so that the total weight on the five replicas was 450 g. This was all placed in an oven at 200°C for 60 min. To separate the replicas and moulds, they were immersed in a large volume of cool (≈ 15°C) dH_2O.

Later this technique was modified so that the total weight on the polystyrene was only 200 g, the oven temperature 160°C, the heating time 90 min, and the replicas were left to cool at room temperature without removing the weight.

2.6 Leaf surfaces

Differentiation *in vivo* was investigated on host leaf segments. Leaves of each cereal were taken from 4 week old plants and 60 mm long sections from each leaf were excised. These were placed in Petri dishes, abaxial side uppermost, with the cut ends under wet filter paper.

2.7 Staining

Cell walls and septa were stained by floating substrata spore side down in 0.01% Calcofluor MRT fluorescent brightener (Sigma) which binds to β-glucans (Butt *et al.*, 1989). This was followed by floating spore side down in a large volume of dH_2O to wash away unbound stain by dilution.

When grown on leaf surfaces the germlings had a tendency to be washed off when immersed in water. To prevent displacement from leaves, germlings and leaves were fixed by adding 3 ml of 3:1 acetic acid : ethanol to the filter paper in the bottom of the Petri dish (not directly on the leaf) and the Petri dish left sealed in a refrigerator (5°C) for 24 h. The germlings were fixed by the fixative in the vapour form and also as a liquid by diffusion through the leaf from the underside. This procedure also bleaches the chlorophyll in the leaf (which reduced autofluorescence) and reduces the hydrophobicity of the cuticle making hydration of the specimen for microscopy much easier. The germlings were stained by lightly misting the leaf with 0.05% Calcofluor and mounted in 0.01% Triton X-100. Detergent was used to reduce surface tension and therefore reduce any potential stripping effect of a meniscus of liquid flowing across the leaf. The dye was visualised with the Reichert Polyvar U1 dichroic block (excitation filter = 330-380 nm; dichroic mirror = 420 nm; long pass filter = 418 nm).

Nuclear staining with DAPI was performed prior to wall staining with Calcofluor. DAPI binds to AT and GC rich regions of double stranded DNA (Butt *et al.*, 1989). Substrata were floated spore side down in 0.01 μg/ml DAPI (Sigma) for 30 sec only before transfer to a large volume of dH_2O to wash off excess dye. Overstaining was a major problem since DAPI also binds to microtubules and tubulin (Bonne *et al.*, 1985; Heusele *et al.*, 1987). The dye was visualised with the Reichert Polyvar U1 dichroic block.

A 50:50 mix of 20 μg/ml propidium iodide and 20 μg/ml fluorescein diacetate was

used as a 'live/dead' stain to test viability (Butt *et al.*, 1989). Propidium iodide is not membrane permeant and can only stain cells which have lost membrane integrity (i.e. are dead). FDA is readily membrane permeant and can load readily into cells regardless of their viability. However it is only live cells which hydrolyse the FDA to the fluorescent fluorescein. Substrata were mounted in the stain mixture and viewed after 5 min. The dye was visualised with the Reichert Polyvar B1 dichroic block (excitation filter = 450-495; dichroic mirror = 510 nm; long pass filter = 520 nm).

Cutin on leaf surfaces was stained with 5 μg/ml Nile red (9-diethylamino-5*H*-benzo[*a*]phenoxinazine-5-one) (Sigma) in 100% methanol by immersion in the dye for 30 sec. The leaves were then washed in a large volume of dH_2O and mounted in dH_2O. Nile red is almost non-fluorescent in water but undergoes a large absorption and emission shift to shorter wavelength in a lipophilic solvent. The nature of the shift is dependent on the solvent polarity. The dye was visualised with the Reichert Polyvar B1 dichroic block.

Leaves were mounted in 0.1 M NH_4OH and UV (360 nm) induced autofluorescence was visualised using the B1 filter block. Ammonia enhanced autofluorescence is indicative of phenolic acids (Palevitz, 1981).

Fluorescence micrographs were recorded on TMAX 400 35 mm film. Brightfield micrographs were recorded on TMAX 100 35 mm film.

2.8 Measurement of cytosolic pH

The dye carboxy-SNARF-1 exhibits a pH-dependent shift in its emission spectrum and can be used to image and quantify cytosolic pH. When excited at 514 nm, the dye exhibits two peaks in its emission spectrum: at 588 nm and 614 nm. The 588 nm emission intensity increases with decreasing pH; the emission at 614 nm increases with increasing pH. Measurement of the emission intensities at the two separate

wavelengths (588 nm and 643 nm) allows quantification of the pH of the dye solution by dividing the intensity of emission at one wavelength by the intensity at the second wavelength. This is the basis for ratio imaging because the ratios measured are independent of the amount of dye sample.

2.8.1 Preparation of the dye solution

The SNARF-1-AM was dissolved in a solution of the detergent Pluronic F-127. The detergent was made as a 25% pluronic/DMSO w/v stock which was diluted with distilled water 1:50 to give the working solution. The stock of SNARF-1-AM was made at 20 mM and stored at -70°C. A sub-stock was made at a concentration of 200 μM by dilution with the 10 mM MES/HEPES buffer in which the spores were germinated (at pH 6.2 or 7.2). The final concentration of SNARF-1-AM used was 2 μM, the sub-stock having been diluted in the 10 mM MES/HEPES. The final concentrations of Pluronic and DMSO were < 0.01% and < 0.1% respectively.

2.8.2 Staining of germlings with SNARF-1 AM

Spores were inoculated onto glass coverslips (Chance Proper No. 0, Warley, UK) and floated spore side down on 10 mM MES/HEPES pH 6.2 or 7.2. After 1-3 h, coverslips were taken and floated on a 1 ml drop of 2 μM SNARF-1-AM for 8 min. The coverslip was then transferred to another volume of 10 mM MES/HEPES at the test pH to wash off excess dye. This was then transferred to the microscope stage with a drop of fresh 10 mM MES/HEPES at the test pH.

2.8.3 Imaging SNARF-1

The dye was imaged with a BioRad MRC 600 laser scanning confocal microscope (Bio-Rad Microscience Ltd., Hemel Hempstead, UK) controlled by CoMOS (v. 6.03) software and attached to a Nikon Diaphot inverted microscope. Image analysis was performed with CoMOS, MPL (v. 1.01) and TCSM (v. 1.1c) software. The

conditions of imaging are detailed in Table 2.2.

Table 2.2. Details of the settings for the BioRad MRC600 confocal microscope used to image SNARF-1.

Neutral density filter for laser light	2
Objective	×60 oil Plan Apo (NA 1.4)
Pinhole	7
Gain channel (1 & 2)	6.7 & 7.1
Black level channel (1 & 2)	5.0 & 5.1
Zoom	4.0
Scan speed	slow (F1)
Acquisition	Kalman ×2

Simultaneous dual emission imaging was used to collect the images for ratio analysis. The fluorescent signal from the specimen was split with a custom made SNARF-1 filter block (excitation filter = 514 nm, dichroic mirror = 540 nm, long pass filter = 550 nm). The signal at 580 ± 15 nm was collected by one photomultiplier (channel 2) and the signal at 640 ± 18 nm was collected by a second photomultiplier (channel 1). 'Background' images were obtained at each wavelength with the specimen not being scanned by the laser. These images represent the 'noise' from the photomultipliers and other spurious signals which made up the 'background' image.

After pH_{cyt} of germlings had been measured, the responsiveness of the intracellular dye was determined using the membrane permeant weak acid, propionic acid. Extracellular pH and pH_{cyt} were equilibrated with 50 mM propionate at either pH 6.2 or pH 7.2. A stock solution of 500 mM sodium propionate (Sigma) was made in distilled water. This was diluted in water to 50 mM and the pH adjusted with 2 M KOH.

2.8.4 Image processing

The background images were arithmetically subtracted pixel-by-pixel from the specimen images. The background-corrected specimen image from channel 2 was divided pixel-by-pixel with the background-corrected image collected at channel 1 to obtain ratio values for each pixel of the image. Each ratio value was assigned a pixel intensity between 0 and 255 and a greyscale image was thus constructed. A pseudocolour image was created from this greyscale image by assigning a colour to each pixel intensity with look-up tables created by Richard Parton (Institute of Cell and Molecular Biology, University of Edinburgh). Mean pixel intensities were taken from areas of the tips of the imaged cells using the CoMOS *histogram* command.

2.9 Inhibitor experiments

Inoculated substrata were floated spore side down on 3 ml of each inhibitor solution in a 5 cm diameter, Parafilm-sealed Petri dish and incubated at 22°C in the dark for 24 h. The inhibitors used are detailed in Table 2.3.

Table 2.3. Details of inhibitors and the stocks used.

Inhibitor	Action	Stock
Calmidazolium (Sigma)	Calmodulin antagonist	100 mM in DMSO
W-7 (Sigma)	Calmodulin antagonist	100 mM in DMSO
W-5 (Sigma)	Calmodulin antagonist	10 mM in 10% DMSO
trifluoperazine (Sigma)	Calmodulin antagonist	100 mM in DMSO
RGD (Sigma)	Integrin-binding inhibitor	1 mM dH_2O
H-7 (Sigma)	Protein kinase C inhibitor	100 mM DMSO
D_2O (Sigma)	Microtubule stabiliser	none
$AgNO_3$ (Sigma)	Ethylene response inhibitor	1 mM in 10 mM MES/HEPES

2.9.1 Chemical induction of appressoria

Inoculated substrata were floated spore side down on 3 ml of each chemical in a 5 cm diameter, Parafilm-sealed Petri dish and incubated at 22°C in the dark for 24 h.

For the dose-response curves, the volatile alcohols *trans*-2-hexen-1-ol (Sigma) and *cis*-3-hexen-1-ol (Aldrich) were made up as 10 mM stock in dH_2O (11.2 µl in 10 ml dH_2O). The solution was rigorously stirred for 30 min. 10 ml of the experimental concentrations were made from the stock. In subsequent experiments in which only 1 mM *trans*-2-hexen-1-ol was used, the solution was made without a stock (i.e. 11.2 µl *trans*-2-hexen-1-ol in 100 ml solution).

Stocks of 100 mM p-coumaric acid (Sigma), ferulic acid (Sigma), stearic acid (Sigma), palmitic acid (Sigma), linoleic acid (Sigma) and oleic acid (Sigma) were made up in 100% ethanol. The stocks were diluted to 100 µM in 10 mM MES/HEPES buffer at pH 7.0. Diferulic acid was obtained from Dr Graham Wallace (Institute of Cell and Molecular Biology, University of Edinburgh), and made up as 20 mM in 100% ethanol before dilution in 10 mM MES/HEPES (pH 7.0) to a final concentration of 20 µM.

2.10 Measurement of cell junction spacing

2.10.1 Confocal analysis of intact leaves

Measurements of wheat leaf surface topography were performed with a BioRad MRC 600 LSCM. The leaf was stained with 5 µg/ml Nile red before being washed and gently blotted dry. Immersion oil was dropped onto the leaf surface and the objective focused directly onto the leaf without a coverslip. This was done to reduce optical artefacts in the z-axis arising through differences between the refractive indices of air, water and glass. The immersion oil took up the dye, resulting in a fluorescing sea of dye on the leaf surface, fluorescence stopped at the cuticle

allowing indirect visualisation of the leaf surface topography.

An optical section was then taken of the surface of the leaf by creating a line scan z-series. Settings for the imaging are detailed in Table 2.4. Optical sections were taken at 90° to the long axis of the leaf and were placed randomly along the longitudinal axis of the stomatal complex. The zoom was adjusted so that the pixel size of the image was the same as the z-step, i.e. each line of the image was 0.1 μm wide and each plane of focus was 0.1 μm apart. This ensured that the image had not been stretched or compressed in the z-axis. Using the CoMOS line command, measurements of the linear distance and 3-dimensional distance between cell junctions were made.

Table 2.4. Details of the settings for the BioRad MRC 600 LSCM used to image Nile red stained wheat epidermis cells.

Neutral density filter for laser light	2
Objective	×60 oil Plan Apo (NA 1.4)
Pinhole	5
Gain	6.1
Black level	5.2
Zoom	2.75
Scan speed	slow (F1)
Acquisition	Direct

2.10.2 Confocal analysis of hand sections

Confocal microscopy allows thin optical sections to be imaged from thick hand sections. Hand sections of wheat leaves were made with a double-sided razor blade. The sections were stained in 5 μg/ml Nile Red and washed in dH_2O. The sections were then mounted in dH_2O and imaged with the LSCM (details in Table 2.5). Measurements of cell junction spacings were made with the integral *line* command.

Table 2.5. Details of the settings for the BioRad MRC 600 LSCM used to image Nile red stained wheat epidermis cells.

Neutral density filter for laser light	2
Objective	×60 oil Plan Apo (NA 1.4)
Pinhole	5
Gain	variable
Black level	5.2
Zoom	2.75
Scan speed	slow (F1)
Acquisition	Direct

2.10.3 Scanning electron microscopy

Segments of 4 week old wheat leaves were prepared for low temperature electron microscopy using the EMscope SP2000 cryopreparation system (Beckett & Read, 1986) interfaced with a Cambridge S250 scanning electron microscope. Samples were mounted on standard EMscope LTSEM stubs using Tissue Tek O.C.T. compound (Miles Laboratories Inc., Illinois, USA) as a cryoadhesive. Leaf segments were mounted flat on the stub within 1 minute of excision from the plant to minimise desiccation. The stub with 1–3 mounted leaf segments was cryofixed by plunging into sub-cooled nitrogen under argon gas. Specimens were examined partially freeze-dried after warming to -65 to -70°C on the microscope cold stage. All material was finally sputter-coated with gold and examined below -155°C with accelerating voltages between 5 and 7 kV. Scanning electron micrographs were recorded on TMAX 100 120 roll film.

Leaf replicas were sputter coated with gold and analysed without drying by ambient-temperature scanning electron microscopy (Jeffree & Read, 1991).

Images were digitally captured and analysed with I-Scan (ISS group, Manchester, UK), a PC based slow-scan image acquisition system. Measurements were made with the integral *line* command. Measurements were taken at 90° to the long axis of the leaf and were placed randomly along the long axis of each stomatal complex.

2.10.4 Measurement of leaf and leaf replica hydrophobicity

A Nikon Optiphot photomicroscope was laid on its back and a plastic block (2 cm × 2 cm × 1 cm) attached to the stage (Figure 2.4). Leaf segments and leaf replicas were placed on the block and a 25 µl drop of dH_2O was carefully placed on the surface of the leaf/leaf replica. Images were obtained with a Sony CCD camera linked to a PC via a frame grabber (DT55, Data Translation Berkshire, UK). Measurements of contact angles were made with Optimas v. 4.0 (Optimas corp., Seattle, USA) image analysis package.

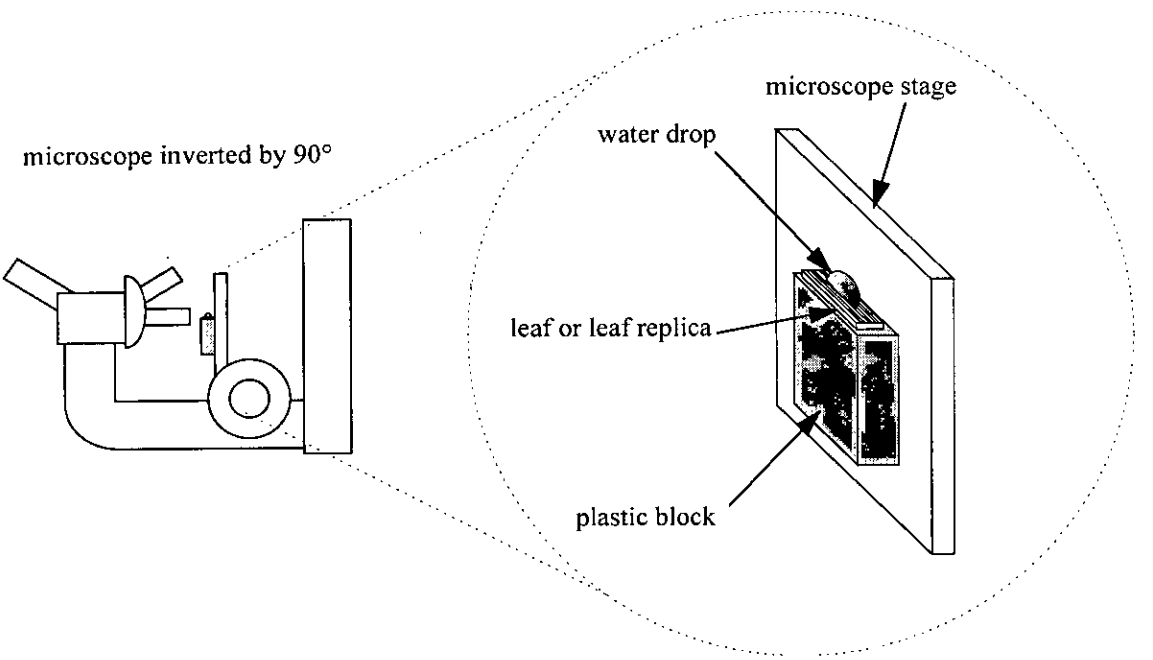

Figure 2.4. Apparatus for measuring leaf and leaf replica surface hydrophobicity

2.10.5 Quantification

Percentage appressorium formation was determined from at least 3 experiments, with 3 replicas per experiment. Typically, at least 100 germlings were counted per replica, however, in some cases, e.g. high inhibitor concentrations, germination was inhibited and fewer than 100 germlings were counted on each replica. An appressorium was identified as a pronounced terminal swelling delineated by a septum and containing the cytoplasm which had migrated into it. Nuclei were stained with 0.1 µg/ml DAPI

(Sigma) for 20 sec.

Primary germ tube lengths were quantified as means of at least 50 germlings but more typically 100 germlings per experiment. Each experiment was carried out at least twice. Length measurements were made on a Nikon Diaphot inverted microscope with a Sony CCD camera linked to a frame grabber (DT55, Data Translation Berkshire, UK) controlled by Optimas v. 4.0 image analysis software. Data were directly exported from Optimas v. 4.0 to Microsoft Excel v. 4.0. Germlings were measured with a ×10 or ×40 Plan Apo objective and appressorium and stomatal dimensions were measured with a ×60 Plan Apo objective. Plan objectives were used to minimise length measurement errors due to spherical aberration. Only the primary germ tube was measured; branching was not assessed.

Statistical analyses were performed with either Fig. P for Windows v. 2 (Biosoft, Cambridge, UK) or Microsoft Excel v. 4.0.

3. TOPOGRAPHICAL INDUCTION OF APPRESSORIA IN THE CEREAL RUSTS

3.1 Introduction

Early work by Sidney Dickinson (Dickinson, 1949a; Dickinson, 1970; Dickinson, 1971) showed that several cereal rust species form appressoria in response to irregular, undefined substrata. More recently Read *et al.* (1996) have observed that the cereal rusts *P. graminis* f. sp. *tritici* and *P. hordei* can be induced to form appressoria by multiple, closely spaced ridges. Of the topographies studied, the optimal for both rusts was ridges 2.0 μm wide, 2.0 μm high spaced 1.5 μm apart. The yellow rust of barley, *P. striiformis* f. sp. *hordei* did not form infection structures in response to topographical stimuli (Kellock, 1994).

The first aim of the work presented in this chapter was to determine the inductive topographic signals for six cereal rusts by growing them on microfabricated substrata with 1.5 μm spaced ridges of different heights. The significance of topographic sensing *in vivo* was investigated by analysing appressorium formation on host leaves and host leaf replicas.

For *P. hordei* and *P. graminis* f. sp. *tritici*, appressorium induction was greater on ridges spaced 1.5 μm than 2.5 μm (Read *et al.*, 1996). The second aim of the work was to determine the optimal spacing of ridges for induction of differentiation in *P. graminis* f. sp. *tritici*.

The close spacing of the ridges is thought to mimic the close spacing of the cell junctions associated with the stomatal complex. The third aim of the work was to correlate the spacings of inductive ridges *in vitro* with topographic features observed on the epidermis of wheat leaves.

The topography may be initially perceived as the duration between the germling encountering ridges (temporally encoded) or as the physical distance between the ridges (spatially encoded). The fourth aim of the work was to address how the signal is encoded. *Puccinia graminis* f. sp. *tritici* was incubated at different temperatures to alter the growth rate on inductive substrata. If the signal is encoded by the physical separation of the ridges, alteration of the growth rate should have no effect on the response to the topography.

3.2 Results

3.2.1 Cereal rust response to ridges of different heights

Six cereal rusts were inoculated onto polystyrene replicas of microfabricated silicon wafers with 2.0 μm wide ridges spaced 1.5 μm and 50 μm apart (referred to from now on as multiple ridges and single ridges, respectively). The topographies used had ridges of heights: 0.116, 0.223, 0.55, 0.72, 1.2, 1.5, 2.0 and 2.4 μm. The inoculated substrata were incubated in 100% RH at 22°C for 24 h in the dark.

All rusts were induced to form appressoria and subsequent infection structures (sub-stomatal vesicles and infection hyphae) by topographical signals alone. Undifferentiated germlings had two nuclei with the cytoplasm distributed towards the apex of the germ tube. Cytoplasmic migration into the appressorium (Figure 3.1A), nuclear division and septation (Figure 3.1B) occurred upon appressorium formation. A further round of mitosis occurred in the substomatal vesicle (SSV) (Figure 3.1C).

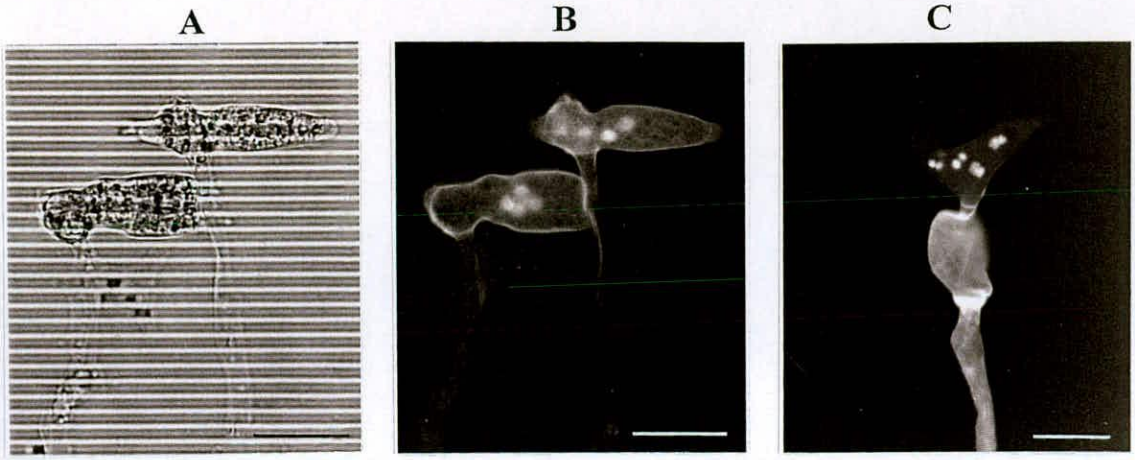

Figure 3.1. *Puccinia graminis* f. sp. *tritici* appressoria induced by multiple ridges showing **A:** cytoplasmic migration (DIC) and **B:** nuclear division and septation (Calcofluor and DAPI). **C:** Appressorium and sub-stomatal vesicle induced by artificial topography showing 4 pairs of nuclei (Calcofluor and DAPI). Bars = 15 µm.

The morphology of the infection structures induced by artificial substrata varied between rust species (Figures 3.2 & 3.3). *Puccinia graminis* had more-or-less oval appressoria with aseptate, oval to fusiform, bipolar SSVs (Figures 3.2A & 3.3). Typically a single IH grew from one pole of the SSV. Occasionally two infection hyphae were produced, one from each pole of the SSV. The IH and SSV were separated by a septum.

Puccinia recondita produced irregular, circular appressoria and SSVs which were elongate with a single pole (Figures 3.2B & 3.3). The SSVs were tapered with the narrowest end nearest the appressorium. There was a single septum between the SSV and appressorium. A single IH was produced, which occasionally branched.

Puccinia coronata formed more-or-less circular appressoria, slightly wider at the apex (Figures 3.2C & 3.3). The SSV was ellipsoid and bipolar, with an infection hypha growing from each pole. The SSV had a central septum and a septum between it and the appressorium.

