F-actin rearrangements and analysis of physical environment of invasive hyphal growth.

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Master of Science in Biochemistry

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Abstract

Invasive growth through a substrate requires a massive amount of penetrative force, and this is generated in the space of a few microns in a growing tip. This process is known to be critical in the root hair, pollen tube, rhizoids, and the topic of this thesis, hyphal growth. However defining the mechanisms underlying the tip growth remains a contentious issue. Shortcomings in control of direction and regulation of growth began to undermine early turgor-based theories, and the cytoskeletal protein actin, ubiquitous in nature and with crucial roles in structure and motility became a target for investigation. A major breakthrough came with the discovery that a characteristic actin depleted zone (ADZ) occurs at the growing tip of hyphae during invasive but not non-invasive hyphal growth. The ADZ is likely to have an important role in generating the greater protrusive force required for invasive growth. However, since its discovery, little has been determined about the characteristics of the ADZ. Uncertainty in the description of the physical environment the hyphae face adds a layer of complexity to interpretation of results.

This thesis aims to address this issue, studying the impact of increasing agarose substrate concentration on the presence and dimensions of the ADZ in the oomycete *A. bisexualis*. Furthermore, agarose is examined by compression and imaging to compare the physical characteristics of the agar samples over the range of concentrations, and determine whether increasing agarose concentration influences agarose gel structure.

Results suggest a difference in the number of ADZ observed in non-invasive compared with invasive samples, however no significant differences in the number or dimensions of ADZ were found amongst the 1-4% w/v agarose concentrations. The 0% sample showed 20.7 percent of hyphae exhibited depleted zones, while 1, 2, 3 and 4% samples showed 56.9%, 48.8%, 40.9% and 54.2% respectively. ADZ dimensions did not correlate with agarose concentration. The average ADZ area:hyphal diameter ratio was 0.634, 0.526, 0.430, 1.09, and 0.65 for 0-4% agarose concentrations respectively. Additionally, investigation of gel compression forces revealed gel strength increases with agarose

concentration. The force required to compress the agarose increased from 1.85 Psi in 1% agarose to 4.85, 7.09 and 12.22 Psi in 2, 3 and 4% agarose concentrations respectively. SEM imaging, however, suggests heterogeneity of the fibrous interconnected network of agarose gels at a microscopic scale with variable porous structure at all agarose concentrations. This scale is relevant to hyphal tip growth. In combination, these results suggest F-actin depletion may be a response mechanism to provide greater force for invasive growth. Additionally, this response is not dependent on the concentration of the agarose media, possibly due to the variability encountered within the media. These results contribute another important step forward in unraveling the elusive mechanism of tip growth.

List of Abbreviations

ABP	Actin-binding protein
ADP	Adenosine diphosphate
ADZ	Actin Depleted Zone
AP	Allexa Phalloidin
Ar	Argon
ATP	Adenosine triphosphate
Au	Gold
BF	Bright field
$[Ca^{2+}]$	Calcium concentration
C.I.	Confidence Interval
DIC	Difference Interference Contrast
EG	Endoglucanase
K^{+}	Potassium
LMP	Low melting point (agarose)
Mg^{2+}	Magnesium
Na^{+}	Sodium
P	Phosphate (free)
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)

PMSF Phenylmethanesulfonylfluoride

PYG Peptone-Yeast-Glucose

SEM Scanning electron microscope

TBE Tris-borate-EDTA (buffer)

TL Transmitted light

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Chapter 1:

Introduction

1.1 Tip Growth

Tip growth is a crucial strategy employed by a variety of species to fulfill a diverse range of needs. These range from penetration and anchorage to foraging mechanisms in colony expansion. Cellular expansion via tip growth is important in colonization and growth through substrates and in the cases of hyphae, root hairs and rhizoids it is a vital resource acquisition strategy. Tip growth is also employed by pollen tubes in plant fertilisation.

Filamentous fungi and oomycetes need to be able to colonise new substrates and survive changes in their physical environment via hyphal tip growth. Tips sense areas of high nutrient content and alter their growth strategies to utilize these areas. Expanding fungal colonies have the ability to increase branching when conditions are favourable, in order to obtain maximum value from areas of high nutrient availability (Bowen et al, 2007). Conversely, they can increase the ratio of longer, unbranched hyphae as a foraging mechanism when environmental conditions are unfavourable, in order to maximize the likelihood of extension of the colony into a more nutrient rich environment (Markham, 1992). Tip extension can thus influence the direction of colonisation of an entire mycelium. Algal rhizoids can grow via tip extension, and in this form can rapidly recover from damage to the cell (Yoshida and Schimmen, 2009). Root hairs seek access to water and nutrient resources for plants. In plant fertilization, pollen tubes grow up a chemical gradient toward ovules, and have the ability to abort in cases of incompatibility (Geitmann et. al., 2001)

There is one fundamental theme without which tip growth could not succeed. This is responsiveness to the surrounding environment. In oomycetes and fungi, extending hyphal tips can sense areas of high nutrient content and adjust their growth accordingly. In plants, root hairs can sense areas of higher water content and algal rhizoids can detect appropriate substrates for growth. Pollen tubes can detect and follow chemical gradients. In all cases, the cells adapt to variation in the substrate. Thus tip growth is a dynamic and responsive process. Under conditions of stress tip extension can reduce or cease, but restart when conditions again become favourable. Extension rates can even decline temporarily to allow for major events of the cell cycle such as sporulation to occur.

Despite this diversity of functions, the purpose underlying tip growth remains the same: directional expansion of the cell. Accordingly, researchers continue to seek underlying mechanisms common in tip growth. There are substantial evolutionary distances between plants, filamentous fungi, and oomycetes, all of which have growing