Puccinia hordei had more-or-less oval appressoria similar to *P. graminis* (Figures 3.2D & 3.3). The SSV for this rust was bipolar and ellipsoidal with the centre 'pinched' at the point of a septum. Infection hyphae grew from both poles of the SSV.

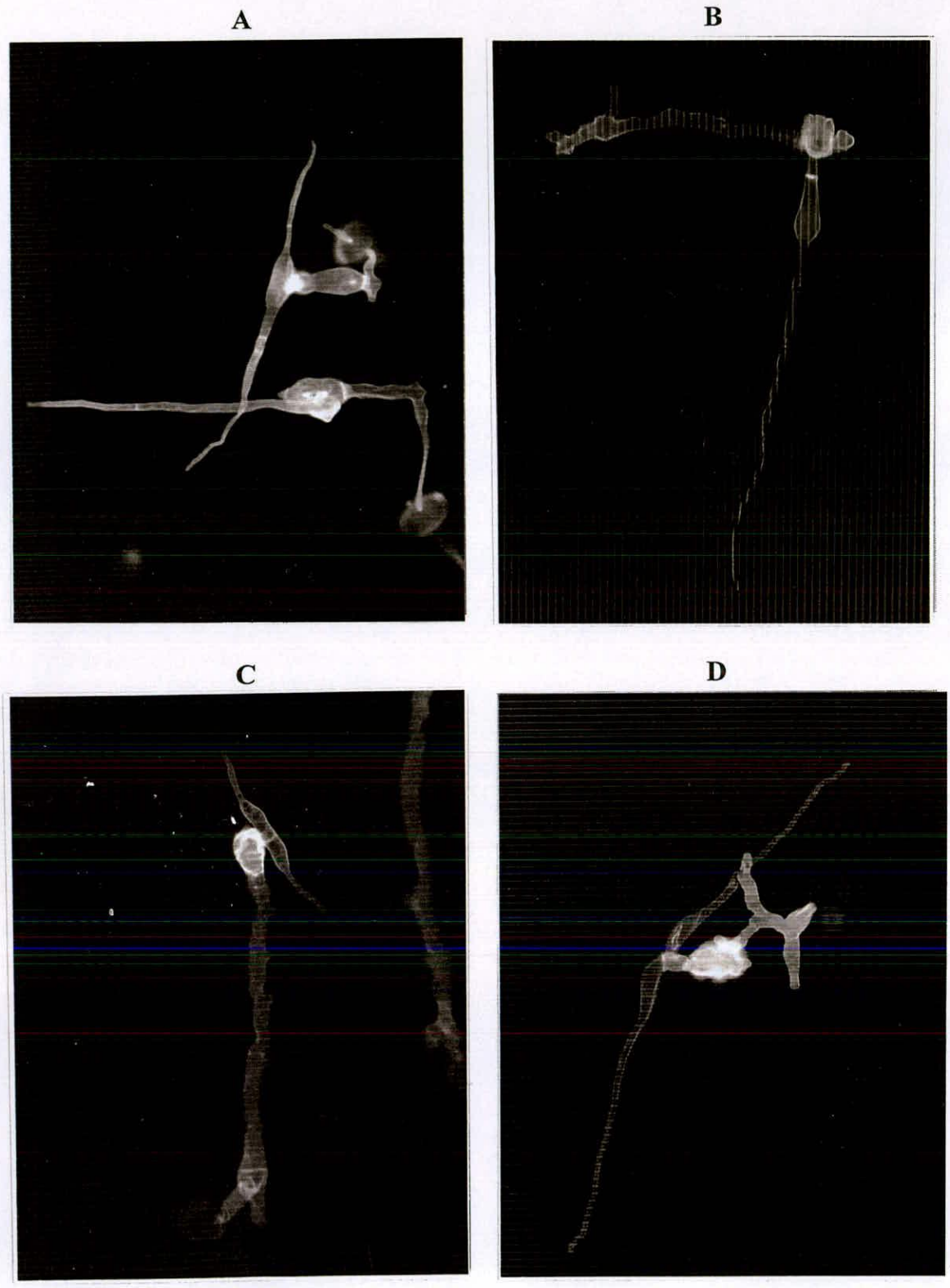

Figure 3.2. Infection structures induced by microfabricated polystyrene substrata. **A**, *P. graminis* f. sp. *tritici*; **B**, *P. recondita* f. sp. *tritici*; **C**, *P. coronata* f. sp. *avenae*; **D**, *P. hordei*. Bar = 100 µm.

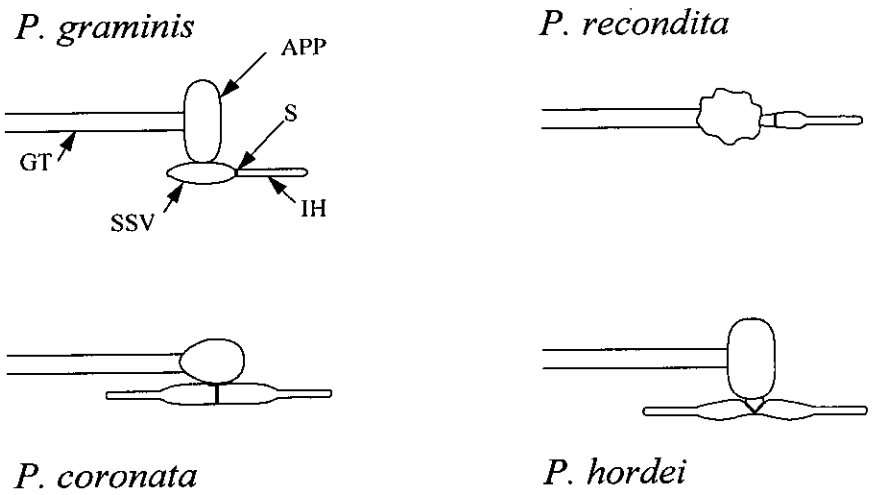

Figure 3.3. Diagram of the typical morphology of the infection structures induced in the four cereal rust species by topographical signals. **GT**, germ tube; **APP**, appressorium; **SSV**, sub-stomatal vesicle; **IH**, infection hypha; **S**, septum.

All rusts formed significantly more appressoria on multiple ridges of some heights than on flat surfaces. There were three patterns of response by the six rust species to multiple ridges (Figure 3.4 A-F).

P. graminis f. sp. *tritici* (Figure 3.4A) and *P. graminis* f. sp. *avenae* (Figure 3.4E) formed increasing numbers of appressoria in response to increasing height of ridges. The highest ridges tested induced maximum or near maximum levels of differentiation (approximately 70% for both rusts).

The second type of response to the multiple ridges was shown by *P. recondita* f. sp. *tritici* (Figure 3.4B) and *P. coronata* f. sp. *avenae* (Figure 3.4F). These rusts had two optimal ridge heights, 0.55 μm and 2.0 μm. On the highest ridges tested (2.4 μm) differentiation in this class was very low. Optimum differentiation for *P. coronata* f. sp. *avenae* (73.4 ± 5.3%) was much higher than *P. recondita* f. sp. *tritici* (32.8 ± 1.9%). *Puccinia hordei* (Figure 3.4D) showed a similar 'two-height-optimum' response although the second optimal height (2 μm) was less obvious. Optimum differentiation for *P. hordei* was 35.8 ± 3.9% on 0.55 μm high ridges.

For *P. graminis* f. sp. *secalis* (Figure 3.4C) differentiation was high even on the lowest (0.116 μm) multiple ridges tested and optimal on 0.55 μm high ridges (88 ± 3%). Differentiation decreased slightly with increasing ridge height beyond 0.55 μm.

Of the six species examined, only *P. graminis* f. sp. *secalis* (Figure 3.4C) and *P. recondita* f. sp. *tritici* (Figure 3.4B) formed significantly ($p < 0.05$) greater numbers of appressoria on 50 μm spaced ridges than on flat surfaces. *Puccinia recondita* f. sp. *tritici* formed significantly ($p < 0.05$) more appressoria on single ridges 0.55 μm high (8.7 ± 2.6%) than on flat surfaces (1 ± 1%). For *P. graminis* f. sp. *secalis* the optimal height of single ridges was also 0.55 μm, with 65% ± 4% germling differentiation. Ridges higher than 0.55 μm were also inductive but, as with induction on multiple ridges in this rust, differentiation decreased slightly with increasing ridge height beyond 0.55 μm.

All rusts grew to varying distances prior to differentiation on each inductive topography.

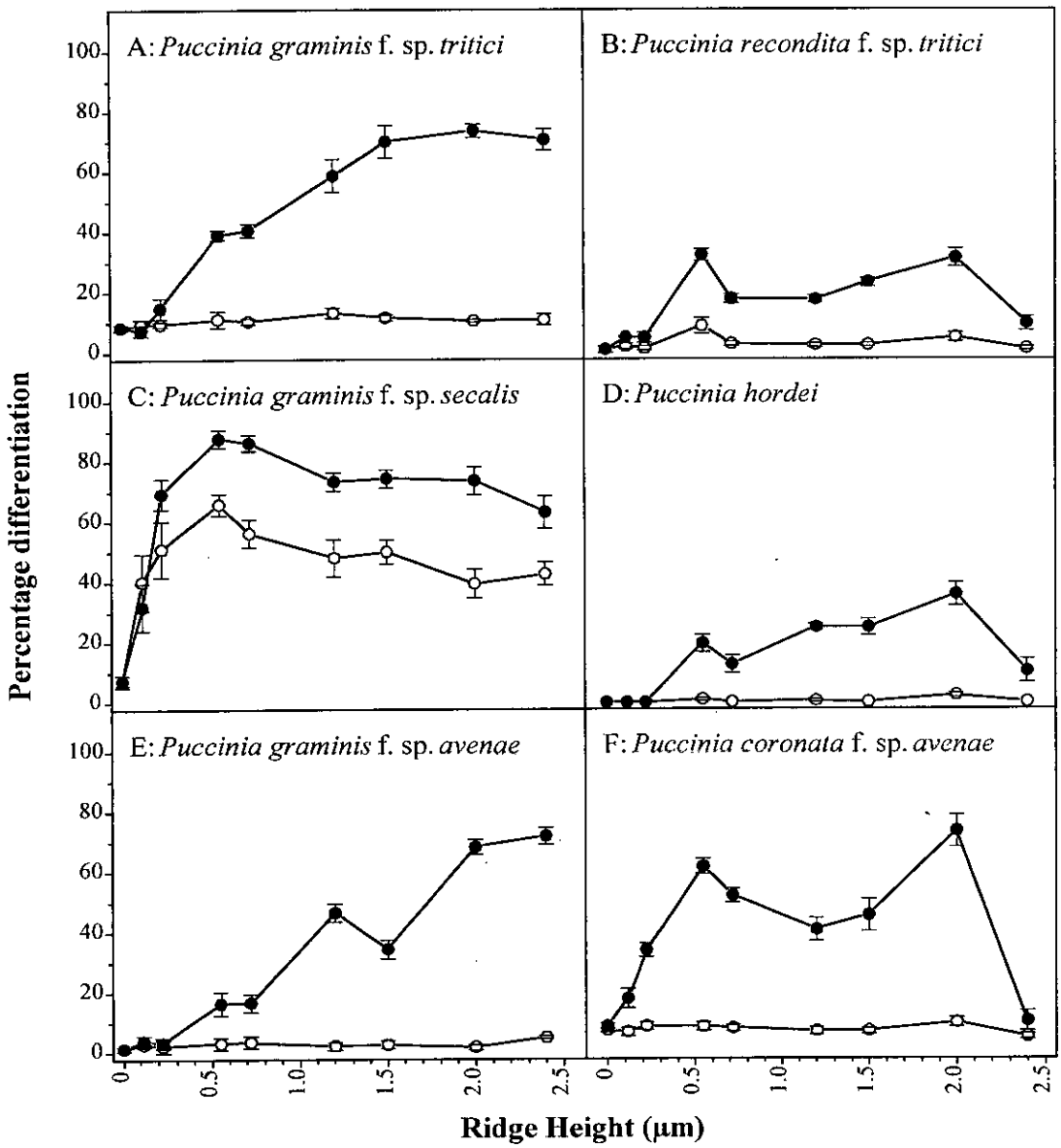

Figure 3.4. Percentage of germlings forming appressoria on polystyrene replicas of micro-fabricated silicon wafers with 2.0 µm wide ridges of various heights, spaced 1.5 µm (●) or 50 µm (○) apart. Means ± sem.

3.2.2 P. graminis *f. sp.* tritici *on ridges of different spacings*

Uredospores of *P. graminis* f. sp. *tritici* were inoculated onto polystyrene replicas of microfabricated silicon wafers with ridges which were spaced 1.5, 2.5. 7.5, 15 and 50 µm apart, and were 2 µm high, 2 µm wide. Flat polystyrene (ridge height = 0 µm) was used as a control. The substrata were incubated at 15, 20 and 25°C for 24 h in

100% RH in the dark. Percentage germling differentiation, and the length of germling growth prior to differentiation, were measured.

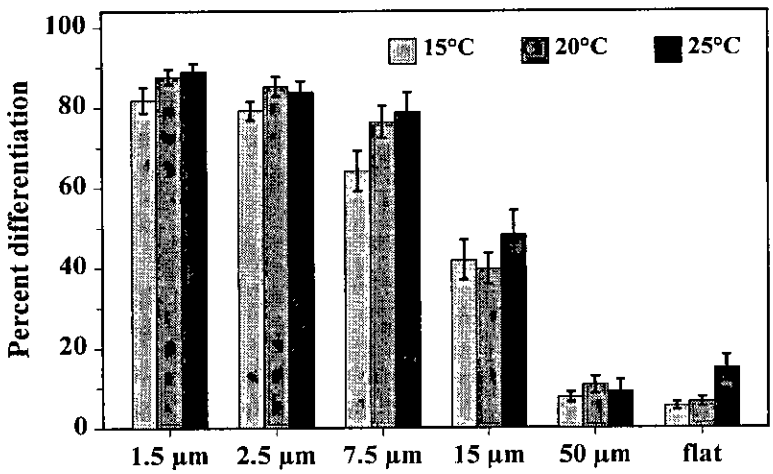

Figure 3.5. Percentage *P. graminis* f. sp. *tritici* germling differentiation on 2.0 µm high, 2.0 µm wide ridges spaced 1.5, 2.5, 7.5, 15 and 50 µm apart and on flat surfaces. Means ± sem.

Table 3.1. t-test analysis of percentage *P. graminis* f. sp. *tritici* germling differentiation on 2.0 µm high, 2.0 µm wide ridges spaced 1.5, 2.5, 7.5, 15 and 50 µm apart, and on flat surfaces (N = not significantly different, degree of significance given).

Ridge spacings compared	15°C	20°C	25°C
1.5 µm v 2.5 µm	N	N	N
2.5 µm v 7.5 µm	$p < 0.02$	N	N
7.5 µm v 15 µm	$p < 0.005$	$p < 0.0001$	$p < 0.002$
15 µm v 50 µm	$p < 0.001$	$p < 0.0001$	$p < 0.005$
50 µm v flat	N	N	N

Percentage appressorium differentiation on a particular topography was not statistically different at the three incubation temperatures (Figure 3.5). Topographies with 1.5 µm or 2.5 µm spaced ridges 2.0 µm high induced maximum levels of differentiation (approximately 70%) at all three temperatures. At 20°C and 25°C, differentiation on 7.5 µm spaced ridges was the same as differentiation on the ridges

spaced 2.5 μm apart. At 15°C, however, differentiation on 7.5 μm spaced ridges was significantly less than differentiation on ridges spaced 2.5 μm apart. For all temperatures, differentiation on 15 μm spaced ridges was less than differentiation on 7.5 μm spaced ridges but higher than differentiation on flat surfaces and 50 μm spaced ridges. Differentiation on flat surfaces and 50 μm spaced ridges was the same at all temperatures.

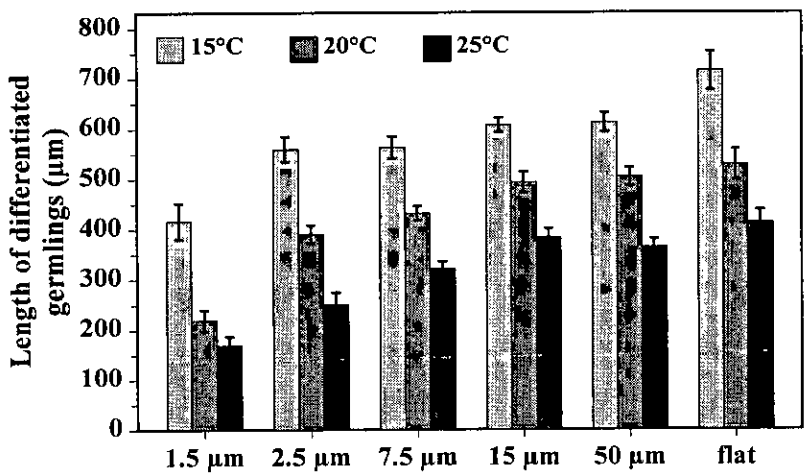

Figure 3.6. Length of differentiated germ tubes of *P. graminis* f. sp. *tritici*, induced by 2.0 μm high, 2.0 μm wide ridges spaced 1.5, 2.5, 7.5, 15 and 50 μm apart and on flat surfaces. Means ± sem.

Table 3.2. t-test analysis of differentiated germ tube lengths of *P. graminis* f. sp. *tritici*, induced by 2.0 μm high, 2.0 μm wide ridges spaced 1.5, 2.5, 7.5, 15 and 50 μm apart and on flat surfaces (N = not significantly different, degree of significance given).

Ridge spacings compared	15°C	20°C	25°C
1.5 μm v 2.5 μm	$p < 0.02$	$p << 0.0001$	$p < 0.01$
2.5 μm v 7.5 μm	N	N	$p < 0.02$
7.5 μm v 15 μm	N	$p < 0.02$	$p < 0.02$
15 μm v 50 μm	N	N	N
50 μm v flat	N	N	N

Growth prior to differentiation on ridges spaced 1.5 μm was significantly ($p < 0.05$) shorter than growth prior to differentiation on ridges spaced 2.5 μm at all three incubation temperatures (Table 3.2).

At 15°C, growth prior to differentiation on ridges spaced 2.5 μm, 7.5 μm, 15 μm and 50 μm was not significantly different. Growth prior to differentiation on flat surfaces was not significantly different to that on 50 μm spaced ridges (Figure 3.6).

Incubation at 20°C resulted in no significant difference in growth prior to differentiation on ridges spaced 15 μm, 50 μm and flat surfaces. Germlings differentiating on ridges spaced 7.5 μm were shorter than those induced on ridges spaced 15 μm. There was no significant difference in growth prior to differentiation on ridges spaced 2.5 μm and 7.5 μm.

At 25°C, the length of germlings prior to differentiation was shortest on ridges spaced 1.5 μm. Growth prior to differentiation on ridges spaced 2.5 μm was significantly ($p < 0.05$) shorter than growth on 7.5 μm spaced ridges, which in turn was shorter than growth on ridges spaced 15 μm. Growth prior to differentiation on ridges spaced 15 μm, 50 μm and flat surfaces were not significantly different.

The lengths of germlings prior to differentiation was affected by temperature on all topographies tested. For all ridge spacings tested, germling growth prior to differentiation at 15°C was significantly ($p < 0.05$) greater than germling growth at 20°C. At 15°C, two individual germlings grew over 1000 μm prior to differentiation. Growth prior to differentiation at 20°C was significantly greater on all ridge spacings tested than growth at 25°C, with the exception of ridges spaced 1.5 μm.

3.2.3 Time course of P. graminis *f. sp.* tritici *growth*

Uredospores of *P. graminis* f. sp. *tritici* were inoculated onto flat polystyrene and incubated in 100% RH for 24 h in the dark at 15, 20 and 25°C. Growth of the

primary germ tube was quantified by measuring 50 individual germlings which had been fixed at certain time points over the 24 h incubation period.

The growth curves for the three incubation temperatures (Figure 3.7) were very similar. After 24 h, the germlings incubated at 15°C and 20°C were the same length; the germlings incubated at 25°C were significantly shorter than those incubated at 15°C and 20°C ($p < 0.05$). At 12 h, germling growth at 25°C was significantly ($p < 0.02$) less than growth at 20°C, but not significantly different to growth at 15°C. Growth at 15°C at 12 h was not significantly different to growth at 20°C. During the part of the growth curve from 2 h to 12 h the linearly regressed rate of growth at 15°C (41.2 ± 5.1 μm/h; $r^2 = 0.96$) was the same as at both 20°C (43.5 ± 5.6 μm/h; $r^2 = 0.95$) and 25°C (35.7 ± 4.4 μm/h; $r^2 = 0.96$) incubations.

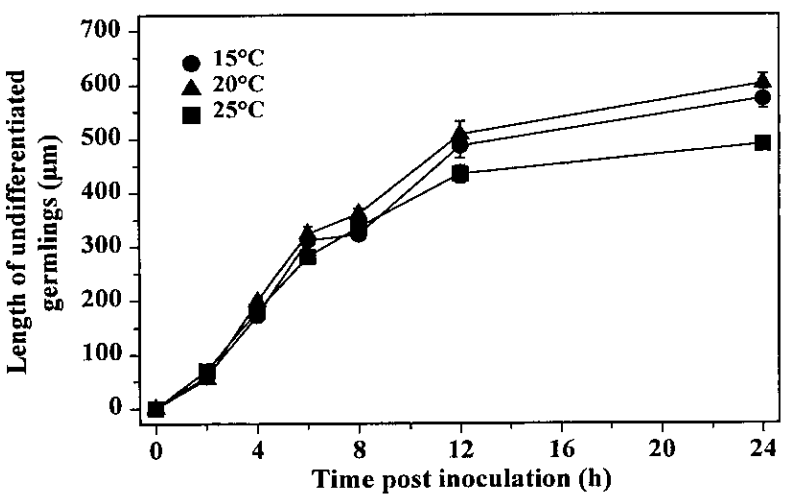

Figure 3.7. Lengths of primary germ tubes of *P. graminis* f. sp. *tritici* with time of incubation on flat polystyrene at 15°, 20° and 25°C. Means ± sem.

3.2.4 Differentiation on leaves and leaf replicas

Uredospores of each species were inoculated onto leaves and leaf replicas of their respective hosts and incubated in 100% RH at 22°C for 24 h in the dark. Differentiation was quantified by calculating differentiation over stomata as a percentage of the total number of germlings encountering stomata. The values were

therefore solely a reflection of the ability of the germlings to recognise a stoma, not the ability to locate a stoma.

The percentage of germlings that encountered a stoma varied from 60% to 80%. On host leaves, for all six rusts, nearly all germlings which encountered stomata formed appressoria over these stomata (88%–98%) (Figure 3.8). On host leaf replicas, approximately 25% of *P. graminis* f. sp. *tritici*, *P. graminis* f. sp. *avenae* and *P. hordei* germlings that encountered a 'stoma' formed appressoria over a stoma (Figure 3.8). *Puccinia coronata* f. sp. *avenae* was more responsive to the leaf replicas with 45% of germlings forming appressoria upon encountering a leaf replica stoma (Figure 3.8). *Puccinia graminis* f. sp. *secalis* was the most responsive to leaf replicas with nearly two thirds (62%) of the germlings that encountered stomata forming appressoria over them (Figures 3.8 & 3.9). *Puccinia recondita* f. sp. *tritici* formed the least appressoria (4%) in response to leaf replica stomata (Figure 3.8).

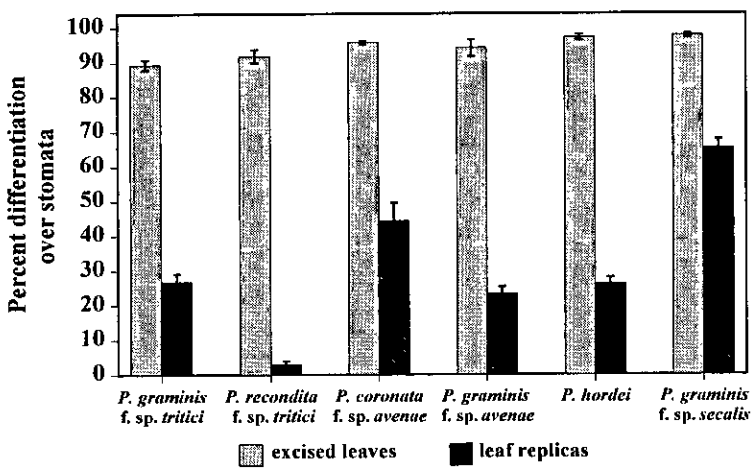

Figure 3.8. Differentiation over stomata on host leaves and polystyrene replicas of host leaves. Mean ± sem.

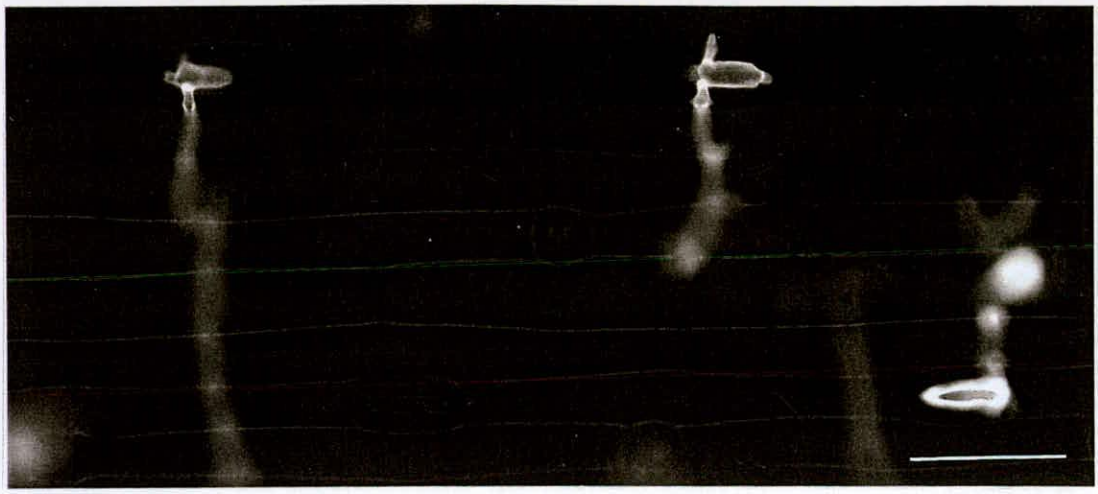

Figure 3.9. Appressoria of *P. graminis* f. sp. *secalis* over stomata on rye leaf replica imaged with combined UV and brightfield illumination (Calcofluor). Bar = 100 μm

Wheat leaf replicas were made from plants grown in continuous bright light and replicas made from plants kept in the dark. This was done in order to compare differentiation on leaf replicas with predominately open stomata to replicas with predominately closed stomata. Differentiation of *P. graminis* f. sp. *tritici* was not significantly different on the two types of leaf replica. Experiments using leaves with open stomata were not possible because it would have introduced further variables, e.g. light or fusiccoccin, making interpretation of any results difficult.

Appressorium morphology of each species on leaves and leaf replicas was the same as observed on the microfabricated substrata (Figures 3.3 & 3.4).

3.2.5 Comparison of leaf and leaf replica physical features

Wheat leaves and leaf replicas were compared to assess how well the physical features of the host were reproduced. Scanning electron microscopy revealed that there was little replication of the wax crystal microstructure (Figures 3.10A & B). On some replicas, stomatal apertures had globular structures associated with them (Figure 3.10C). The linear distance between cell junctions, however, was not significantly different on leaf replicas and host leaves (Table 3.3).

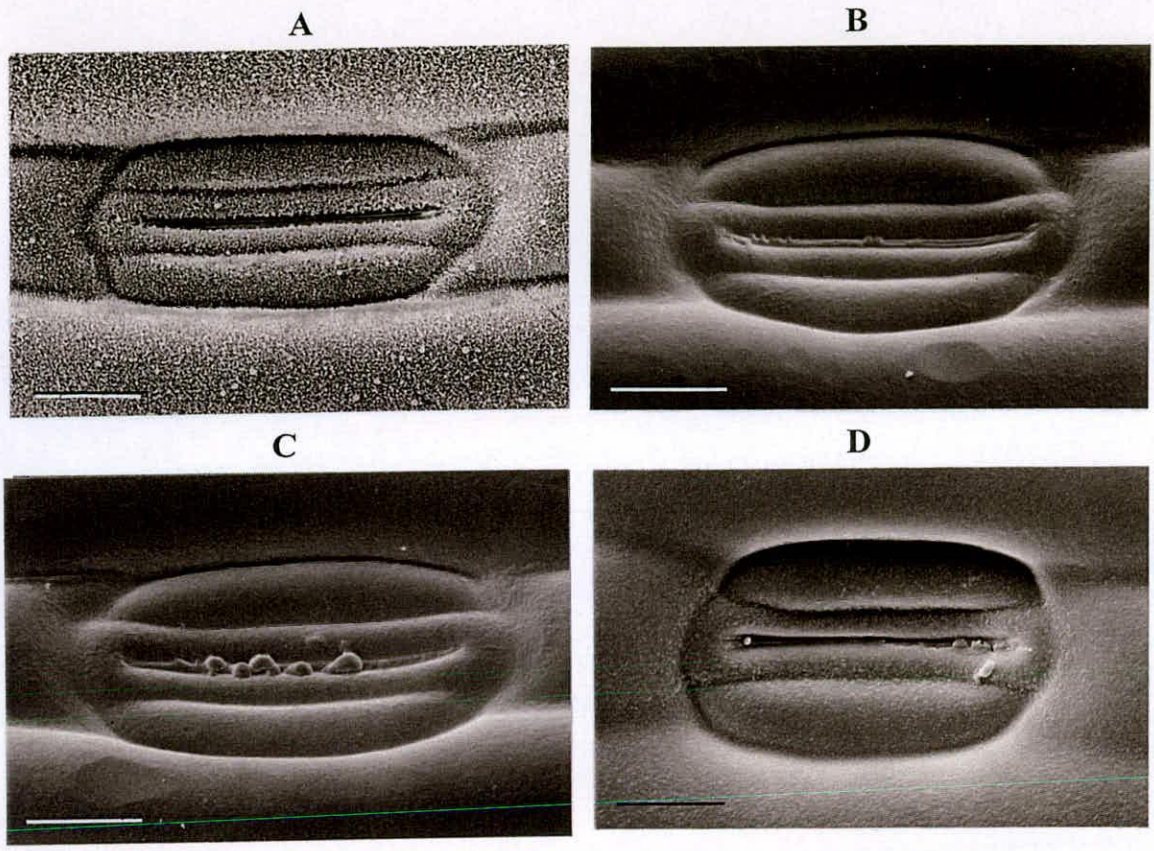

Figure 3.10. **A.** Wheat leaf stoma. Note the crystalline epicuticular wax. **B:** Wheat leaf replica stoma made with original method (Section 2.5) showing no replication of the structure of crystalline wax. **C:** Wheat leaf replica stoma made with original method illustrating globular structures between guard cells. **D:** Wheat leaf replica stoma made with modified method illustrating the improved reproduction of the wax microstructure (Section 2.5). Bar = 20 μm.

Table 3.3. Measurements of the widths (cell junction spacings) of epidermal cells, guard cells and companion cells on wheat leaves and wheat leaf replicas examined with brightfield microscopy. Means ± sem.

Cell type	Width on wheat leaf	Width on wheat leaf replica
Epidermal cell	25.0 ± 0.9 μm	23.5 ± 0.8 μm
Companion cell	11.0 ± 0.3 μm	11.1 ± 0.2 μm
Guard cell	4.7 ± 0.1 μm	4.5 ± 0.1 μm

Refinement of the method of leaf replication (see Section 2.5) resulted in better reproduction of the wax microstructure (Figure 3.10D) but, for *P. graminis* f. sp.

tritici on wheat leaf replicas, no corresponding increase in differentiation.

An attempt was made to obtain precise 3-dimensional measurements of wheat stomatal complexes in the z-axis by 'optical-sectioning' of intact wheat leaves using confocal microscopy and 3-D imaging techniques. This approach had several advantages over more conventional sectioning techniques. It allowed imaging of fresh tissue, definition of the position of the optical-section along the length of the stomatal complex, and ensured that the optical-section was at 90° to the long axis of the leaf.

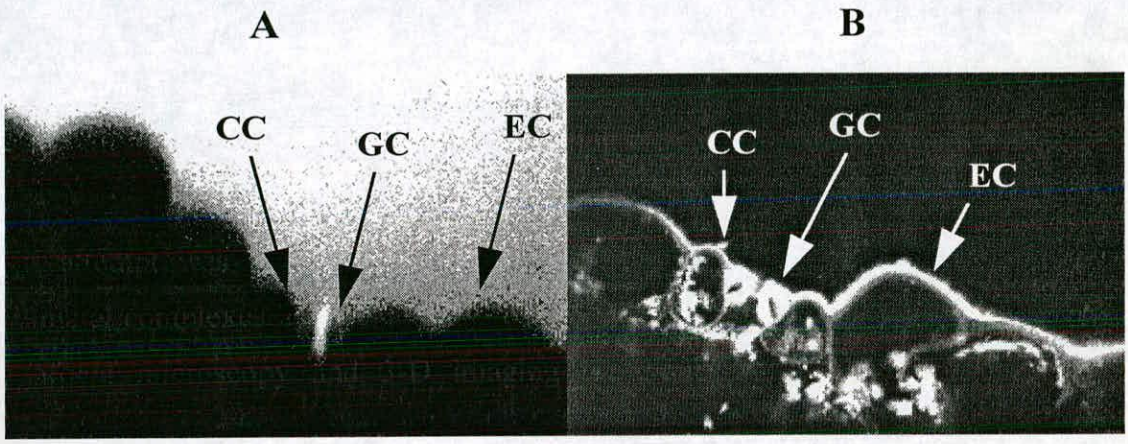

Figure 3.11. Z sections of stomatal complex obtained by: **A.** 3-D reconstruction of a series of x-y confocal optical sections; **B.** a single x-y confocal optical section of a thick hand cut section. **EC**, epidermal cell; **GC**, guard cell; **CC**, companion cell. Bar = 15 μm.

Although the resolution of the confocal optical section image was good enough for measurement of epidermal cell junction spacings, the resolution of the image around the stomatal complex was fairly poor and made measurements unreliable (Figure 3.11A). To determine whether any gross artefacts were being introduced to the confocal optical section image, hand sections of wheat were imaged. Using the confocal microscope, 1 μm optical sections of hand sectioned wheat leaves were made. Hand sectioned wheat leaves showed more detail of the cell junctions associated with the stomatal complex, but the cells had collapsed, altering their

surface topography (Figure 3.11B). Hand sectioning was useful as a comparison with the confocal optical sectioned images, confirming that optical artefacts associated with 3-dimensional microscopy were minimal.

Ratios of 3-dimensional and 2-dimensional cell widths were calculated for individual cells. The 2-dimensional measurement of epidermal cell junction spacings was approximately a 25% underestimate of the actual cell junction spacing. Guard cells were 32% underestimated and companion cells were 22% underestimated although the poor image quality makes these values estimations (Table 3.4).

Table 3.4. 2-Dimensional and 3-dimensional widths (see Fig. 3.12), and the ratio of the two values, of wheat epidermal, companion and guard cells measured by confocal optical sectioning. Mean ± sem.

Cell types	2-D widths (μm)	3-D widths (μm)	ratio 3-D : 2-D
Guard cells	5.1 ± 0.1	6.7 ± 0.2 [a]	1.32 ± 0.03 [a]
Companion cells	9.0 ± 0.3	11.0 ± 0.3 [a]	1.22 ± 0.02 [a]
Epidermal cells	19.7 ± 0.4	24.5 ± 0.6	1.25 ± 0.02

[a] these values are only estimates due to the poor image quality of the optical sections of the stomatal complexes.

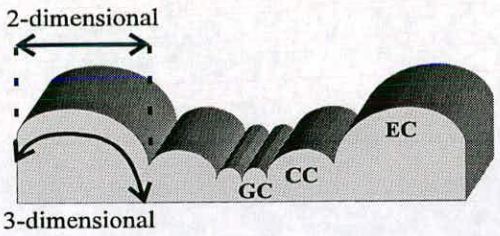

Figure 3.12. Illustration of the 3-D topography of a stomatal complex demonstrating 2-dimensional and 3-dimensional measurement of cell junctions.

The relative hyrophobicities of wheat leaves and leaf replicas were compared by assessing the contact angle of a 20 μl drop of distilled water. The contact angle of the wheat leaf (162° ± 2) was much greater than the contact angle made on the leaf replica (94° ± 7), demonstrating that the wheat leaf (Figure 3.13A) was more

hydrophobic than the wheat leaf replica (Figure 3.13B).

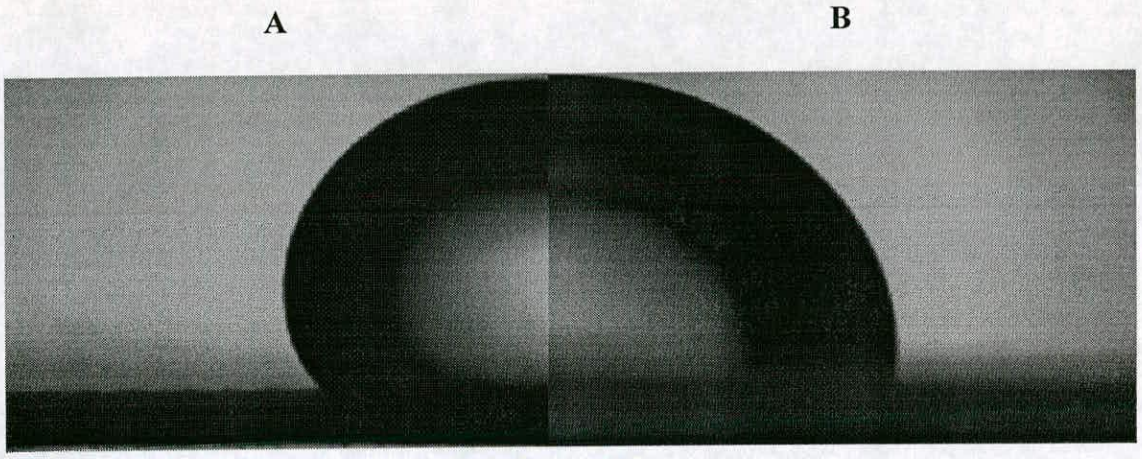

Figure 3.13. Micrographs illustrating the difference in the contact angle of a drop of water on **A:** wheat leaf and **B:** wheat leaf replica. Bar = 500 μm

3.3 Discussion

3.3.1 Differentiation on microfabricated topographies

All the cereal rusts studied were induced to form significant numbers of appressoria on 1.5 μm spaced ridges of certain heights. These results together with those reported by Read *et al.* (1996) show that induction of reproducibly high levels of appressorium differentiation on micro-fabricated substrata is a common feature of the cereal rusts.

Appressoria, when fully differentiated on artificial substrata, had undergone cytoplasmic migration, nuclear division and septation as described for *Uromyces appendiculatus* (Kwon *et al.*, 1991a). There was also a further round of mitosis in the substomatal vesicle. The morphology of infection structures varied between the species of rust (Figure 3.1A-D). The morphology of the SSV produced by all four species on microfabricated substrata was similar to those observed *in vivo* (Niks, 1986; Swertz, 1994). The morphology of the appressoria induced by microfabricated substrata for each species was the same as that seen over host stomata. These

observations suggest that the fungi were forming infection structures *in vitro* as they would *in vivo*.

The patterns of appressorium formation in response to multiple ridges of different heights varied between and even within rust species, as illustrated by the *forma specialis* of *P. graminis* (Figure 3.1A, C & E). The pattern of induction also did not correlate with the host species; the two rusts which infect wheat (*P. recondita* f. sp. *tritici* and *P. graminis* f. sp. *tritici*) had different responses, as did the two rusts which infect oat, *P. graminis* f. sp. *avenae* and *P. coronata* f. sp. *avenae*. This suggests that different rust species on the same host may respond to different inductive signals *in vivo*.

The pattern of response to microfabricated topographies for the isolate* of *P. graminis* f. sp. *tritici* (isolate 84, NIAB, Cambridge) tested by Read *et al.* (1996) was very similar to that observed in the race used here (race 32). One difference was that isolate 84 formed no appressoria on flat surfaces whilst race 32 formed approximately 10% (Figure 3.1A). The significance of this low level of spontaneous differentiation is unclear, but it was also seen in the isolate of *P. coronata* f. sp. *avenae* tested here (Figure 3.4F).

The response to different heights of multiple ridges by the race of *P. hordei* used here (race 32) showed the same overall pattern as the race of *P. hordei* (octal 1653) tested by Read *et al.* (1996). However, the maximum percentage differentiation was significantly different (race octal 1653 = 83% differentiation; race 32 = 36% differentiation), suggesting possible differences in topographical induction between races of this rust.

* The term isolate is used because it was not determined whether the spores were of uniform race.

The only cereal rusts which were induced to form significant numbers of appressoria on single ridges of any height were *P. graminis* f. sp. *secalis* and, to a much lesser extent, *P. recondita* f. sp. *tritici.* Allen *et al.* (1991a) also found that *P. recondita* f. sp. *tritici* could be induced to form appressoria in response to single ridges. One isolate tested (DB-66) formed up to 30% appressoria on optimal (0.5–0.7 μm high) single ridges. The response by a second isolate (89-516-YC1313) tested by Allen *et al.,* (1991a) was similar to that observed with the isolate tested here: maximum differentiation of about 10%. The inductive range of heights for the second isolate of Allen *et al.* (1991a) was, however, wider (0.5–0.9 μm) than that observed here (only 0.5 μm high ridges were inductive). The isolates used by Allen *et al.* (1991a) showed marked differences between each other and our results were different again. Differences in topographical induction of appressoria were also seen between races of *U. appendiculatus.* Forty races of *U. appendiculatus* showed similar patterns of response to single ridges of different heights, with the optimal height being 0.3–0.5 μm, but the mean percentage differentiation of each race varied between 100% and 47% (Allen *et al.,* 1991b). The variation seen in response to topography by the 40 races of *U. appendiculatus*, two races of *P. hordei* and the three isolates of *P. recondita* f. sp. *tritici* may demonstrate a general phenomenon between races of a particular rust. This variation in topographical sensing may create difficulties in developing host resistance based on a non-inductive leaf topography.

For *P. graminis* f. sp. *secalis*, the pattern of differentiation in response to single ridges of increasing height was similar to that seen in *P. hieracii*, *P. arachidis*, *P. sorghi*, *P. menthae*, *P. polysora* and *Physopella zeae* (Figure 1.4 from Allen *et al.*, 1991a). The species *P. graminis* therefore contains a sub-specific taxon which responds to single ridges and two others that only respond to multiple ridges. This suggests that the mechanism behind induction of appressoria by multiple ridges has fundamental similarities with induction by single ridges. For *P. graminis* f. sp. *secalis*, multiple ridges may have been slightly more inductive than single ridges because multiple ridged topography has more inductive single ridges.

For *P. graminis* f. sp. *tritici*, optimal percentage germling differentiation (approximately 70%) was induced by ridges spaced 1.5, 2.5 and 7.5 μm apart (Figure 3.5). Since germlings grow for varying periods prior to differentiation, the inductive nature of a topography can also be considered in terms of the time in which the fungus responds. The longer, in terms of length and/or duration, the germling grows over an inductive topography prior to differentiation, the less inductive the topography may be considered to be. On this basis, the optimal spacing of ridges was 1.5 μm, because germlings which had undergone differentiation were shortest on this topography at a given temperature (Figure 3.6). Since this was the smallest ridge spacing studied, the optimal spacing may be less than 1.5 μm. However, the percentage differentiation peaked at 7.5 μm spaced ridges; closer spaced ridges did not induce significantly higher differentiation. This suggests that ridges spaced closer than 1.5 μm may result in a shorter growth period prior to differentiation rather than a higher percentage of germlings differentiating.

Germlings grew for varying lengths prior to differentiation on a particular inductive topography, so differentiation was not induced by the same number of ridges for all germlings. This may be because some ridges are over-grown and presumably not sensed (Read *et al.*, 1992). Alternatively, it may be because individual germlings require slightly different inductive topographic signals. The width of guard cells ranged from 2.5μm to 6.1 μm. This range of widths was from a population of stomata from the same part of the leaf, the leaves having been grown at the same temperature and being the same age. In the field, where conditions are not so uniform, it is possible that the dimensions of stomata will vary much more. Variations in the dimensions of inductive topographical signals within a population may be an advantage to the fungus. The variation in response by a population to a particular signal has been a point of discussion in the field of plant growth regulators for quite some time. The premise is that individuals in a population have different thresholds for a given plant growth regulator. The range of thresholds, i.e. sensitivities, in a population accounts for the variation seen in dose response curves for the given plant growth regulator. It is proposed that variations in sensitivity,

rather than being experimental artefacts, have a physiological significance and provide "built-in plasticity to match the short term chaotic variations in environmental conditions" (Bradford & Trewavas, 1994). The variation in response to topographical features may not be an *in vitro* artefact but may represent a physiologically significant adaptation to the variable environment of the host leaf.

Another possibility is that some individuals in the population may rely on topographic signals alone to initiate appressorium induction, whilst other individuals may rely on alternative signals. The majority of the population may lie somewhere in between these two extremes, relying to a greater or lesser extent on each of several signals.

3.3.2 The effect of temperature on induction by topography

The different growth temperatures produced no differences in the percentage of germlings differentiating on the differently spaced ridges but did affect the length of germ tube growth before induction. On the multiple ridges there was very little growth in length prior to differentiation at 25°C; at 15°C, however, there was considerable growth (over 1000 μm in two instances).

From the calculated growth rate at each temperature, the mean time of differentiation can be estimated. At 25°C, germlings were on average induced to form appressoria approximately 4.5 h after incubation, whilst at 15°C, germlings were induced to form appressoria on average 11 h after incubation. These are only estimated times for several reasons. On multiple ridges 2-dimensional growth was measured, but the germlings were actually growing 3-dimensionally into grooves, making the linear measurement of growth an underestimate of the actual germ tube length. Also, it is unclear how the ridges affect the normal tip growth of germlings; growth rates were calculated from growth on flat surfaces and not multiple ridges. Germlings reorientate the direction of growth so that a germ tube leaves the encountered ridge at 90° to the direction of the ridge. This process may involve the reorganisation of the

cytoskeleton each time a ridge is encountered, which may slow growth significantly. Furthermore, the length of the primary germ tube will be affected by the extent of branching of the germling. On topographies with ridges spaced 1.5 μm apart there was no branching of the primary germ tube, whilst there was considerable branching (but no directional growth) on topographies with no ridges or ridges spaced 50 μm apart. The reason the experiments measuring growth over time were performed on flat surfaces was to ensure that growth was continuous over 24 h. If the experiment had been carried out on multiple ridges, a high percentage of germlings would have terminated linear growth when they differentiated. The estimated average time of induction at 25°C (4.5 h) may not be very accurate, but it is clear that most induction will have occurred by 12 h. Since it was only after 12 h that differences in growth between the incubation temperatures became apparent, the differences in induction of appressoria at the three incubation temperatures cannot be attributed to differences in growth rate.

Adhesion of the fungus to the substratum is critical for sensing the topographic signal (Epstein *et al.*, 1987). If adhesion was adversely affected by low temperatures, then more ridges might have been overgrown and not sensed before the inductive sequence of ridges was encountered. However, this does not explain why germlings which form appressoria on flat surfaces in the absence of topographic signals show a similar temperature dependent induction of appressoria. Approximately 10% of *P. graminis* f. sp. *tritici* germlings form appressoria in the apparent absence of inductive topographic features on glass, polystyrene, wheat leaves and leaf replicas and also in axenic culture (B. Moerschbacher, personal communication). Adhesion of the germling is dependent on the hydrophobicity of the germling (Clement *et al.*, 1994) and so the diverse nature of these substrata suggests that spontaneous induction is not likely to be dependent on adhesion of the fungus. Since induction on flat polystyrene surfaces was affected by temperature in a similar manner to differentiation induced by topographic signals, i.e. percentage differentiation is unaffected but growth prior to differentiation is longer at 15°C than at 25 °C (Figures 3.5 & 3.6), it seems that the

effect of temperature was not on adhesion to the substratum.

It has been suggested that MS ion channels are involved in both fungal tip-growth (Levina *et al.*, 1994) and induction of differentiation in *Uromyces appendiculatus* (Zhou *et al.*, 1991). In onion epidermis MS channels are also temperature sensitive (Ding & Pickard, 1993). The observed effect of temperature on topographical induction of appressoria may possibly be as a result of a similarly temperature sensitive MS channel.

The earlier induction of appressoria at higher temperatures (and hence shorter lengths of differentiated germ tubes) may be a result of the involvement of other signal(s) for appressorium induction. *Puccinia graminis* f. sp. *tritici* can be induced to form appressoria by a 30°C heat shock (Wanner *et al.*, 1985). Incubation at 25°C was not sufficient alone to induce appressoria (Figure 3.5) but may sensitise the germlings to the topographic stimulus sufficiently to result in a shorter growth period prior to differentiation. Other temperature dependent signals may include a metabolic product of the germlings. Acrolein, a volatile compound is associated with uredospores of *P. graminis* f. sp. *tritici*; it induces appressoria at very low concentrations (Macko *et al.*, 1978). It is possible that at the higher temperatures, inductive levels of acrolein, or other inductive metabolites, are reached sooner than at lower temperatures. The metabolite may act synergistically with topography making induction quicker at higher temperatures.

The aim of using different incubation temperatures was to determine whether the fungus recognises the topographical signal as either a temporally encoded or spatially encoded stimulus. This question can not be answered unambiguously from the results here since incubation temperatures between 15 and 25°C did not affect the growth rate of the fungus over the first 12 h. Differences were seen in induction of appressoria at the three incubation temperatures in terms of the length of growth prior to differentiation. If the signal was spatially encoded, there should have been no difference in induction of appressoria at the three incubation temperatures. Also, if

the signal was temporally encoded and since the growth rate was constant at the three temperatures, there should have been no differences in the response to topography at the three temperatures.

The fact that a 10°C temperature range (from 15° to 25°C) did not affect growth rate is quite unusual. The rusts *Hemileia vastatrix* (De Jong *et al.*, 1987) and *Tranzschelia discolor* (Ellison *et al.*, 1990) show temperature dependent germ tube growth over the temperature range 10–30°C. It is unclear as to why *P. graminis* f. sp. *tritici* should not show temperature dependent growth. What is apparent is that a constant growth rate would mean that the duration of growth between two topographic signals will be constant, irrespective of ambient temperature between 15°C and 25°C. The optimal temperature for development of wheat stem rust disease is 15°C to 30°C (Roelfs, 1992).

One curious aspect of the data was that after 24 h the length of the undifferentiated germlings at 15°C (575 μm) was shorter than the differentiated germlings (700 μm). However, undifferentiated germlings at 20°C and 25°C were on average longer than differentiated germlings. Germlings which undergo differentiation and stop linear growth are expected to be shorter than germlings which do not differentiate and continue to grow.

Whether the effect of temperature on differentiation has any significance *in vivo* is hard to say since topography may not be the only signal involved in appressorium induction *in vivo* (Grambow, 1977; Grambow & Reidel, 1977; Grambow & Grambow, 1978; see Chapter 4) and other mechanisms may compensate for poorer topographic induction at lower temperatures. Appressorium formation by *P. coronata* f. sp. *avenae in vivo* was not affected by temperature over the range 10–26.5°C (Politowski & Browning, 1975).

3.3.3 Differentiation on host leaves and leaf replicas

Unlike *U. appendiculatus* and *P. sorghi*, where differentiation on leaf replicas was the same as on host leaves (Wynn, 1976), differentiation of appressoria over stomata by cereal rusts was much lower on the leaf replicas than on leaves. The only rust to form numbers of appressoria over replica stomata close to the numbers observed over host stomata was *P. graminis* f. sp. *secalis*. This rust had a very different pattern of response to microfabricated topographies compared with the other cereal rusts, but a similar pattern of response to single ridges as *P. sorghi* (Allen *et al.*, 1991a). The patterns of induction of differentiation by microfabricated substrata with single ridges suggest that *P. graminis* f. sp. *secalis* was responding to a different topographic signal to the other cereal rusts studied, a signal that may have been better reproduced by the methods employed.

The low percentage of germlings forming appressoria over leaf replica stomata may have been because only a small percentage of the germlings were competent to respond to topography. However, this does not seem to be true since a high percentage of germlings were induced to form appressoria on microfabricated substrata (Figure 3.4).

The low response to replica stomata may be due to poor replication of the physical features of the host leaf to which germlings would normally respond. Electron microscopic analysis of wheat leaves and leaf replicas showed that the wax crystal microstructure was not reproduced in the leaf replicas. Also, globular structures were present between some guard cells. These structures are thought to have arisen through transpiration gases leaking through the stomata and into the polysiloxane polymer before it had set. It is possible that some of these bubbles may have interfered with the detection of the stomata by some germlings. However, the infrequency and small size of the bubbles makes it unlikely that they contributed significantly to the failure of germlings to respond to the leaf replica stomatal topography.

Another physical difference between the two substrata was their hydrophobicity, host leaves were much more hydrophobic than polystyrene leaf replicas. It has been shown that increasing the hydrophobicity of a substratum improves the response of *U. appendiculatus* to inductive topographic signals on that substratum (Terhune & Hoch, 1993). The very high hydrophobicity of wheat leaves is due to the chemistry of the epicuticular waxes. Also, the physical shape of the crystalline epicuticular waxes increases the hydrophobicity (measured by contact angle of a water droplet) of the leaf (Holloway, 1969).

A subsequent refinement to the method of making leaf replicas has allowed the wax crystal microstructure to be better reproduced (Figure 3.10D). However, there was no corresponding increase in differentiation over stomata for *P. graminis* f. sp. *tritici*.

Despite these differences between leaves and leaf replicas, the replication method does seem to be an improvement on the method employed by Wynn (unpubl. reported in Allen *et al.*, 1991c), where no differentiation of *P. graminis* f. sp. *tritici* was observed on leaf replicas.

An attempt was made to determine whether induction of *P. graminis* f. sp. *tritici* appressoria by stomatal topography varied with open or closed stomata. The results here show that *P. graminis* f. sp. *tritici* forms the same number of appressoria on leaf replicas prepared from plants with predominately closed stomata and predominately open stomata. This may have been because the replication technique did not reproduce the substomatal cavity, which might be an important topographical feature of an open stoma. Germination and growth of *P. graminis* f. sp. *tritici* are inhibited by light (Knights & Lucas, 1980), so appressorium formation *in vivo* probably occurs predominately over closed stomata.

Another reason for the low differentiation on leaf replicas may be that the signal(s) provided by the host is a combination of chemical features and topography. There is evidence that certain volatile compounds associated with the 'green-odour' of leaves,

notably *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol, can induce infection structures in *P. graminis* f. sp. *tritici* (Grambow, 1977). It seems likely that there are a range of signals provided by the host, including topographical and chemical signals, which, in combination, ensure that stomata are accurately recognised by germlings of cereal rusts (see Chapter 4).

3.3.4 Correlation of in vitro *and* in vivo *topographies*

Light microscopy measurement of cell junction spacings on leaf epidermis was a simple method for comparison of leaves and leaf replicas, but it only provided a 2-dimensional measurement of the cell junction spacings and not an accurate measurement of the 3-dimensional cell junction spacing. Confocal microscopic optical sectioning provided a non-invasive technique to determine 3-dimensional features. For measurements of leaf surface topography it had the advantage over conventional fixation, embedding and sectioning, in that the 'cross-section' could be precisely aligned before the section was taken. This ensured that the measurements were not taken obliquely to the longitudinal axis of the stomatal complex and, since the tissue was fresh, tissue shrinkage due to fixation and dehydration was avoided. There were problems with resolution of the stomatal apertures due to the high fluorescent signal from the guard cell lips (see Section 4.2). Other possible artefacts were refraction effects as the signal passed through surrounding cells. This was primarily a problem with guard cells since these were generally sunken in grooves. Measurements of epidermal cells where these problems appeared to be minimal (Figure 3.11) showed that the linear measurement of cell junctions was approximately a 25% underestimate of actual cell junction spacing (Table 3.4). More detailed analysis of the stomatal complex topography is required using confocal optical sectioning and conventional sectioning techniques.

To correlate the *in vitro* topographical signals with those found *in vivo*, the *in vitro* topographic signal must be defined. Defining the precise dimensions of the inductive microfabricated topographic feature which provides the inductive signal for *P.*

graminis f. sp. *tritici* is not possible for several reasons. The germlings grew for different lengths prior to differentiation, growing over a variable number of ridges. Calculation of the number of ridges encountered by a germling which has differentiated does not provide a satisfactory definition of the inductive topographic signal because the germling may not grow into the groove associated with two closely spaced ridges, and therefore not sense the ridges (Read *et al.* 1992). Low angle SEM would allow quantification of the number of grooves into which the germling grew prior to differentiation, and therefore provide a better indication of the precise inductive sequence of ridges.

However, the ridges of the artificial topographies are comprised of four right angles; it is thought that right angles, not ridges *per se*, are the topographical signals for *U. appendiculatus* (Hoch *et al.*, 1987b). Although the ridges on the topographies are spaced equally, each ridge is made up of four right angles, so for a given topography there is a number of sequences of right angles on the substratum.

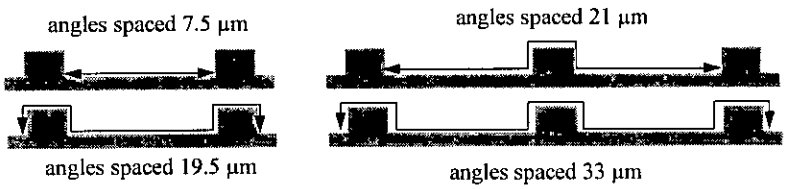

Figure 3.14. Illustration of the different spacings of pairs of right angles in the 7.5 μm spaced, 2.0 μm wide, 2.0 μm high ridge topography.

For example, two ridges spaced 7.5 μm apart there are two right angles spaced 7.5 μm, two spaced 19.5 μm and a number of different spacings in between (Figure 3.14). These are the two extremes of right angle spacing for two ridges on this topography. The spacings of angles within single ridges are non-inductive for *P. graminis* f. sp. *tritici* (Figure 3.4A). Subsequent ridges on the topography increase the range of topographic signals further (Figure 3.14). This assumes that the inductive signal is only two right angles, but it is possible that three or more right

angles provide the inductive signal increasing the permutations of right angles further. All this assumes that the germling follows the contours of the substratum exactly, which was not always the case (Allen *et al.*, 1991a; Read *et al.*, 1992).

The closer spaced ridges may be most inductive because there is a greater variety of sequences of right angles per length of germ tube growth. For example if a germling grows for 50 μm on 1.5 μm spaced ridges, it will overgrow approximately 56 right angles compared to 12 right angles on a topography with 15 μm spaced ridges.

Since we cannot precisely define the *in vitro* topographic signal, accurately correlating it with the *in vivo* topography was not possible. However, a rough correlation between *in vitro* and *in vivo* topographies can be made. The artificial topography with 7.5 μm spaced ridges was the artificial topography most closely resembling the dimensions associated with any single stomatal topographic feature, namely guard cell junctions (spaced 5.9 ± 0.16 μm). The 7.5 μm spaced ridges were also the widest ridge spacing tested that induced optimal percentage differentiation. The ridges spaced 15 μm apart induced significant differentiation (approximately 40%). The dimension of the 15 μm spaced ridges corresponds most closely with the sum of the companion cell junction spacing (approximately 11 μm) and the guard cell junction spacing (approximately 6 μm), i.e. the distance between the outer companion cell junction and the stomatal aperture (6 + 11 = 17 μm). Differentiation induced by 15 μm spaced ridges was sub-optimal, but so was induction by leaf replicas, where only 25% of germlings formed appressoria over stomata.

It is thought that directional growth in the cereal rusts increases the probability of stomatal location since stomata on grasses are arranged in staggered parallel rows. The morphology of cereal stomata, i.e. long and thin with respect to the longitudinal axis of the leaf, means that they would be present a larger target to be encountered 'broad-side' by a approaching germ tube. Also it is possible that, if the germling did not show directional growth, it would be trapped into following the paths of the epidermal cell junctions. If the fungus was trapped into following cell junctions, then the arrangement and shape of epidermal cells on a cereal leaf would make it unlikely that a germ tube would encounter a stomatal aperture. Furthermore, if the spacing of cell junctions associated with the stomata is the topographical signal for appressorium induction, then contact-mediated directional growth may play a role in ensuring that the signal is correctly perceived. If the germling did not show directional growth, it would approach a stoma at a random angle. The distance between cell junctions would then be dependent on the angle of approach to the stoma (Figure 3.15). For example, a germling growing across a wheat guard cell at 90° to the longitudinal axis of the leaf perceives the guard cell junction spacing correctly as approximately 6 μm. A germling growing across the guard cell at 15° to the longitudinal axis of the host, however, perceives the cell junction spacing as 23 μm [= 6 μm ÷ sin(15°)], which is equal to the distance between epidermal cell junctions. Directional growth will therefore ensure that the distance between cell junctions is only dependent on epidermal cell type, not on the angle at which the cell is approached.

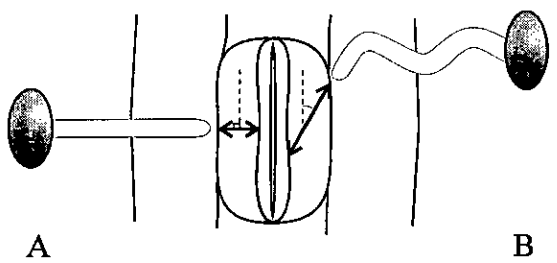

Figure 3.15. Diagram illustrating how the perceived distance between cell junctions varies with the angle of approach to the stoma. Spore **A** approach is at 90° to the longitudinal axis of the leaf; spore **B** is at an acute angle.

The shortcomings of the 'square' ridge artificial topographies have been highlighted. For more certain definition of the precise spacing of the topographic signal, very

large single ridges are required (possibly 15 μm high) but this is not practicable with the technique used to manufacture the silicon wafers. Alternatively, a topography with ridges with only one acute angle per ridge (Figure 3.16) could be microfabricated.

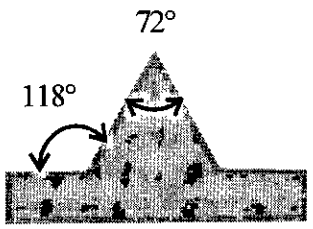

Figure 3.16. Diagram of a single ridge with a single acute angle (72°) manufactured by anisotropic etching of a silicon (100) wafer.

This is possible with the technique of anisotropic etching. This process is fundamentally the same as the technique used to manufacture the wafers with 'square' ridges (isotropic etching), but uses a different form of silicon wafer [Silicon (100) wafers]. The shape of an anisotropically etched groove is determined by the crystalline orientation of the wafer surface (Brunette, 1986).

4. INDUCTION OF APPRESSORIA BY HOST CHEMICAL SIGNALS

4.1 Introduction

Grambow and co-workers have shown that *P. graminis* f. sp. *tritici* can be induced to form appressoria by the alcohols *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol on agar in the presence of boiled leaf extract, a range of salts, sucrose and casein (Grambow & Reidel, 1977; Grambow, 1977). The first aim of the work presented in this chapter was to determine whether *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol were inductive in solution in the absence of the complex culture medium. The second objective was to determine whether these chemicals induce appressoria when in a volatile form. Since *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol are synthesised in the mesophyll, but would act as inducers of appressorium differentiation at the stomatal aperture, then they must be active as volatile compounds. The third objective was to investigate the interaction of chemical and topographic signals to determine whether they act synergistically or independently. A fourth objective was to determine whether other chemical factors were localised in the region of the stomatal aperture and therefore might potentially provide further signals for appressorium induction.

4.2 Results

4.2.1 *Induction of appressoria by liquid* cis-*3-hexen-1-ol and* trans-*2-hexen-1-ol*

Uredospores were inoculated onto flat polystyrene substrata which were inverted (spore side down) onto solutions of *cis*-3-hexen-1-ol or *trans*-2-hexen-1-ol and incubated for 24 h at 22°C in the dark.

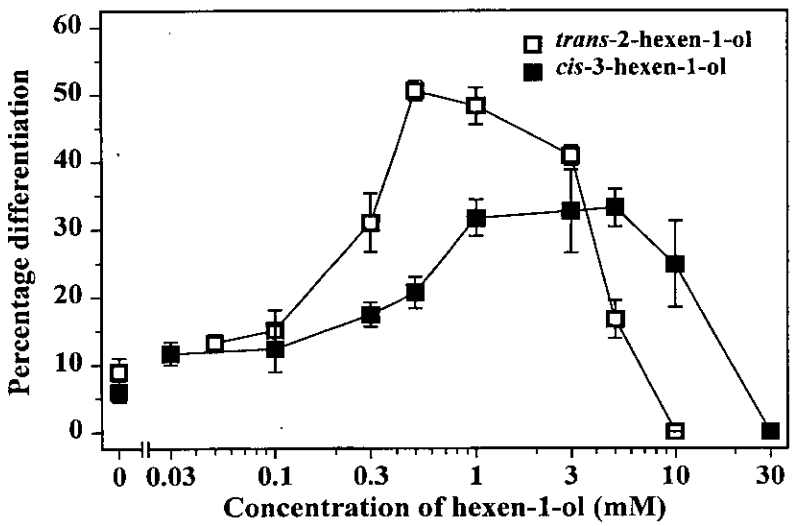

Figure 4.1. Appressorium formation by *P. graminis* f. sp. *tritici* on flat polystyrene in response to *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol solutions. Means ± sem.

Both *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol induced appressoria on flat polystyrene surfaces after 24 h incubation. Induction was optimal in the range 0.5–1 mM for *trans*-2-hexen-1-ol (approximately 48 % induction) and the range 1–10 mM for *cis*-3-hexen-1-ol (approximately 30% induction) (Figure 4.1). Further experiments used *trans*-2-hexen-1-ol since it was more inductive than *cis*-3-hexen-1-ol as a liquid. Growth and germination were inhibited at concentrations above 2 mM for *trans*-2-hexen-1-ol and above 5 mM for *cis*-3-hexen-1-ol.

4.2.2 Induction of appressoria by trans*-2-hexen-1-ol vapour*

To determine whether *trans*-2-hexen-1-ol was inductive as a vapour, solutions of *trans*-2-hexen-1-ol in small Petri dish lids were placed in sealed Petri dishes next to inoculated flat polystyrene substrata. Only *trans*-2-hexen-1-ol was tested for activity as a vapour since it was more inductive as a liquid than *cis*-3-hexen-1-ol (Section 4.2.1). Raoult's law states that the vapour pressure of a component of an ideal solution is equal to the product of its mole fraction and the vapour pressure of the pure liquid. Whether *trans*-2-hexen-1-ol is an ideal liquid needs to be determined empirically, but the different concentrations of *trans*-2-hexen-1-ol will have had different partial pressures of *trans*-2-hexen-1-ol.

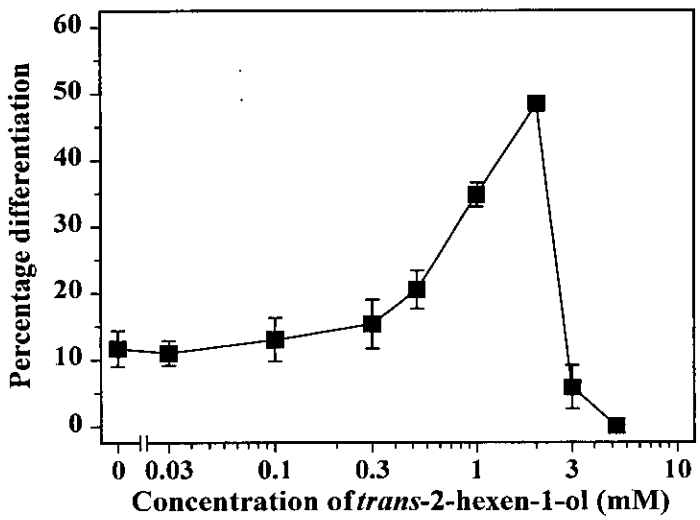

Figure 4.2. Appressorium formation by *P. graminis* f. sp. *tritici* on flat polystyrene induced by vapour from *trans*-2-hexen-1-ol solutions. Means ± sem. Control was 0 mM *trans*-2-hexen-1-ol, which was distilled water.

Appressoria were induced by vapour from *trans*-2-hexen-1-ol solutions between 0.5 mM and 2.0 mM. At the optimal concentration of *trans*-2-hexen-1-ol solution (2 mM) 48.5 ± 0.5% of germlings formed appressoria (Figure 4.2). Inhibition of growth and differentiation occurred at concentrations above 2 mM.

4.2.3 Topography and trans*-2-hexen-1-ol in combination*

Microfabricated substrata were inoculated with uredospores and incubated spore-side down in *trans*-2-hexen-1-ol solutions. Three treatments were applied. The first combined optimal inductive topography (1.5 μm spaced, 2.0 μm high ridges) with optimal inductive *trans*-2-hexen-1-ol solution (1 mM). The second combined sub-optimal inductive topography (0.5 μm spaced, 2.0 μm high ridges) with sub-optimal inductive *trans*-2-hexen-1-ol solution (0.3 mM). The third treatment combined topography with ridges 0.223 μm high (the height just below the lowest inductive ridges, Figure 3.4A) with 0.1 mM *trans*-2-hexen-1-ol, which was just lower than the minimum inductive concentration (Figure 4.1). The aim of this experiment was to determine whether the two signals had synergistic or independent effects.

The combination of optimal topography with the optimal concentration of *trans*-2-hexen-1-ol resulted in 85% of germlings forming appressoria after 24 h (Figure 4.3A). Alone, the topography induced 60% differentiation and the optimal concentration of *trans*-2-hexen-1-ol induced 42% differentiation.

Combination of sub-optimal topography and sub-optimal *trans*-2-hexen-1-ol treatments resulted in a percentage differentiation equivalent to the sum of the percentage differentiation induced by each treatment alone (Figure 4.3B):

% differentiation induced by combination	≈60%
% differentiation induced by *trans*-2-hexen-1-ol	≈25%
% differentiation induced by topography	≈40%

Non-inductive topographic and *trans*-2-hexen-1-ol signals remained non-inductive even when combined (Figure 4.3C).

One mM *trans*-2-hexen-1-ol, optimal for induction of *P. graminis* f. sp. *tritici* appressoria, failed to induce appressoria in *P. recondita* f. sp. *tritici* and did not enhance appressorium formation in response to inductive topography (1.5 μm

spaced, 2.0 μm high, 2.0 μm wide ridges) (Figure 4.3D).

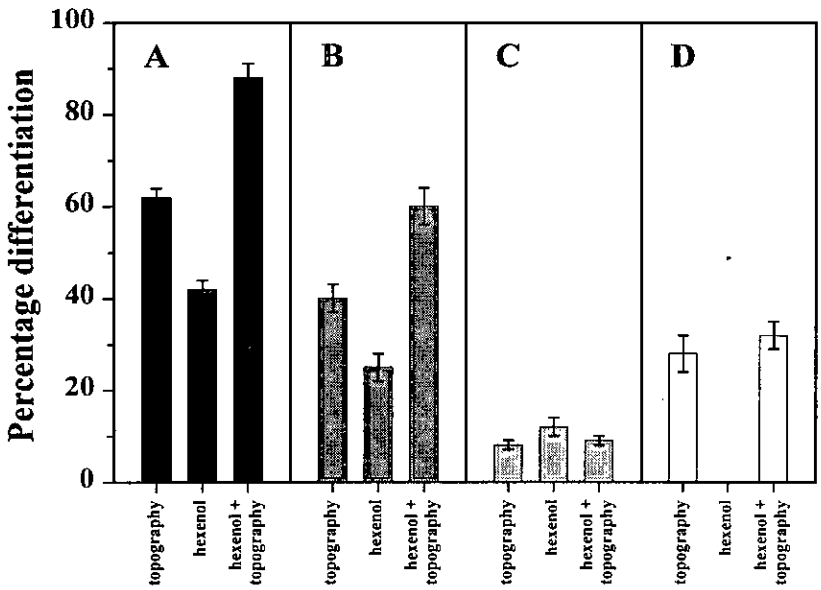

Figure 4.3. Induction of appressoria of *P. graminis* f. sp. *tritici* by *trans*-2-hexen-1-ol, topography and the two combined. **A.** 1 mM *trans*-2-hexen-1-ol and 2.0 μm high, 1.5 μm spaced ridges (optimal conditions); **B.** 0.3 mM *trans*-2-hexen-1-ol and 0.5 μm high, 1.5 μm spaced ridges (sub-optimal); **C.** 0.1 mM *trans*-2-hexen-1-ol and 0.223 μm high, 1.5 μm spaced ridges (sub-inductive). **D.** Differentiation of *P. recondita* f. sp. *tritici* in response to 1 mM *trans*-2-hexen-1-ol and 2.0 μm high, 1.5 μm spaced ridges. Means ± sem.

The time course of differentiation induced by either optimal topography or 1 mM *trans*-2-hexen-1-ol was similar. Differentiation started after 4 h and increased over the 24 h period (Figure 4.4). During the linear phase of appressorium formation (4–8 h) appressoria were induced at a rate of 10.0 ± 1.2 % h^{-1} (r^2 = 0.98) by *trans*-2-hexen-1-ol. The linear phase of appressorium formation lasted longer for topographical induction (4–12 h), and occurred at a slower rate (5.1 ± 1.1 % h^{-1}, r^2 = 0.99).

When the topography and *trans*-2-hexen-1-ol were combined, however, there was differentiation (12%) within 2 h and 85% of germlings formed appressoria within 4 h. There was no significant differentiation after 4 h (Figure 4.4).

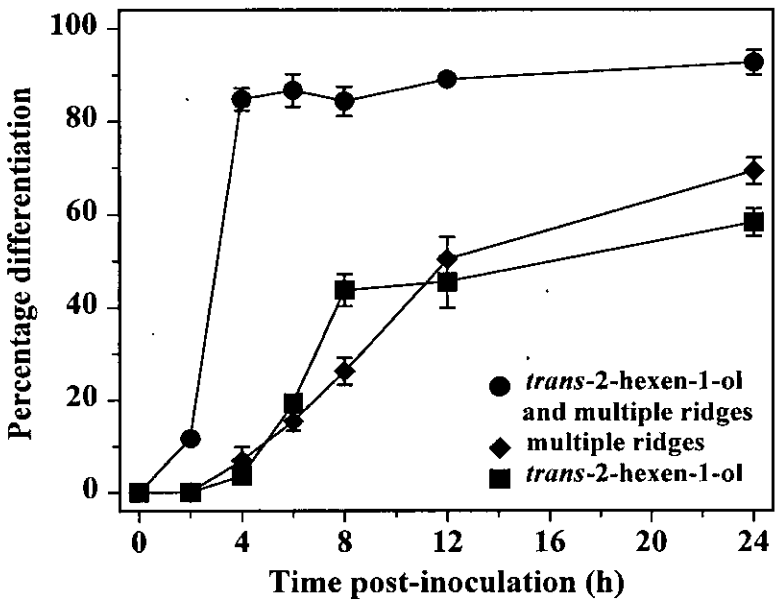

Figure 4.4. Time course of differentiation of *P. graminis* f. sp. *tritici* induced by topography, 1 mM *trans*-2-hexen-1-ol and the two treatments combined. Means ± sem.

After 24 h, FDA/PI live/dead staining showed that very few germlings (5.0 % ± 1.8) which had not undergone differentiation were still alive, whilst a third of germlings (32.3% ± 3.6) which had differentiated were still alive. Therefore, further significant differentiation of germ tubes after 24 h was not likely to occur.

4.2.4 Leaf replicas with trans*-2-hexen-1-ol*

Uredospores were inoculated onto wheat leaf replicas and incubated in either dH_2O or 1 mM *trans*-2-hexen-1-ol for 24 h in the dark at 22°C. Appressorium formation over guard cells (correctly placed) and epidermal cells (misplaced) was quantified. The percentage of the leaf area occupied by guard cells was calculated by determining the average area of a pair of guard cells and the stomatal density for each replica.

Table 4.1. Differentiation of *P. graminis* f. sp. *tritici* appressoria on leaf replicas in the presence and absence of 1 mM *trans*-2-hexen-1-ol. Means ± sem.

Treatment	Appressoria over guard cells	Appressoria over epidermal cells
dH_2O	27.6 ± 1.9%	14.7 ± 2.5%
trans-2-hexen-1-ol	29.3 ± 3.4%	58.7 ± 3.7%

Differentiation over guard cells was not significantly different in the presence or absence of *trans*-2-hexen-1-ol (Table 4.1). Differentiation over the rest of the epidermis was significantly greater in the presence of *trans*-2-hexen-1-ol. The guard cells accounted for 2.1 ± 0.2% of the leaf replica area. If the differentiation induced by *trans*-2-hexen-1-ol was random, then it would be expected that 2.1% of the total 'random' differentiation (58.7%) to be over guard cells. This would contribute another 1.3% differentiation over stomata in the *trans*-2-hexen-1-ol treatment compared to the dH_2O treatment, making the total expected differentiation over stomata 28.9%. This expected value is within the 95% confidence limits of the observed differentiation over stomata in the presence of *trans*-2-hexen-1-ol. There was therefore no significant synergy between *trans*-2-hexen-1-ol and leaf replica topography.

Appressoria which were not formed over the stomatal aperture tended to be formed in epidermal cell junctions.

4.2.5 Appressorium morphology

Lengths (perpendicular to the direction of growth) and widths (at 90° to the direction of growth) of appressoria induced by optimal topography, optimal *trans*-2-hexen-1-ol and the two treatments combined were made.

Appressoria induced by *trans*-2-hexen-1-ol and topography had the same morphology (≈ 15 × 23 μm) but those induced by topography were oriented at 90° to the direction of growth whilst those induced by *trans*-2-hexen-1-ol were oriented in

the direction of growth (Figure 4.5). Topographically induced appressoria were wide (23.2 ± 2.0 μm) and short (16.1 ± 1.3 μm), whilst chemically induced appressoria were long (23.3 ± 1.2) and thin (13.3 ± 1.4 μm) (Figure 4.5). The mean length of appressoria induced by topography was not significantly different from the mean width of appressoria induced by *trans*-2-hexen-1-ol. Similarly, the length of appressoria induced by *trans*-2-hexen-1-ol was not significantly different from the width of appressoria induced by topography. Appressoria induced by topography and *trans*-2-hexen-1-ol in combination were significantly wider (38.2 ± 1.1 μm) than appressoria induced by topography alone but the same length (13.5 ± 1.6 μm) (Figure 4.5).

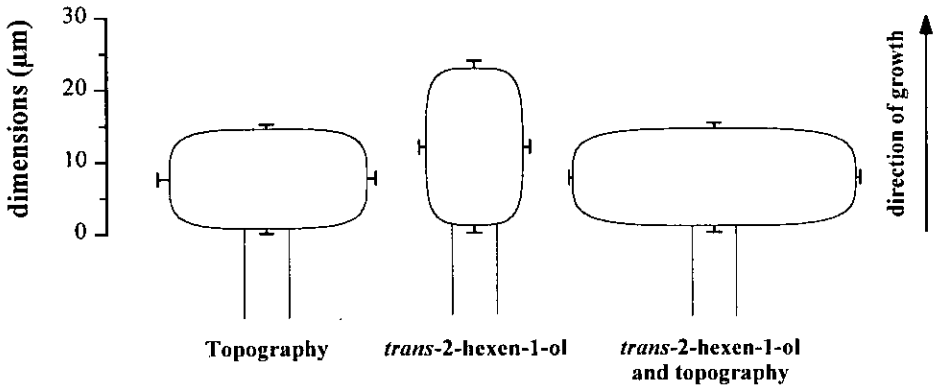

Figure 4.5. Diagrams representing the morphology of *P. graminis* f. sp. *tritici* appressoria induced by topography, *trans*-2-hexen-1-ol and the two treatments combined. Means ± sem.

It was also noted that appressoria induced by *trans*-2-hexen-1-ol on a non-inductive, 2.0 μm high, single ridge were short and wide unlike those induced by *trans*-2-hexen-1-ol alone but similar to those induced by topography alone (Figure 4.6). Appressoria induced on epidermal cells of leaf replicas in the presence of *trans*-2-hexen-1-ol were also similar in morphology to appressoria induced by topography alone.

Appressoria induced by *trans*-2-hexen-1-ol formed normally, with cytoplasmic migration into the swollen germ tube apex, nuclear division and septation (Figure 4.7).

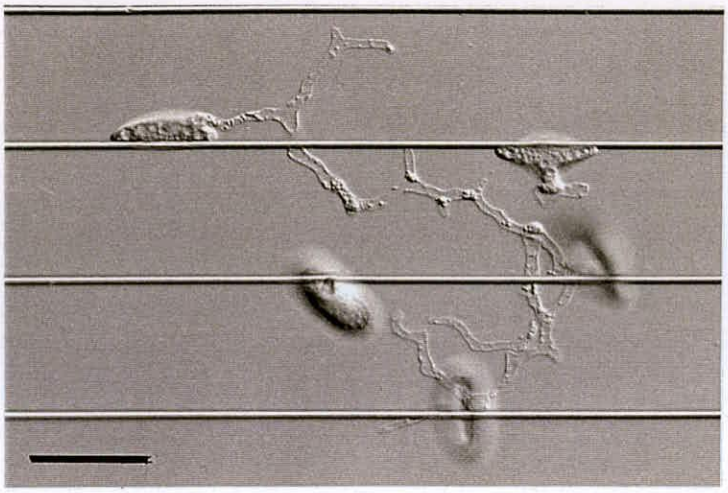

Figure 4.6. Appressoria of *P. graminis* f. sp. *tritici* induced by 1 mM *trans*-2-hexen-1-ol when germ tubes grew over or against a non-inductive single ridge 2.0 μm high. Bar = 30 μm.

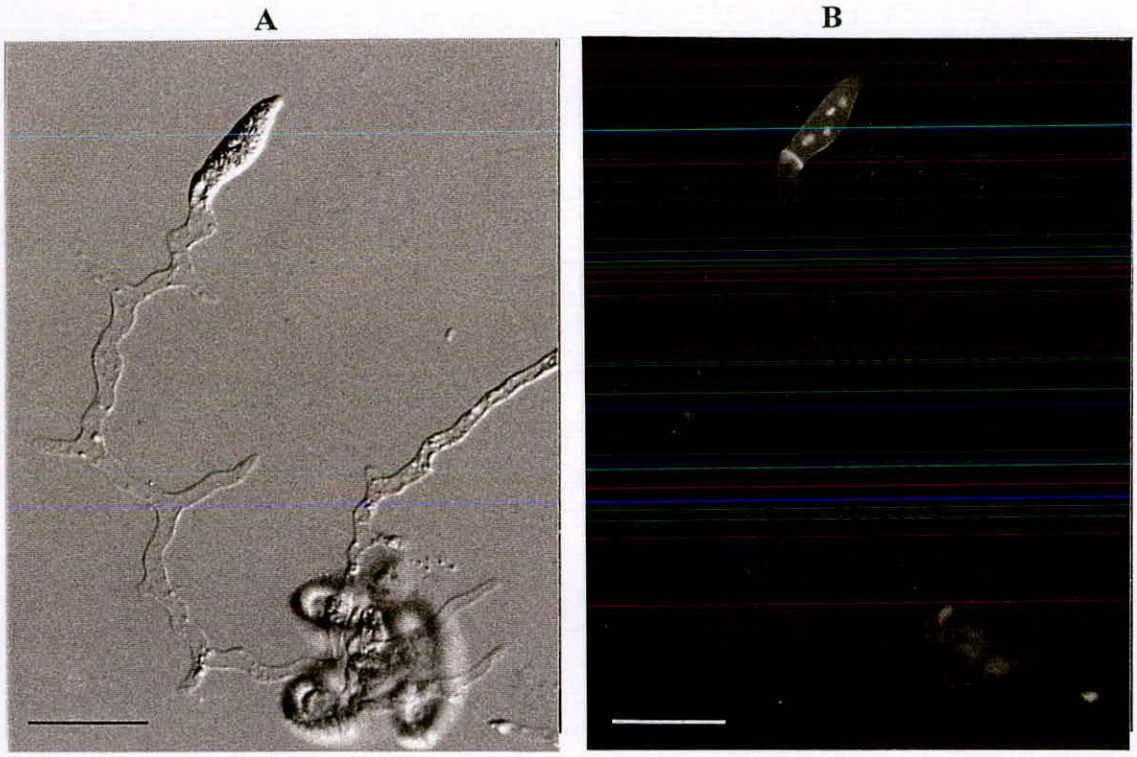

Figure 4.7. Appressorium of *P. graminis* f. sp. *tritici* induced by 1 mM *trans*-2-hexen-1-ol showing (**A**) cytoplasmic migration into the swollen germ tube apex (DIC), (**B**) nuclear division and septation (Calcofluor and DAPI). Bars = 30 μm.

4.2.6 Differential staining of wheat cuticle

Staining of the leaf surface with Nile red showed clear differences between the cuticle associated with the guard cell ledges and the rest of the cuticle. This was not due to accumulation of dye in the stomatal aperture since open stoma clearly show the two ledges associated with them (Figure 4.8). This staining is thought to indicate the presence of cutin (Palevitz, 1981).

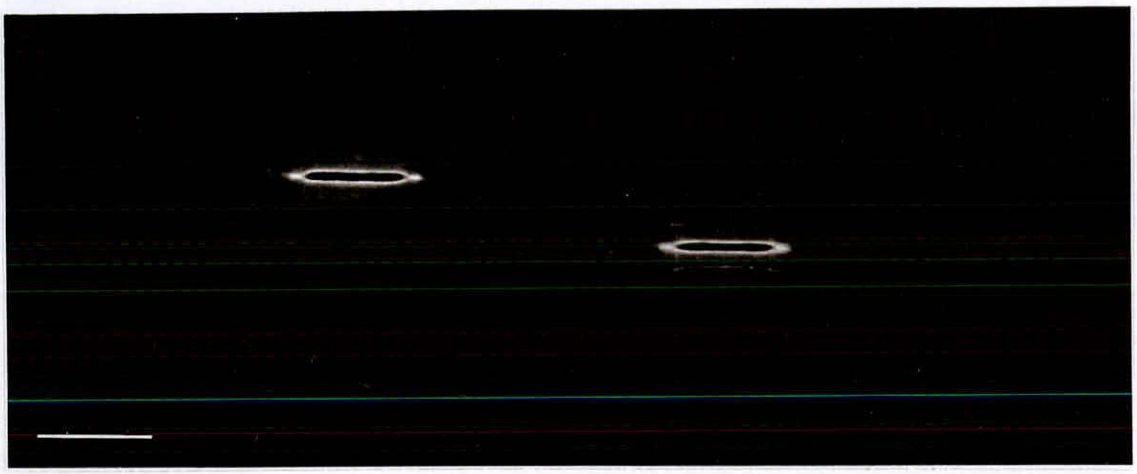

Figure 4.8. Wheat stomatal complex stained with Nile red and viewed with UV epifluorescence. Note the staining of guard cell ledges only. Bar = 50 μm

Some cutin monomers (oleic acid, stearic acid, linoleic acid and palmitic acid) were tested for induction of appressoria on flat polystyrene substrata by incubation in 100 μM of each monomer in MES/HEPES buffer at pH 7.0. No monomer was found to induce appressoria. At 100 μM linoleic acid inhibited growth and germination and was subsequently tested for inductive activity at 10 μM. At this concentration there was no inhibition of growth, nor any induction of appressoria.

There was little autofluorescence associated with guards cell in distilled water, but there was autofluorescence from chlorophyll in the mesophyll cells. Ammonia enhanced autofluorescence, indicative of esters of phenolic acids; this was evident on guard cell cuticles (Figure 4.9). This enhanced autofluorescence was reversible by removing the ammonia solution, distinguishing it from autofluorescence of lignin (Palevitz, 1981). The phenolic acids, *p*- coumaric, ferulic and diferulic, all

associated with the cell walls of cereals, had no inductive activity at 100, 100 and 20 μM respectively, either alone or in combination with inductive topography.

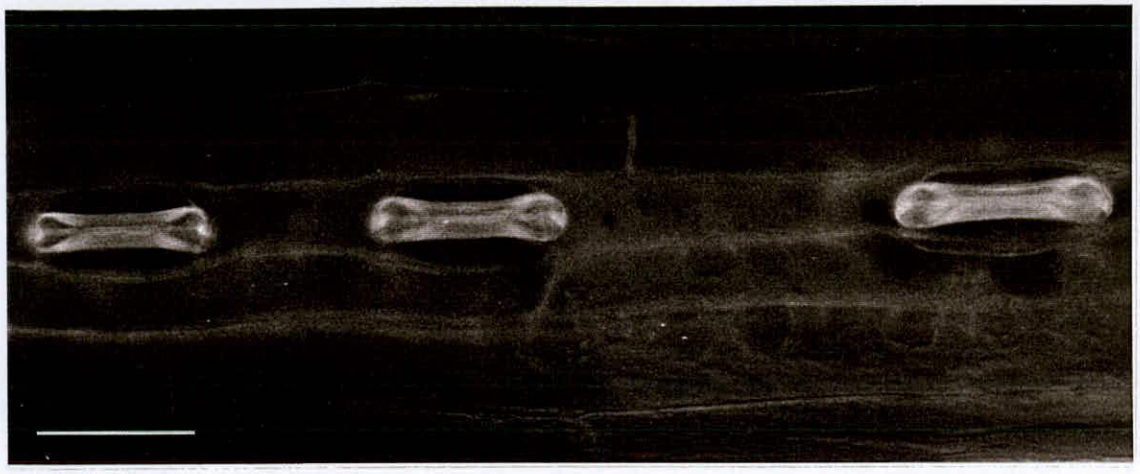

Figure 4.9. Ammonia enhanced UV induced autofluorescence of wheat epidermis. Bar = 100 μm.

4.3 Discussion

4.3.1 trans-*2-hexen-1-ol and* cis-*3-hexen-1-ol*

Grambow (1977) found that the volatile compounds *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol induced appressoria, although their activity was greatly enhanced in the presence of leaf extract. It is unclear why the leaf extract had an effect. It is possible that the leaf extract contained these compounds and simply increased the concentration of each of the volatile compounds present. However, the extract may also have contained other chemical factors which acted in synergy with the volatile alcohols.

The induction of appressoria by *trans*-2-hexen-1-ol in axenic culture raised questions as to whether the high nutrient environment influenced appressorium induction (Grambow, 1977). It has been shown for *U. appendiculatus* that sugars and inorganic ions affect appressorium formation (Kaminskyj & Day, 1984a; Kaminskyj & Day, 1984b; Hoch *et al.*, 1987a). The high nutrient environment of the *in vitro* assay in

axenic culture compared to the relatively nutrient poor environment of the phyllosphere raised questions as to the significance of the results *in vivo*. Work here shows that neither the leaf extract nor complex culture medium is required for the induction of appressoria by *trans*-2-hexen-1-ol or *cis*-3-hexen-1-ol.

Both *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol induced appressorium formation *in vitro* in the absence of topographical or other host signals. Induction by *trans*-2-hexen-1-ol was significantly higher than induction of appressoria by *cis*-3-hexen-1-ol and so *trans*-2-hexen-1-ol was used for subsequent experiments. At concentrations above that which was optimal for induction, differentiation, germination and germ tube growth were inhibited. *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol are two of the C6 derivatives from the lipoxygenase pathway in plants, part of which is shown in Figure 4.4.

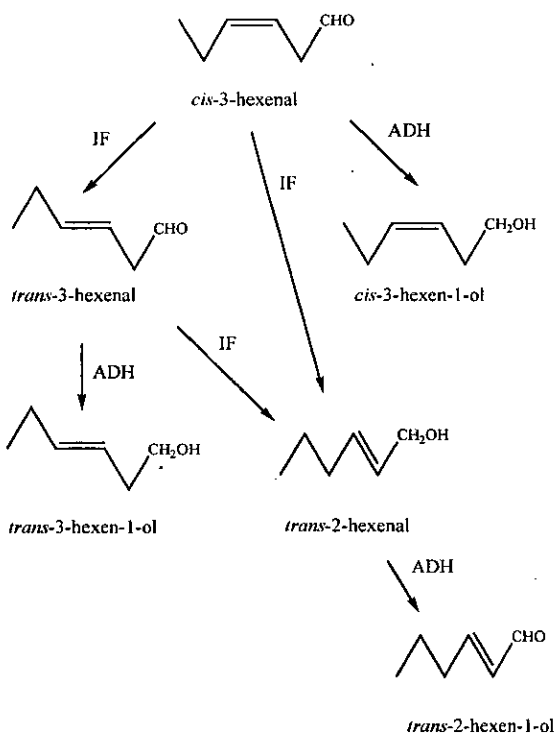

Figure 4.10. Part of the lipoxygenase pathway illustrating how the six C6 volatile derivatives are synthesised from each other. **IF**, isomerisation factor; **ADH**, alcohol dehydrogenase.

Lipoxygenase (linoleate : oxygen oxidoreductase, E.C. 1.13.11.12) (abbreviated to LOX) is a term describing a number of enzymes which catalyse the oxygenation

of fatty acids containing a *cis, cis*-1, 4-pentadiene system with molecular oxygen. LOX catalyses the oxygenation of linolenic acid with the resultant production of two C18 hydroperoxides (Galliard & Chan, 1980). These undergo further reactions producing C9 and C6 compounds. It was shown that *Phaseolus vulgaris* produced C6 derivatives of the lipoxygenase pathway only after a challenge with the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* (Croft *et al.,* 1993). Lipoxygenase activity in wheat also increased with attack by an avirulent pathogen or fungal elicitor (Ocampo *et al.,* 1986) and is thought to have a role in resistance of oats to *P. coronata* f. sp. *avenae* (Yamamoto & Tani, 1986).

In plants, the lipoxygenase pathway is thought to be involved in the synthesis of 'the wound hormone' (Galliard & Chan, 1980) and some of the C6 volatile compounds have been shown to have anti-pathogen activity. The aldehyde *trans*-2-hexenal, another derivative of the lipoxygenase pathway, is inhibitory to *P. syringae* pv *phaseolicola* (Croft *et al.*, 1993) and the fungal plant pathogens *Colletotrichum truncatum, Rhizoctonia solani, Sclerotium rolfsii*, (Vaughn & Gardner, 1993) *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp *et al.*, 1992) as well as *P. graminis* f. sp. *tritici* (Grambow, 1977). Since experimental work assessing biological activity of volatile compounds has been carried out using a number of techniques (e.g. in agar, in liquid, as a vapour), it is difficult to directly compare concentrations used in one set of experiments with others. Using a range of experimental methods, Croft *et al.* (1993) assessed the inhibition of *P. syringae* pv *phaseolicola* by *trans*-2-hexenal and also *cis*-3-hexen-1-ol. It was found that 100 µm *trans*-2-hexenal in the liquid medium inhibited growth of *P. syringae* pv *phaseolicola*. In another experiment, with the volatile dissolved in the nutrient agar, it was shown that *trans*-2-hexenal was 20× more inhibitory than *cis*-3-hexen-1-ol (Croft *et al.,* 1993). It may be predicted that in liquid culture, *cis*-3-hexen-1-ol would be inhibitory for *P. syringae* pv *phaseolicola* at approximately 2 mM (20 × inhibitory concentration of *trans*-2-hexenal), which is the concentration found to inhibit growth of *P. graminis* f. sp. *tritici.*

Induction of appressoria by *trans*-2-hexen-1-ol was optimal at 1 mM. This suggests that the concentration of *trans*-2-hexen-1-ol at the stomatal aperture must be approximately 1 mM if induction by *trans*-2-hexen-1-ol alone was significant *in vivo*. If the concentration of *trans*-2-hexen-1-ol is less than 1 mM then, on its own, it would cause little induction. If the concentration of *trans*-2-hexen-1-ol is greater than 1 mM over the stomatal aperture, then, due to diffusion, there will be inductive concentrations of *trans*-2-hexen-1-ol away from the stomata. If however, there is 1 mM *trans*-2-hexen-1-ol at the stomatal aperture, then it is probable that the concentration in the intercellular spaces of the leaf is higher. This means that when *trans*-2-hexen-1-ol is at an inductive concentration at the stomatal aperture, it is probably at an inhibitory concentration in the intercellular spaces of the leaf. It is possible that inhibition of growth of *P. graminis* f. sp. *tritici* by *trans*-2-hexen-1-ol may be due to over-stimulation of the signal transduction mechanism. If this is the case, and the signalling mechanism is deactivated upon appressorium formation since it may be no longer required, the germling may no longer be susceptible to inhibition by *trans*-2-hexen-1-ol, i.e. infection hyphae may not be inhibited by *trans*-2-hexen-1-ol and could grow in the intracellular spaces despite the high *trans*-2-hexen-1-ol concentration.

The reliability of a volatile derived from an induced metabolic pathway as a stomatal recognition signal is questionable. The precise concentration of *trans*-2-hexen-1-ol signal at the stomatal aperture and over the epidermis will probably vary significantly with the size of the stomatal aperture, activity of the lipoxygenase pathway and prevailing climatic conditions (still air will reduce mixing of the boundary layer of air).

Estimations of C6 volatile compounds in uninfected, undamaged wheat (Table 4.2) showed that *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol accounted for less than 2% of the 2 mg of volatile oil isolated from 1 kg of fresh aerial parts of wheat (Hamilton-Kemp & Anderson, 1984; Hamilton-Kemp *et al.*, 1987). If it is assumed that 1 kg wheat contains 1 litre of cellular contents, then cellular concentrations of *trans*-2-

hexen-1-ol and *cis*-3-hexen-1-ol are approximately 0.3 μM and 0.02 μM respectively, approximately 4 and 5 orders of magnitude lower than concentrations inductive for *P. graminis* f. sp. *tritici* appressorium formation *in vitro*.

Table 4.2. Estimation of the cellular concentrations of some C6 volatile compounds in wheat. (Data from Hamilton-Kemp & Anderson, 1984; Hamilton-Kemp *et al.* 1987).

C6 compound	% composition of volatile oil [1]	Mass in 1 kg wheat (mg)	MW	Estimation of cellular concentration (μM) [2]
trans-2-hexenal	11.1	0.222	98	2.27
trans-2-hexen-1-ol	1.7	0.034	100	0.34
cis-3-hexen-1-ol	0.1	0.002	100	0.02

[1] % composition of the 2 mg of volatile oil isolated from 1 kg fresh wheat material.
[2] concentration estimation based on 1 kg wheat having a volume of 1 l cellular water.

This large difference between inductive concentrations of *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol and the actual concentration measured in the host leaf cannot be explained by experimental errors in the measurements of the volatile concentrations (e.g. inefficiency of extraction, loss of volatile during extraction and poor detection methods). In fact, since LOX is involved in the synthesis of a wound hormone, the protocol for volatile extraction (maceration of tissue) would, if anything, be expected to increase the amount of LOX derivatives.

These measurements of LOX derivatives refer to concentrations in the leaf. What is significant, however, is the concentration of these volatile compounds at the stomatal aperture, which will be lower than the cellular concentration due to diffusion effects. If the stomata are closed then it is possible that the concentration in the sub-stomatal cavity will equilibrate with the cellular concentration, but will never exceed it. The only way for the concentration of C6 compounds to be higher at the stomatal aperture than the average leaf concentration is if only guard cells have LOX activity. There is no evidence that this is true and no obvious reason why only guard cells should

synthesise wound hormone.

It has been shown for several rusts, including *P. graminis* f. sp. *tritici*, that a heat shock treatment of 30°C will induce appressorium formation (Wanner *et al.*, 1985; Deising *et al.*, 1991). Heat shock induction of appressoria is probably an artefact, having no significant role in the initiation of penetration of the host. If appressoria can be induced by an environmental stress such as heat shock, and LOX derivatives have anti-pathogen activity, then induction of appressoria by *trans*-2-hexen-1-ol may be a similar stress-induced event with no significance *in vivo*.

It is possible that *trans*-2-hexen-1-ol mimics another chemical signal, or that other chemical signals are at active concentrations when *trans*-2-hexen-1-ol is at a non-inductive concentration. It has been shown that *P. graminis* f. sp. *tritici* forms appressoria in response to a number of short chained, unsaturated, aliphatic carbonyl compounds (Macko *et al.*, 1978); *trans*-2-hexen-1-ol may possibly be mimicking one of these, or similar, compounds.

Interestingly, wheat leaf rust, *P. recondita* f. sp. *tritici*, was not induced to form infection structures in response to the concentration of *trans*-2-hexen-1-ol that was optimal for *P. graminis* f. sp. *tritici*. This means that two rusts, infecting the same host, respond to different topographic and chemical signals. Other plant pathogens form infection structures in response to other, mostly non-volatile host chemicals (Grover, 1971; Swinburne, 1976; Harper & Swinburne, 1979; Parberry & Blakeman, 1978; Podila *et al.*, 1993; Flaishman & Kolattukudy, 1994). It is possible that *P. recondita* f. sp. *tritici* utilises other chemical signals associated with stomata (see Section 4.3.4). Other differences between the two wheat rusts have also been documented in that penetration by *P. graminis* f. sp. *tritici* appears to be inhibited by light and high CO_2, whilst penetration by *P. recondita* f. sp. *tritici* is not (Yirgou & Caldwell, 1968).

4.3.2 Interaction of topography and trans*-2-hexen-1-ol*

It is difficult to analyse the interaction of topography and *trans*-2-hexen-1-ol based on percentage appressorium formation. Unless more appressoria are induced by the two signals in combination than the total of the two signals alone, interpretation of the results is not possible. The results here show that differentiation induced by the combination of signals at 24 h was equal to or less than the sum of the two treatments alone (Figure 4.4). There are two possibilities. Firstly, there may be two types of germling: those which form appressoria only in response to topography and those which only form appressoria in response to *trans*-2-hexen-1-ol, and if the second signal is present, it has no effect (i.e. the two signals act independently). Alternatively, there may be some germlings which form appressoria in response to either topography or *trans*-2-hexen-1-ol alone. If this was the case then induction by topography and *trans*-2-hexen-1-ol in combination would result in differentiation significantly less than the sum of the two treatments alone. Since this is not the case, it suggests that there may be some germlings which have formed appressoria only when topography and *trans*-2-hexen-1-ol were combined (i.e. the two signals act synergistically). It is also possible that in the population there is a combination of these two effects.

Since it was not possible to determine which of these two signals was inducing individual germlings, it is not possible to say whether germlings formed appressoria in response to topography plus *trans*-2-hexen-1-ol but would not have formed appressoria in response to either signal alone. It has been shown for *U. appendiculatus* that protein expression is different in appressoria induced by heat shock and topography (Staples *et al.*, 1989). Based on this observation, it may be possible to isolate proteins unique to either topographical or *trans*-2-hexen-1-ol induced appressoria. Antibodies raised against these proteins may allow identification of appressoria induced by each signal. If, however, the induction of appressoria by heat shock is an *in vitro* artefact, it is possible that no 'signal specific' proteins will be found.

The time course of differentiation induced by *trans*-2-hexen-1-ol or topography alone (Figure 4.4) suggests that induction occurs relatively slowly. Since very few stomata were overgrown by germ tubes *in vivo* (Figure 3.8), induction on the host leaf occurs quickly in response to an inductive signal. *In vitro*, induction by the combination of topography and *trans*-2-hexen-1-ol is only rapid if the two signals are combined (Figure 4.4). Since appressorium induction after 4 h by the two signals combined is greater than the sum of induction by either signal alone at this time, it can be said that the two signals are acting synergistically in terms of the rate of induction.

If the time course of appressorium development after induction in *P. graminis* f. sp. *tritici* is similar to that in *U. appendiculatus* (Kwon *et al.*, 1991a) then septation (i.e. completion of the appressorium) is complete after 1 h (Table 1.1). Therefore, since germination started after approximately 30 min (Collins, T. J. unpubl.), some appressoria may have been induced after 30 min growth on the inductive substrata, making induction by topography and *trans*-2-hexen-1-ol in combination highly efficient. This synergy will ensure induction of appressoria is only very efficient when topographical and chemical signals are combined. *In vivo*, this is suggested to occur only at the stomatal aperture.

This raises the question as to whether, as suggested above, the chemical signal needs to be localised over the stoma, since the topographical signal is located at the stoma. The experiments with leaf replicas and *trans*-2-hexen-1-ol showed that a uniform concentration of *trans*-2-hexen-1-ol across the epidermis topography resulted in high numbers of misplaced appressoria and no increase in response to the stomatal topography. For the induction of appressoria by *trans*-2-hexen-1-ol to be significant *in vivo* the signal would need to be inductive only over the stomatal aperture. It is therefore a requirement that there is constantly 1 mM *trans*-2-hexen-1-ol in close proximity to the stomatal aperture.

Lack of synergy between leaf replica topography may be because the leaf replicas do not faithfully reproduce the inductive host topography (see Section 3.3.4).

Alternatively, *P. graminis* f. sp. *tritici* may rely on a combination of at least two signals for initiation of penetration. But why should induction rely on two signals in combination? If induction by topography or *trans*-2-hexen-1-ol alone is inefficient, then the fungus is buffered against spurious high concentrations of *trans*-2-hexen-1-ol or any unusual topography (e.g. veins) on the epidermis. Only when there is inductive topography and inductive *trans*-2-hexen-1-ol will the fungus rapidly differentiate. The observation that induction by topography is inefficient and requires prolonged growth over the inductive topography may explain the poor response of germlings to leaf replicas (25%) compared to microfabricated substrata (70%). On leaf replicas the putative inductive topographies (i.e. stomata) are only encountered infrequently and then only briefly as the fungus grows on, whilst on microfabricated substrata, the fungus is continually overgrowing an inductive topography.

4.3.3 Appressorium morphology

The appressoria induced by *trans*-2-hexen-1-ol were formed correctly with nuclear division, nuclear and cytoplasmic migration into the swelling tip and correct formation of the septum. This is in contrast to appressoria of *Uromyces* spp. induced by other chemical signals, which were formed aerially (Hoch *et al.*, 1987a) or without correct cytoplasmic migration (K. Taylor & N. D. Read, unpubl.). The appressoria induced by topography expanded along the direction of the topographical features whilst appressoria induced on flat surfaces had no topography to form along and were long and thin. Also, appressoria induced by *trans*-2-hexen-1-ol on non-inductive single ridges expanded along the ridge and were short and wide with respect to the direction of growth (Figure 4.6). On the leaf the appressoria are shaped like those induced by topography, forming along the stomatal aperture slit (Figure 3.9). The differences in morphology between appressoria induced by topography and *trans*-2-hexen-1-ol illustrate a further type of contact-mediated response.

The appressoria induced by a combination of topography and *trans*-2-hexen-1-ol were wider than those induced by topography alone. This may be because the

appressoria induced by topography plus *trans*-2-hexen-1-ol are induced quicker and so have utilised less internal reserves, with perhaps more cytoplasm to be accommodated in the appressorium.

This work has shown certain characteristics of the biological activity of *trans*-2-hexen-1-ol *in vitro*, namely action as a volatile, synergy with topography and induction of correct appressorium formation. These characteristic are consistent with *trans*-2-hexen-1-ol (or a chemical with a similar mode of action) having a role in initiation of appressorium formation *in vivo*. It can also be argued that reliance on more than one signal to initiate host infection, a critical stage of the pathogen's life cycle, would be an advantage to the fungus. However, analysis of other characteristics of *trans*-2-hexen-1-ol as a potential stomatal location signal raises some doubts to its suitability *in vivo*. Firstly there is the fact that it has been shown, along with other derivatives of the lipoxygenase pathway, to have antifungal activity. Secondly, the signal is required to be at a specific concentration over the stomatal aperture, which seems unlikely. Thirdly, and perhaps most significantly, *in vivo* concentrations of *trans*-2-hexen-1-ol appear to be much lower in healthy leaves than concentrations found to be inductive *in vitro*.

4.3.4 Other inductive chemical signals

Analysis of the cuticle revealed differences in the chemistry of the cuticle of the wheat stomatal complex and epidermal cells. Nile red only stained the guard cell ledges (Figure 4.8). This is where the epicuticular wax ends, exposing the heavily cutinised guard cell lip/ledge (Palevitz, 1981). Similar staining of the guard cell ledges was observed in broad bean and cowpea, which are hosts to *U. appendiculatus* and *U. vignae* respectively (Collins, T., unpubl.). Cutin is an amorphous polymer of a variety of long-chain hydroxylated fatty acids, typically C16 and C18 monomers. The C16 monomers are typically based on palmitic acid, 16-hydroxypalmitic acid and 10, 16-dihydroxypalmitic acid. The C18 monomers are typically comprised of stearic acid, oleic acid, linoleic acid, 18-hydroxyoleic acid, 9, 10-epoxy stearic acid

and 9, 10, 18-trihydroxystearic acid (Kolattukudy, 1980). Cutin also contains a number of phenolics including *p*-coumaric acid and smaller amounts of ferulic acid (Riley & Kolattukudy, 1975). Rust fungi have been shown to produce cutinases and esterase (Deising *et al.*, 1992) which would liberate cutin monomers from the guard cell ledge and degrade epicuticular waxes. The role of these enzymes is thought to be in germling adhesion, but they may also have a role in liberating chemical signals from the cuticle associated with the stomata. Most cutin monomers have to be synthesised or extracted. Since this was beyond the scope of my research project the biological activity of many cutin monomers was not tested. The compounds stearic acid, palmitic acid, linoleic acid and oleic acid were readily available but were found to have no biological activity. The biological activity of cutin extracted from wheat needs to be assessed.

Wheat guard cells showed enhanced autofluorescence when treated with ammonia. This is due to esters of phenolic acids, such as *p*-coumaric, ferulic and diferulic acid (Palevitz, 1979). These phenolics are thought to add to the mechanical strength of the wall (Palevitz, 1981). It has been proposed that the extracellular enzymes secreted by rusts would degrade the cuticle, exposing the rust to the chemistry of the epidermal cell walls (Edwards & Bowling, 1986).

A phenolic fraction of wheat has been shown to induce appressoria in *P. graminis* f. sp. *tritici*. The phenolic acids tested (*p*-coumaric, ferulic and diferulic acid) had no biological activity, in agreement with the findings of Grambow (1978). Esters of these acids are released from grass cell walls by cellulase treatment and may be the active fraction for *P. graminis* f. sp. *tritici*. For the unidentified compound to be a significant inductive signal *in vivo* it must be present only at the stomatal complex, and the observations that the guard cell wall is the predominant location of phenolic acid suggest that this may be the case.

5. TRANSDUCTION OF INDUCTIVE SIGNALS

5.1 Introduction

There is evidence that the transduction of topographical signals for induction of appressoria in rusts involves Ca^{2+} (Hoch *et al.*, 1987a; K. Taylor & N. D. Read, unpubl.; C. Bauch; S. Horne, B. Myer, S. Ruffert, T. J. Collins, B. Moerschbacher & N. D. Read, unpubl.). In the cell, Ca^{2+} commonly acts via the calcium binding protein calmodulin (CaM). CaM has been implicated in the transduction of signals for a wide range of fungal cell processes including cell proliferation, cell cycle control and nuclear division (Gadd, 1995). In this chapter, research is described in which the role of CaM in the transduction of the topographic signals for appressorium induction in *P. graminis* f. sp. *tritici* was investigated with a range of CaM antagonists. The effect of these inhibitors at their IC_{50} concentrations on appressorium induction by *trans*-2-hexen-1-ol, was determined.

Mechanical signals in animal and plant cells can be transduced via integrin proteins (Ingber, 1991). The role of integrins in topographical induction of appressoria in *P. graminis* f. sp. *tritici* was investigated here using the integrin binding tripeptide arginine-glycine-aspartic acid (RGD) which competitively inhibits the binding of integrins to ECM glycoproteins (Hynes, 1992).

External pH (pH_{ext}) has been shown to affect the topographical induction of appressoria in *U. appendiculatus* (Stumpf *et al.*, 1991). The effect of pH_{ext} on appressorium formation in *P. graminis* f. sp. *tritici* induced by topography and *trans*-2-hexen-1-ol was thus determined. In *Candida albicans*, increased pH_{ext} induces the dimorphic transition between germ tube and yeast forms (Monk *et al.*, 1993) by inducing a rise in cytosolic pH (pH_{cyt}) (Stewart *et al.*, 1988; Yokoyama *et al.*, 1994). A rise in pH_{cyt} also triggers conidiation in *Penicillium* (Roncal *et al.*, 1993). Using

the pH sensitive ratio dye SNARF-1, the effect of changes in pH_{ext} on pH_{cyt} of *P. graminis* f. sp. *tritici* was analysed.

In *U. appendiculatus* appressoria can be stimulated by K^+ and also by heat shock. It has been shown in *U. appendiculatus* that D_2O, which stabilises microtubules, inhibits K^+ and heat-shock induced appressoria, but not topographically induced appressoria (Hoch *et al.*, 1986). The effect of D_2O on the topographical and *trans*-2-hexen-1-ol induction of appressoria in *P. graminis* f. sp. *tritici* was thus determined.

Nothing is known about the signal transduction pathways of *trans*-2-hexen-1-ol in *P. graminis* f. sp. *tritici*. Plants and *Colletotrichum gloeosporioides* both respond to the gaseous plant growth regulator ethylene. For these organisms the signal transduction of the gaseous signal can be inhibited by Ag^{3+} and the protein kinase C inhibitor H-7 (Kolattukudy *et al.*, 1995; Raz & Fluhr, 1993; Beyer, 1976). The effects of these inhibitors on *trans*-2-hexen-1-ol induction of appressoria in *P. graminis* f. sp. *tritici* was therefore investigated here.

5.2 Results

5.2.1 CaM antagonists

Uredospores were inoculated onto polystyrene substrata with 1.5 μm spaced, 2.0 μm high ridges. The inoculated substrata were floated spore side down on a test solution. The CaM antagonists calmidazolium, trifluoperazine (TFP), W-5 and W-7 were used at various concentrations, ranging from 0.03 μM to 100 μM. After 24 h the lengths of undifferentiated primary germ tubes (as an indication of the extent of germling growth) and percentage germling differentiation was quantified.

Inhibition of differentiation by calmidazolium had an IC_{50} of 0.3 μM and inhibition of growth had an IC_{50} of approximately 2 μM (Table 5.1 & Figure 5.1A). Inhibition of differentiation and germ tube growth by TFP had IC_{50} of 2 μM and 3 μM respectively (Table 5.1 & Figure 5.1B). W-7 significantly ($p < 0.05$) inhibited growth and differentiation at 10 μM and germination was completely inhibited at 30 μM (Table 5.1 & Figure 5.1C). W-5 was less inhibitory than W-7, with no inhibition of growth at 30 μM and complete inhibition of germination at 100 μM. W-5 significantly inhibited growth and differentiation at 60 μM (Table 5.1 & Figure 5.1D). There was no inhibition of differentiation induced by 1 mM *trans*-2-hexen-1-ol by the CaM antagonists at their respective IC_{50} concentrations for topographical induction of appressoria (Table 5.2). Germination, growth and differentiation was not affected by concentrations of DMSO up to 7% (S. Maughan, unpubl.).

Table 5.1: 50% Inhibitory concentrations (IC_{50}) for germling growth and differentiation of CaM antagonists and the affinity constants (K_a) of these inhibitors for CaM.

Inhibitor	Mean IC_{50} for undifferentiated germ tube growth	Mean IC_{50} for appressorium formation	pK_a (μM) [a]
Calmidazolium	2	0.3	0.3
TFP	3	2	1
W-7	10	10	11
W-5	60	60	90

[a] Data from Asano & Stull (1985).

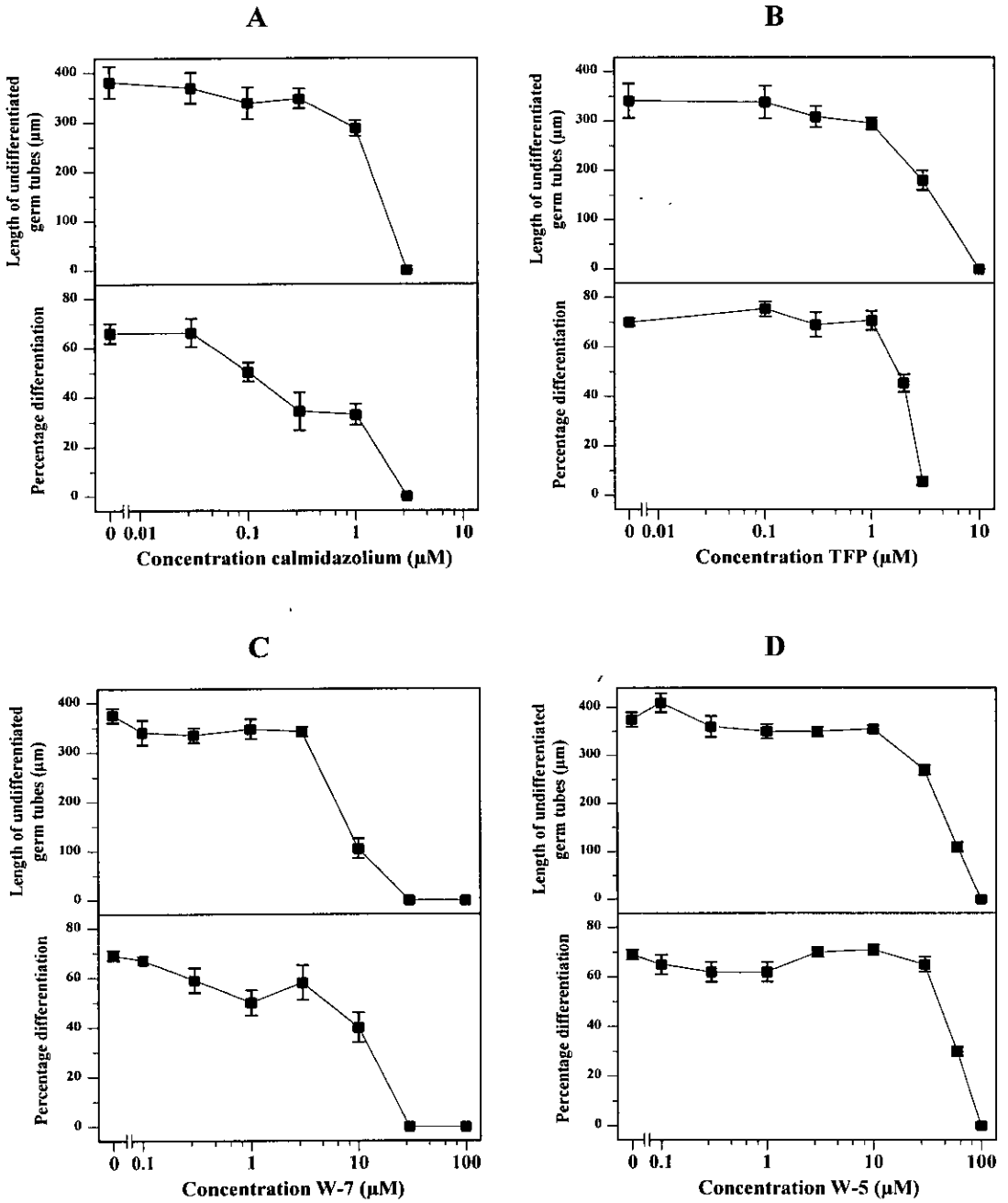

Figure 5.1: Effect of CaM inhibitors on growth and differentiation. **A**, calmidazolium; **B**, TFP; **C**, W-7; and **D**, W-5. Means ± sem.

Table 5.2: Effect of CaM antagonists on induction of appressoria on flat surfaces by 1 mM *trans*-2-hexen-1-ol at their respective IC_{50} concentrations for topographical induction of appressoria. Means ± sem.

Treatment	% Appressorium formation in *trans*-2-hexen-1-ol
Control	38.6 ±1.6
0.3 μM calmidazolium	41.5 ± 1.7
2 μM TFP	43.7 ± 5.8
10 μM W-7	42.3 ± 3.4
60 μM W-5	37.0 ± 5.0

5.2.2 RGD

Uredospores were inoculated onto polystyrene substrata with 1.5 μm spaced, 2.0 μm high ridges topographies and incubated in RGD solutions in distilled water. After 24 h incubation at 22°C appressorium formation on multiple ridges and growth of undifferentiated germlings on flat surfaces was quantified.

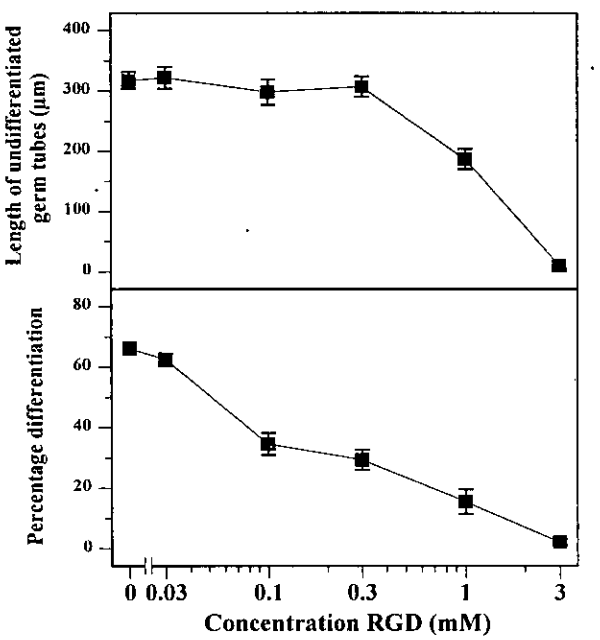

Figure 5.2: Inhibition of growth of undifferentiated germlings and appressorium differentiation on microfabricated topographies by RGD solutions of different concentrations in distilled water. Means ± sem.

Differentiation was inhibited with 0.1 mM RGD, 0.03 mM RGD was not inhibitory. Growth was inhibited by 1 mM RGD and higher. The IC_{50} for inhibition of topographical induction of appressoria was 0.1 mM (Figure 5.2).

The pH of the RGD solutions varied with RGD concentration. To analyse whether the observed inhibition by RGD solutions was due to RGD or the pH of the solution, germlings were incubated under the same conditions except that the RGD solutions had their pH adjusted to 7.0 with KOH.

Inhibition of differentiation by RGD at pH 7.0 was only at 1 mM (Figure 5.3). Growth was affected by RGD at pH 7.0 and unadjusted RGD in a similar manner, with marked growth inhibition occurring at 1 mM (cf. Figures 5.2 & 5.3). Growth was significantly ($p < 0.05$) inhibited by 0.3 mM RGD at pH 7.0. 1 mM RGD at pH 7.0 had approximately 1.3 mM KOH added.

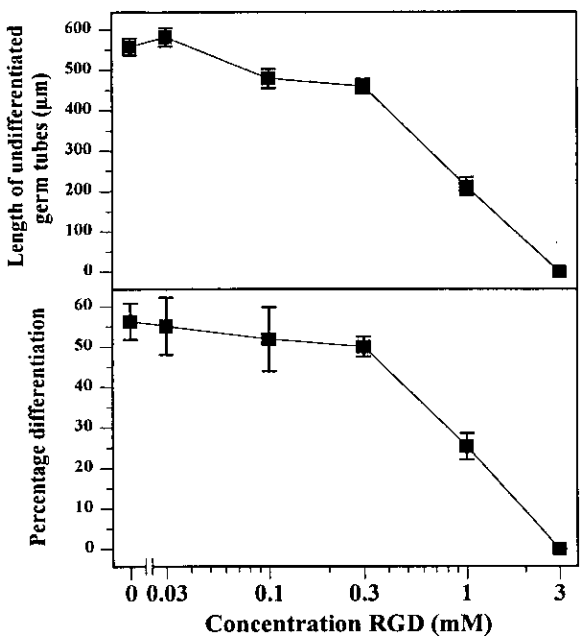

Figure 5.3: Inhibition of appressorium formation induced by topography using RGD solutions, pH adjusted to 7.0 with KOH. Mean ± sem.

Induction of appressoria by 1 mM *trans*-2-hexen-1-ol was not inhibited by 1 mM RGD (pH 7.0).

5.2.3 External pH

Uredospores were inoculated onto microfabricated substrata and incubated in 10 mM MES/HEPES ranging from pH 5.8 to pH 8.0 for 24 h at 22°C. Uredospores were also inoculated onto flat polystyrene and incubated in 1 mM *trans*-2-hexen-1-ol in 10 mM MES/HEPES ranging from pH 5.8 to pH 8.0 for 24 h at 22°C.

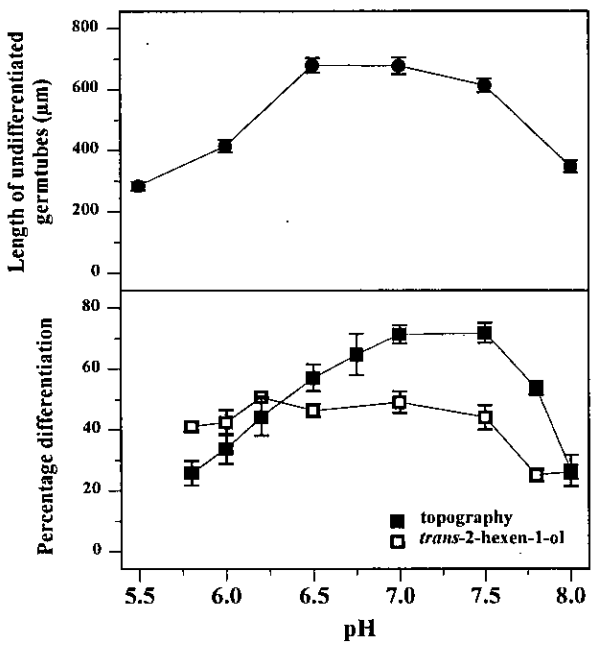

Figure 5.4: The effects of external pH on appressorium induction by topography or 1 mM *trans*-2-hexen-1-ol, and lengths of undifferentiated germ tubes after 24 h on flat surfaces. Means ± sem.

Growth was inhibited below pH 6.5 and above pH 7.5 (Figure 5.4). Over the range pH 5.8 to pH 7.5, induction by 1 mM *trans*-2-hexen-1-ol was unaffected by external pH. Optimum topographical induction occurred between pH 6.75 and pH 7.5. Appressorium formation induced by topography or *trans*-2-hexen-1-ol was reduced

to 50% of the maximum between pH 6.0 and pH 6.2 and between pH 7.8 and 8.0 (Figure 5.4).

To determine whether the observed effects were due to buffer or counter ion concentration ,several controls were performed. 10 mM MES/HEPES adjusted to pH 7.0 had a $[K^+]$ of 6 mM; whilst 10 mM MES/HEPES adjusted to pH 8.0 had a $[K^+]$ of 10 mM. In order to keep $[K^+]$ constant at these two pH values, the 10 mM MES/HEPES at pH 7.0 mM had the $[K^+]$ increased to 10 mM with KCl. Also, 20 mM MES/HEPES was adjusted to pH 7.0 to determine whether the buffer was affecting appressorium induction. The results showed that the induction by topography was the same irrespective of $[K^+]$ or buffer concentration (Figure 5.5). Only pH affected appressorium induction (Figure 5.5).

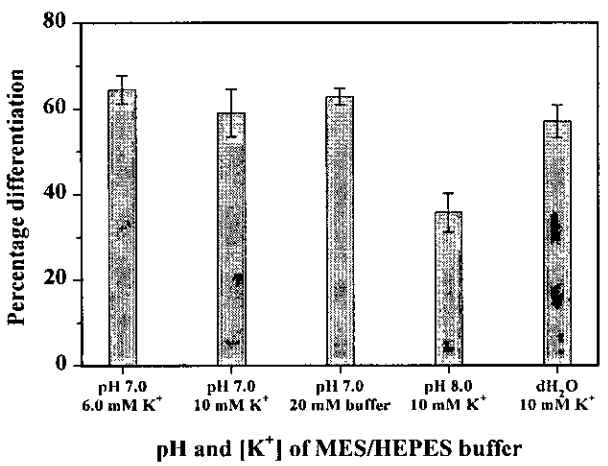

Figure 5.5: The effect of pH, $[K^+]$ and buffer concentrations on topographical induction of appressoria. Means ± sem.

5.2.4 Effect of external pH on cytosolic pH

The pH_{cyt} of germlings growing in pH 6.2 and 7.2 buffers was compared to determine whether the pH_{ext} was affecting appressorium induction by altering pH_{cyt}.

At pH_{ext} 6.2 and pH_{ext} 7.2 the pH_{cyt} was the same. At pH 6.2 the ratio for the cytosol was 54 ± 4 (mean ± sd) and at pH_{ext} 7.2 the ratio values were 49 ± 4 (Figure 5.6A & C).

To confirm that any differences in pH_{cyt} would have been detected, the membrane permeant weak acid, propionic acid was added to the germling to equilibrate pH_{cyt} with pH_{ext}. When 50 mM pH 6.2 propionic acid was added to the growing germling the cytoplasmic ratio changed to 98 ± 7 (Figure 5.6B). Propionic acid at pH 7.2 (or more correctly propionate at pH 7.2) failed to alter the ratio of the cytosol (ratio = 49 ± 4), indicating that this was the approximate value of pH_{cyt} (Figure 5.6D).

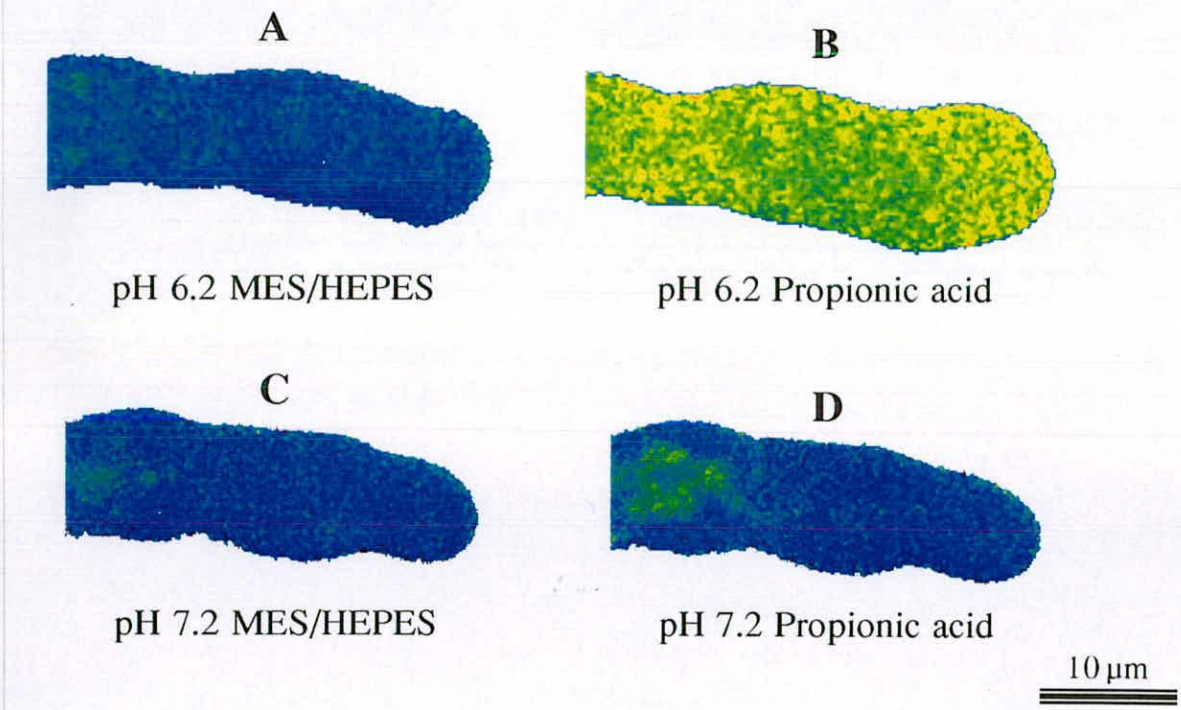

Figure 5.6: Cytosolic pH of germlings reported by ratio imaging of SNARF-1. **A,** germling growing in pH 6.2 MES/HEPES; **B,** the same germling as in **A** but after pH 6.2 propionic acid was added; **C,** germling growing in pH 7.2 MES/HEPES; **D,** the same germling as in **C** but after pH 7.2 propionate was added. Bar = 10 µm.

5.2.5 D_2O

Uredospores were inoculated onto microfabricated polystyrene substrata and incubated in distilled water with varying percentages of D_2O. Uredospores were also inoculated onto flat polystyrene and incubated in a 1 mM solution of *trans*-2-hexen-1-ol solution made up with various percentages of D_2O. Growth of undifferentiated germlings and percentage differentiation after 24 h was quantified.

Germling growth was significantly ($p < 0.05$) inhibited by 20% and higher D_2O concentrations. Topographical induction of appressoria was significantly ($p < 0.05$) inhibited at and above 40% D_2O (Figure 5.7). Induction of appressoria by *trans*-2-hexen-1-ol was not inhibited by D_2O up to 80%. Germination was completely inhibited in 100% D_2O.

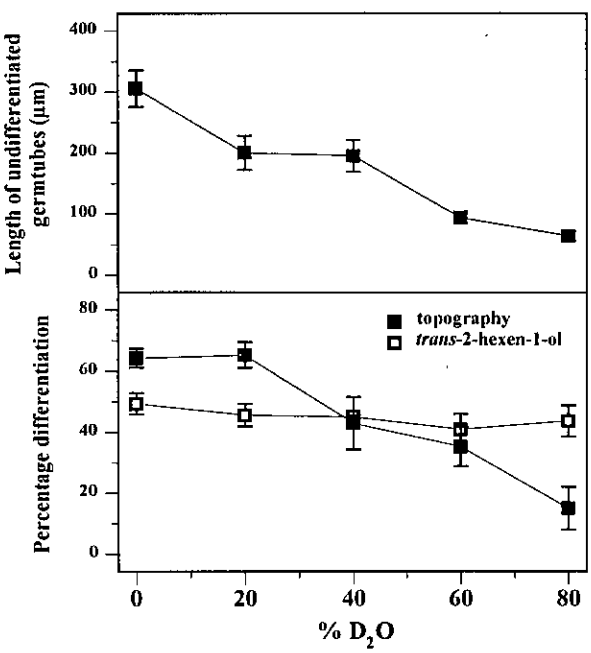

Figure 5.7: Effect of D_2O on appressorium formation induced by topography and *trans*-2-hexen-1-ol and on germ tube growth. Means ± sem.

5.2.6 $AgNO_3$

Uredospores were inoculated onto flat polystyrene and incubated in 1 mM *trans*-2-hexen-1-ol with concentrations of silver nitrate ranging from 0.06 µM to 1 µM. Nitrate has been shown not to affect appressorium formation in response to 1 mM *trans*-2-hexen-1-ol *P. graminis* f. sp. *tritici* over this range (C. Bauch, unpubl.).

Inhibition of *trans*-2-hexen-1-ol induced appressorium formation by $AgNO_3$ had an IC_{50} of 0.1 µM (Figure 5.8) and germination was inhibited by 0.3 µM. At the IC_{50} concentration for *trans*-2-hexen-1-ol induction of appressoria, $AgNO_3$ did not inhibit topographical induction of appressoria (Table 5.3).

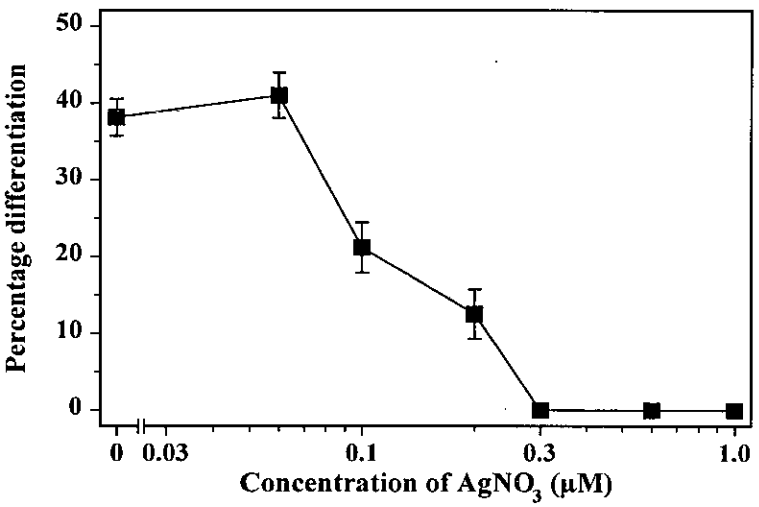

Figure 5.8: Inhibition of 1 mM *trans*-2-hexen-1-ol induced appressorium differentiation in *P. graminis* f. sp. *tritici* by $AgNO_3$. Mean ± sem.

5.2.7 H-7

Uredospores were inoculated onto flat polystyrene and incubated in 1 mM *trans*-2-hexen-1-ol with concentrations of the protein kinase C inhibitor H-7 ranging from 0.1 µM to 100 µM.

Inhibition of *trans*-2-hexen-1-ol induced appressorium formation by H-7 had an IC_{50} of 10 μM (Figure 5.9). There was no inhibition by H-7 of topographical induction of appressoria at this IC_{50} concentration (Table 5.3).

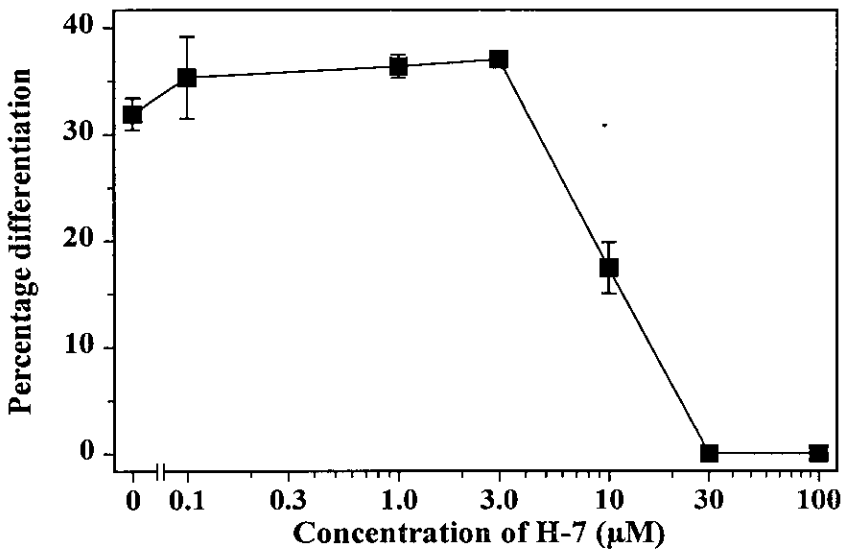

Figure 5.9: Inhibition by H-7 of 1 mM *trans*-2-hexen-1-ol induced appressorium differentiation. Means ± sem.

Table 5.3: Effect of inhibitors of *trans*-2-hexen-1-ol induction of appressoria at their respective IC_{50} concentrations on induction of appressoria by 1.5 μm spaced, 2.0 μm high, 2.0 μm wide ridged topographies. Means ± sem.

Treatment	% Appressorium formation on multiple ridges
Control	68.0 ± 9.3
0.1 μM $AgNO_3$	75.0 ± 7.0
10 μM H-7	65.3 ± 2.6

5.3 Discussion

The results presented here suggest a role for CaM, integrins and microtubules in topographical induction of appressoria but not in *trans*-2-hexen-1-ol induction of appressoria (Table 5.4). The results also suggest a role for an ethylene-type receptor and protein kinase C in *trans*-2-hexen-1-ol induction of appressoria but not

topographical induction (Table 5.4). A highly speculative, schematic diagram of the two signal transduction pathways are detailed in Figure 5.10.

Table 5.4: Summary of results from inhibitor studies. Effects on topographical induction of appressoria, *trans*-2-hexen-1-ol induction of appressoria and germling length after 24 h.

Treatment	Concentration	Topographical induction	*trans*-2-Hexen-1-ol induction	Germ tube length
Calmidazolium	0.3 µM	✗		
TFP	2 µM	✗		✗
W-7	10 µM	✗		✗
W-5	60 µM	✗		✗
RGD	1 mM	✗		✗
$AgNO_3$	0.1 µM		✗	nd
H-7	10 µM		✗	nd
D_2O	40%	✗		✗

✗ : inhibition ($p < 0.05$); nd: not determined.

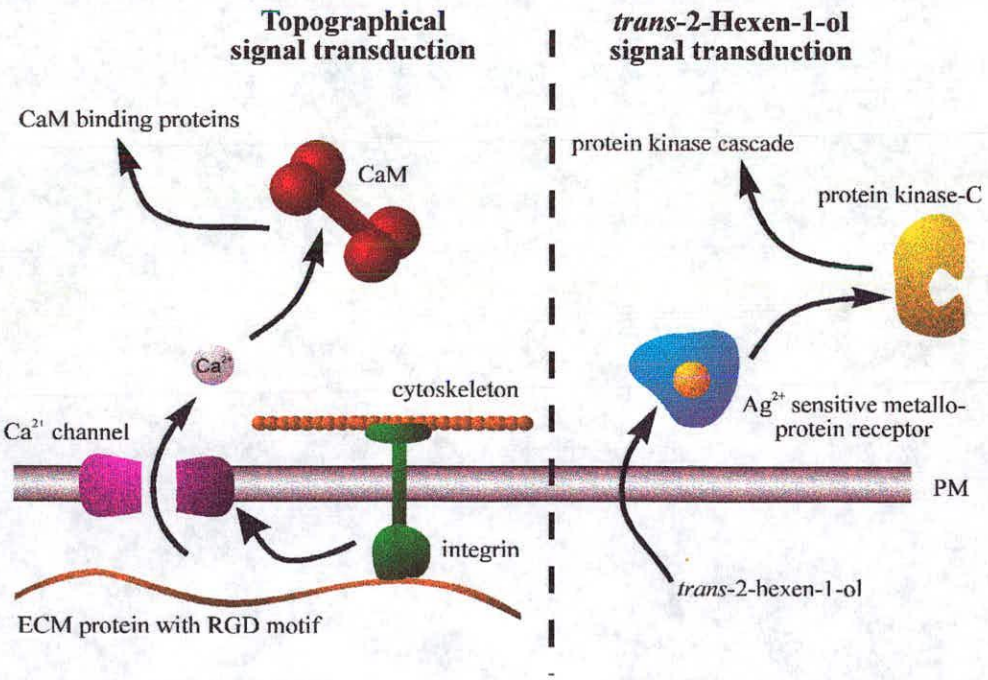

Figure 5.10. Diagram respresenting the two signalling pathways thought to be involved in appressorium induction in *P. graminis* f. sp. *tritici*. Topographical signalling involes the deformation of ECM proteins by topography which induces integrins to activate Ca^{2+} channels, inducing an influx of extracellular Ca^{2+}. This activates CaM which propagates the signal through the cell. The pathway for transduction of *trans*-2-hexen-1-ol involves a metallo-protein receptor which activates protein kinase C.

However, these results are based on inhibitor experiments which have inherent problems. Two main problems are as follows. Firstly there is the issue of specificity. Many inhibitors purporting to be specific, rarely are. Using a range of inhibitors, each with the same target but different mode of action helps overcome this problem. This was attempted here with the CaM antagonists. It may be considered that non-specific effects of an inhibitor are less likely if a low concentration of the inhibitor is used. However, it must be borne in mind that what is actually relevent is the affinity of the inhibitor for the non-specific target. If an inhibitor has a high pK_a for its intended target and a low pK_a for an unintended target, then even at high inhibitor concentrations, there may be low 'non-specific' effects of the inhibitor. If, however, the inhibitor has a low pK_a for its intended target and an even higher pK_a for an unintended target, then even at low inhibitor concentrations there may be considerable 'non-specific' effects of the inhibitor. Since pK_a values are not known for each inhibitor and each potential target, the reliability of an inhibitor experiment is best judged by the similarity of the IC_{50} and the pK_a for the intended target.

With inhibitor experiments there is also the question of where exactly in the signal transduction pathway the inhibitor is acting. Signalling pathways can be broken down into several stages: reception, where the signal is initially perceived; transduction, where the signal is propagated and translocated; and the response itself which may also involve several stages. Inhibition at any one of these stages can have the same observed effect. Likewise, inhibition of a process indirectly related to the signalling pathway may also cause inhibition of the response itself.

Before a signal can be transduced, the cell must be exposed to the signal. This often goes without saying, but for topographical sensing it must be highlighted. If the topographical signal is temporally encoded (see Section 3.1) and therefore dependent on unperturbed growth, then the germling will not be correctly exposed to the inductive signal if growth is affected. It is possible that inhibition of growth will result in no appressorium formation even though the machinery for reception,

transduction and response to an inductive topographic signal is fully functional. It is for this reason that germling growth was assessed when inhibition of topographical induction was studied.

If the inhibitor acts on the appressorium formation process itself, i.e. nuclear division, septation etc., then differentiation will be inhibited even though machinery for reception and transduction of an inductive signal is fully functional. At inhibitor concentrations inhibitory to one signalling pathway (*trans*-2-hexen-1-ol or topography) appressorium formation could still be induced by the other signalling pathway showing that the inhibitors were not acting on the appressorium formation process itself.

5.3.1 CaM antagonists

CaM antagonists inhibited topographical induction of appressoria, but not *trans*-2-hexen-1-ol induction of appressoria. This suggests that CaM is involved in the response to topography but not to *trans*-2-hexen-1-ol.

Apart from inhibition of CaM mediated response, some CaM antagonists have been shown to have other effects. Phenothiazines, such as TFP, have been show to inhibit: mitochondrial ATPase (Ruben & Rasmussen, 1981); electron transport (Dunn *et al.*, 1984); and also Ca^{2+} transport across the inner mitochondrial membrane (Vale *et al.*, 1983). W-5 and W-7 have been shown to inhibit autophosphorylation of a CaM independent protein kinase in soybean (Harmon *et al.*, 1987) and there is also evidence that TFP affects membrane integrity in oat coleoptiles (Gonzalezdaros *et al.*, 1993).

However, the observed inhibition of topographical induction of appressoria in *P. graminis* f. sp. *tritici* seem to be due to CaM antagonism since the pK_a values for the inhibitors correlate well with the inhibitory concentrations (Table 5.1).

Calmidazolium is the strongest inhibitor of CaM (pK_a = 0.3 μM) and had the lowest IC_{50} (0.3 μM) for topographical induction of appressoria in *P. graminis* f. sp. *tritici*. W-5 is the weakest inhibitor of CaM (pK_a = 80 μM) and had the highest IC_{50} (8 μM). Also, the inhibitors are chemically quite diverse but their inhibitory effects were similar, suggesting no significant non-specific effects.

Since *trans*-2-hexen-1-ol induction of appressoria is not inhibited by CaM antagonists (Table 5.2) it seems that the CaM antagonists are inhibiting the transduction of the topographical signal rather than the process of appressorium formation itself.

The failure of the CaM antagonists to inhibit *trans*-2-hexen-1-ol induction of appressoria at their respective IC_{50} concentrations suggest that calmodulin is not involved in the transduction of the *trans*-2-hexen-1-ol signal. Lanthanides (inorganic Ca^{2+} channel blockers) also failed to inhibit *trans*-2-hexen-1-ol induction of appressoria suggesting that extracellular calcium is not required for this pathway (S. Horne, B. Myer, C. Bauch, S. Ruffert, T. J. Collins, B. Moerschbacher, N. D. Read, unpubl.). the lanthanides did inhibit topographical induction of appressoria supporting the Ca^{2+} dependency of this process.

Calmodulin has been shown to be involved in a number of fungal differentiation events. In the dimorphic fungi *Candida albicans* and *Ophiostoma ulmi*, calmodulin antagonists can inhibit the yeast-germ tube transitions (Gadd & Brunton, 1992, Buchan *et al.*, 1993).

In *Z. radicans* appressorium formation was found to involve CaM. Inhibition of differentiation in this fungus by CaM antagonists was at similar concentrations to those founds here for *P. graminis* f. sp. *tritici* (Table 5.5) with calmidazolium being inhibitory at the lowest concentration and W-5 the highest (Magalhães *et al.* 1991).

Table 5.5: Inhibitory concentrations (IC_{50}) of four CaM antagonists for appressorium formation of *Zoophthora radicans* (data from Magalhães *et al.*, 1991) and topographical induction of *P. graminis* f. sp. *tritici* appressoria.

CaM antagonist	IC_{50} for *Z. radicans* appressorium formation(μM)	IC_{50} for *P.graminis* f. sp. *tritici* appressorium formation(μM)
Calmidazolium	0.55	0.3
TFP	3	2
W-7	37	10
W-5	100	60

However, for *P. graminis* f. sp. *tritici*, CaM antagonists also inhibited growth making it difficult to interpret the results presented here with respect to topographical sensing. Calmidazolium inhibited differentiation and not growth at 0.1 μM, but growth was inhibited at 1 μM (Figure 5.1A). It is possible that at 0.1 μM, calmidazolium was affecting growth, possibly by slowing growth, and therefore exerting an indirect affect on topographical sensing. This highlights a limitation with the method used here to assess growth. The length of the germ tube after 24 h is dependent on both growth rate and the duration of growth because the germlings do not grow at a constant rate over the 24 h period of incubation (Figure. 3.7). It is possible that calmidazolium at 0.1 μM slows the rate of growth but also prolongs it, thereby not altering the distance grown after 24 h. The results of all the CaM antagonists taken together suggest that either topographical sensing directly involves CaM or that topographical sensing does not directly require CaM, but does require unperturbed, CaM-regulated growth.

The involvement of CaM in tip growth has been demonstrated elsewhere. In *Fusarium graminiarum* mycelia, CaM inhibition by TFP resulted in decreased tip extension rate but increased branching resulting in no change in the hyphal growth unit (Robson *et al.*, 1991). CaM inhibition has been show to reduce tip extension in *Candida albicans* (Buchan *et al.*, 1993), *Neurospora crassa* (Ortega & Turian, 1987) and also pollen tubes (Steer & Steer, 1989).

It seems well established that a tip-high concentration gradient of Ca^{2+} is required for tip growth in hyphae (See review by Gow, 1995). It is possible that CaM antagonism may disrupt this gradient. In plant protoplasts CaM antagonists induced an elevation in cytosolic free Ca^{2+} thought to be due to inhibition of the CaM-regulated Ca^{2+}-ATPase in the plasma membrane (Gilroy *et al.*, 1987). This highlights another limitation with inhibitor studies: it is difficult to guarantee that observed responses to an inhibitor are due to primary effects rather than indirect, secondary effects.

Germlings incubated in 1 mM *trans*-2-hexen-1-ol are not inhibited by CaM antagonists suggesting CaM is not involved in the transduction of the *trans*-2-hexen-1-ol signal. This result is also consistent with the hypothesis that CaM is not required for appressorium formation but is required for normal growth and that topographical sensing is dependent on normal growth. It may be that unperturbed growth is not required for induction by *trans*-2-hexen-1-ol since, unlike the topographical signal, the germlings are constantly receiving the signal. So, although the CaM antagonists seem to have specifically inhibited CaM, it is not clear whether CaM was specifically involved with the transduction of the topographical signal. It is possible that CaM is regulating another process, such as growth, perturbation of which results in failure of the fungus to receive the topographic signal. It does seem clear, however, that induction of appressoria by *trans*-2-hexen-1-ol is independent of CaM.

In the cell, Ca^{2+}-CaM may act via adenylate cyclase and cyclic nucleotide phosphodiesterase thereby regulating the concentration of cAMP in the cell. cyclicAMP and cGMP have been shown to induce nuclear division in *U. appendiculatus* germlings, albeit at a high concentration (10 mM) (Hoch & Staples, 1984). Induction of nuclear division and DNA synthesis, but not complete appressorium formation, by cyclic nucleotides is an interesting observation. So far we have considered appressorium formation as a "single response", where in fact it is the culmination of "multiple responses" including: septation, change in the polarity of tip growth, cytoplasmic migration, nuclear division and DNA synthesis. Each one

of these individual responses may require an individual intracellular trigger. For nuclear division the trigger may be cAMP. Calmodulin acts on a wide number of targets in the cell. It is possible that several of these targets are activated during differentiation, each triggering a different part of the appressorium formation response.

5.3.2 D_2O

Treatment of germlings with D_2O, a microtubule stabilising agent, resulted in inhibition of topographical induction of appressoria and growth, but no inhibition of *trans*-2-hexen-1-ol induction of appressoria. The results suggest that microtubules are involved in topographical induction of appressoria and growth but not in *trans*-2-hexen-1-ol induction of appressoria.

Failure of D_2O to inhibit chemical induction of appressoria by *trans*-2-hexen-1-ol in *P. graminis* f. sp. *tritici* (Figure 5.7) is in contrast to D_2O inhibition of chemical induction of appressoria by K^+ in *U. appendiculatus* (Hoch et al., 1986). This suggests that the two types of chemical induction are not analogous. *Uromyces appendiculatus* appressoria induced by K^+ were formed by germ tubes which had grown away from the substrata. If the germlings did not grow aerially they were not induced by K^+ to form appressoria (Hoch *et al.*, 1987a). It is possible that the stabilisation of microtubules by D_2O prevented the germlings becoming aerial, thereby indirectly inhibiting appressorium induction. Failure of D_2O to inhibit topographical induction of appressoria at concentrations inhibitory for K^+ induction of appressoria may be because the stabilisation of microtubules is not sufficient to prevent their deformation by a relatively immovable physical stimulus.

It has been shown that heat shock induction of appressoria in both *P. graminis* f. sp. *tritici* and *U. appendiculatus* could be inhibited by D_2O (IC_{50} = 35%; IC_{100} = 60%) (Hoch et al., 1986). It was noted in *Chlorella* cells that growth in media containing

D_2O resulted in decreased induction of heat-shock proteins (Unno & Okada, 1994). It is possible that growth in D_2O also desensitised *U. appendiculatus* to heat shock.

Topographical induction of appressoria was inhibited by D_2O in *P. graminis* f. sp. *tritici* (Figure 5.7). D_2O did not inhibit topographical induction of *U. appendiculatus* appressoria (Hoch et al., 1986). Appressorium induction by topography in *U. appendiculatus* can also be inhibited by the antimicrotubule drugs griseofulvin (IC_{50} $\approx$ 10 μM), vincristine sulphate ($IC_{50} \approx 50$ μM) and nocadazole ($IC_{50} < 5$ μM) (Hoch *et al.*, 1987c). The concentrations of griseofulvin found to inhibit appressorium formation did not significantly inhibit tip growth (at 95% level of confidence). It was suggested that the effect of the anti-microtubule drugs was not on the differentiation event itself since nuclear division, induced by cAMP, was only partially inhibited. The results presented here show that topographical induction of appressoria in *P. graminis* f. sp. *tritici* can be inhibited by D_2O. Inhibition by D_2O is not through inhibition of the appressorium formation process itself because induction of appressoria by *trans*-2-hexen-1-ol was unaffected by up to 80% D_2O (Figure 5.7).

In the work presented here inhibition of growth occurred in 20% D_2O (Figure 5.7) and the inhibition of differentiation may have been a consequence of the affect of D_2O on growth. D_2O is the only inhibitor used in this current work which inhibited growth at a concentration lower (20%) than the concentration (40%) at which it inhibited appressorium induction by topography. This may mean that unperturbed growth is not necessary for sensing the topographical signal and the caution in interpreting results with other inhibitors which affected both topographical induction of appressoria and growth may be unnecessary. However, more inhibitors need to be found which affect growth and not topographical induction of appressoria for this interpretation to be wise. Length of germ tubes after 24 h is dependent on both growth rate and duration of growth. If topographical induction of appressoria is dependent on growth rate, then it is possible that at 20% D_2O growth rate is unaffected, so appressorium formation continues normally, but the duration of

growth is shortened. It is possible that topographical sensing is dependent on growth rate rather than growth duration.

At 20% D_2O, growth of *U. appendiculatus* was significantly inhibited (Hoch et al., 1986), as observed here for *P. graminis* f. sp. *tritici*. Inhibition of growth of *P. graminis* f. sp. *tritici* by 20% D_2O presented here disagrees with the observation by Hoch *et al.* (1986) that *P. graminis* f. sp. *tritici* growth was only inhibited by > 80% D_2O. It is unclear as to why the results here differ from those presented by Hoch *et al.* (1986) for *P. graminis* f. sp. *tritici* since it was the same race (race 32) as used in my study. The experiments carried out here were in dH_2O on polystyrene substrata, whereas the experiments performed by Hoch *et al.* (1986) were on water agar with 50 mM K_2HPO_4-KH_2PO_4. It is possible that the differences seen in inhibition of growth by D_2O may be attributable to the differences in substratum and/or salt concentration. It has been observed that adhesion of *U. appendiculatus* germlings is dependent on substratum hydrophobicity; the higher the hydrophobicity, the greater the adhesion (Terhune & Hoch, 1993). It would therefore be predicted that germlings would adhere more tightly to polystyrene than to agar. The differences seen in response to D_2O may be as a consequence of differences in adhesion.

The differences in response to D_2O are not the only changes seen in race 32 of *P. graminis* f. sp. *tritici* over the years. Wynn (1976) and Allen *et al.* (1991c) reported that race 32 of *P. graminis* f. sp. *tritici* did not form appressoria on leaf replicas and reported no 'spontaneous' differentiation. It is possible that changes have occurred in the race over its long period of cultivation in the laboratory.

Despite the largely accepted view that D_2O acts to stabilise microtubules, it has been shown in *Nitellopsis obtusa* that D_2O can also inhibit Ca^{2+} and Cl^- channels (Andjus *et al.*, 1994). However, the similarity between the effects of D_2O observed in *U. appendiculatus* and another microtubule stabilising drug, taxol (Hoch et al., 1986), suggests that the inhibition by D_2O is due to stabilisation of microtubules.

Inhibition of growth of *P. graminis* f. sp. *tritici* by D_2O adds to the contradictory evidence that microtubules are involved in fungal tip growth. In *Fusarium acuminatum* (Howard & Aist, 1977, 1980) and *Neurospora crassa*, disruption of microtubules results in cessation of tip growth (That *et al.*, 1988). However, tip growth is unaffected by microtubule inhibitors in *Uromyces phaseoli* (Herr & Heath, 1982), *U. appendiculatus* (Hoch *et al.* 1987c), *Phytophthora infestans* (Temperli, *et al.*, 1991) and *Candida albicans* (Yokoyama *et al.*, 1990).

5.3.3 RGD

It was found that RGD inhibited topographical induction of appressoria and growth, but not *trans*-2-hexen-1-ol induction of appressoria at the IC_{50} for topographical induction of appressoria.

The preliminary results with inhibition of appressorium formation by RGD peptide suggested that topographical induction may involve integrin proteins. However, the low pH of the inhibitory RGD solutions raised the possibility that the observed inhibition was due to differences in the pH of the incubation medium rather than presence of RGD itself. All the RGD solutions were adjusted to neutral pH with KOH. The maximum concentration of K^+ used was less than 2 mM, which was not inhibitory to the fungus (Figure 5.5). The inhibition of differentiation by pH 7.0 RGD had a higher IC_{50} than unbufferred RGD indicating that there were some complicating effects of pH. Inhibition of topographical induction of appressoria at pH 7.0 by RGD indicates that integrin or integrin-like proteins may have a role in topographical induction of appressoria. Induction of appressoria by *trans*-2-hexen-1-ol was not inhibited by 1 mM RGD, suggesting that integrin-like proteins are not involved in the *trans*-2-hexen-1-ol signalling

There was also a difference observed in growth of germlings on flat surfaces treated with pH 7.0 RGD and pH-unadjusted RGD. Growth was inhibited by 0.1 mM RGD

pH 7.0 but only at 0.3 mM for pH-unadjusted RGD. Binding of peptides containing the RGD motif has been shown to be pH dependent. Integrin binding to vitronectin in *Candida albicans* yeast cells was optimal around pH 4 and binding to fibronectin around pH 6 (Jakab *et al.*, 1993). Binding of microspheres coated with GRGDS peptide to urothelium cells was found to be pH sensitive with optimal binding at pH 7.2 (See *et al.*, 1992). It is possible that the low pH of the unadjusted RGD solution affected the affinity of the peptide for its target. The differences between the pH adjusted and unadjusted may be due to the interaction of the pH inhibition of appressorium induction (see Section 5.3.4), RGD inhibition of appressorium formation and pH influencing the binding of RGD to its target protein.

The inhibition of growth by RGD in *P. graminis* f. sp. *tritici* is consistent with observations in the oomycete *Saprolegnia ferax.* In *S. ferax*, growth is inhibited by RGD (Bachewich & Heath, 1993) and the integrin density at the apex of *Saprolegnia ferax* hyphae is correlated with growth rate (Kaminskyj & Heath, 1995). The evidence suggests a significant role for integrins in tip growth. This again raises the problem with interpreting the results: is RGD inhibiting the transduction of topographical signals in the rust fungus or preventing the germ tube from receiving the stimulus?

It is unclear whether the observed inhibition by RGD of growth and differentiation is through inhibition of integrin-like binding to extracellular matrix protein(s) or because of a non-specific effect. Use of the control peptide RGE, with the conservative substitution of aspartic acid for glutamic acid, is required as a control to determine possible non-specific effects of RGD in future studies.

Integrins are thought to be activated by 'clustering', i.e. when several integrins come within close proximity to each other (Ingber, 1991). In the case of rust contact sensing, it is easy to see how such clustering of transmembrane proteins may occur as the germ tube encounters a cell junction or right angle in the topography.

5.3.4 pH

External pH was found to affect topographical induction of appressorium formation. pH 6.7-7.5 was optimal for both topographical induction of appressoria and also for maximal undifferentiated germ tube length after 24 h. Appressorium induction by 1 mM *trans*-2-hexen-1-ol was not inhibited over the pH range 5.8–7.5. The inhibition of topographical induction of appressoria in this pH range was therefore not due to inhibition of the appressorium formation process itself. At pH 7.8 and 8.0, appressorium induction by topography and *trans*-2-hexen-1-ol was inhibited, which does not exclude the possibility that the process of appressorium formation itself was inhibited.

Stumpf *et al.* (1991) also observed an effect of pH_{ext} on differentiation induced by topography in *U. appendiculatus*. For that rust the optimum pH for appressorium induction by topography was 5.5–6.5, which is significantly more acidic than the optimum for *P. graminis* f. sp. *tritici*.

Changes in external pH may exert influence on the germ tube in several ways. In *C. albicans*, changes in external pH resulted in changes in cytosolic pH and a change in the dimorphic state (Stewart *et al.*, 1988). The aim of imaging cytosolic pH in the present study was not to precisely quantify it, but to compare the pH_{cyt} in germlings growing in buffer with different pH values to determine whether pH_{cyt} is influenced by pH_{ext}. Addition of the cell permeant propionic acid equilibrated the cytosol with the external pH and confirmed that the method of measurement using SNARF-1 would detect changes in pH_{cyt} if there were any.

Imaging of SNARF-1 showed that the pH_{cyt} was the same at pH_{ext} 7.2 or pH_{ext} 6.2 (Figure 5.6). The differences seen in topographical induction of appressoria at pH_{ext} 6.2 and 7.2 are, therefore, not because of changes in pH_{cyt}. However, the results cannot exclude the possibility that the pH_{ext} is affecting what might be a change in pH_{cyt} upon induction of differentiation because pH was not imaged during the

differentiation process. The low external pH may inhibit a transient cytosolic alkalinisation upon induction of appressoria for instance. The pH_{cyt} of differentiating germ tubes needs to be imaged.

The growth of *P. graminis* f. sp. *tritici* had a pH_{ext} optimum of 6.5 to 7.5. This differs from the observed affect of pH_{ext} on growth of *U. appendiculatus* which had optimal growth at pH_{ext} 5.5 (Stumpf *et al.*, 1991). The optimal growth pH_{ext} is also the optimal pH_{ext} for topographical induction of appressoria for both *U. appendiculatus* and *P. graminis* f. sp. *tritici.*

The growing fungal hypha typically has a positive proton current entering the growing apex (Gow, 1984). It has been suggested that this is involved in the establishment of hyphal polarity, although more recent evidence seems to show that the proton currents arise as a consequence of tip growth rather than tip growth occurring because of the proton influx (Gow, 1995).

The suggested involvement of integrins in tip growth (Kaminskyj & Heath, 1995) raises the possibility that the observed inhibition of tip growth by external pH is through adverse affects on the attachment of integrins to their ECM ligands (see Section 5.3.3).

Also, the effect of pH may be to affect the flux of ions across the plasma membrane. Mechanosensitive Ca^{2+} channel activity in onion root cells (Ding *et al.*, 1993) and *Escherichia coli* (Cui *et al.*, 1995) has been shown to be pH sensitive. It is possible that at low pH the MS channels in the rust germ tube plasma membrane (Zhou *et al.*, 1991) are also inhibited. Mechanosensitive channels have been implicated in the transduction of a wide variety of mechanical signals (Sachs, 1989) including gravitropism in plants (Ding & Pickard, 1993), and thigmosensitive coiling of plant tendrils (Klusener *et al.*, 1995). Mechanosensitive channels have also been shown to

be involved in the regulation of tip growth of the oomycete *Saprolegnia ferax* (Levina *et al.*, 1994).

5.3.5 $AgNO_3$ and H-7

The hypothesis that transduction of *trans*-2-hexen-1-ol in *P. graminis* f. sp. *tritici* may share similarities with the transduction of ethylene siganals in plants and *Colletotrichum gloeosporioides* seems to have been borne out from the data presented in this thesis. Appressorium induction by *trans*-2-hexen-1-ol could be inhibited by both Ag^{3+} and H-7.

It is thought that the inhibition of ethylene responses by Ag^{3+} is due its to substitution with the metal in the ethylene receptor (Beyer, 1976). Ag^{3+} inhibition is not due to ethylene scavenging or irreversible binding, since Hg^{3+} is more efficient than Ag^{3+} at these processes, yet does not inhibit the ethylene response of plants (Beyer, 1976).

Inhibition of appressorium induction by *trans*-2-hexen-1-ol by H-7 suggests that protein kinase C is involved in the signal transduction. H-7 has an IC_{50} value of 9-11 μM for rat brain protein kinase C (Robinson, 1992), a concentration very similar to the IC_{50} of *trans*-2-hexen-1-ol induction of appressoria (10 μM). This suggests that the effect of H-7 on *trans*-2-hexen-1-ol induction of appressoria is specific.

The failure of H-7 to inhibit topographically induced appressoria suggests that topographical induction does not involve protein kinase C, although it does not preclude the involvement of other protein kinases. There was no evidence for the involvement of protein kinase C in appressorium formation in *M. anisopliae* (St. Leger *et al.*, 1990) or *Z. radicans* (Magalhães *et al.*, 1991).

Transduction of ethylene signal in plants, identified by induction of the chitinase gene, could be mimicked by artificially increasing cytosolic Ca^{2+} and inhibited with calcium chelators (Raz & Fluhr, 1992). Induction of appressoria by *trans*-2-hexen-1-ol does not involve CaM (Section 5.3.1) and extracellular calcium is nor apparently extracellular Ca^{2+} (S. Horne, B. Myer, C. Bauch, S. Ruffert, T. J. Collins, B. Moerschbacher & N. D. Read, unpubl.). This illustrates that despite the similarities, there are differences between *trans*-2-hexen-1-ol transduction in *P. graminis* f. sp. *tritici* and ethylene transduction in plants.

6. FUTURE WORK

A) Determine the precise topographical features of the host leaf which induce appressorium formation by using microfabricated substrata, and analyse the three-dimensional topographies of wheat stomatal complexes.

B) Determine whether wheat cutin is inductive for appressorium formation of *P. graminis* f. sp. *tritici*.

C) Determine whether ethylene will induce appressorium formation in *P. graminis* f. sp. *tritici*.

D) Define the two signal transduction pathways resulting from the induction of appressoria by topographical and *trans*-2-hexen-1-ol signals by imaging cytosolic Ca^{2+} in conjunction with inhibitor and caged probe treatments.

E) Investigate the role in integrins in topographical induction of appressoria by the use of peptides with the RGD and RGE motif.

F) Determine, using 2D-gel analysis, the protein signatures of appressoria induced by topographical and chemical signals and use these signatures to identify the two appressorium types selectively induced by photo-activating appropriate caged probes.

G) Develop a novel *in vitro* assay system to screen for fungicides targeting specific signalling events during the pre-penetration phase of cereal rust infection.

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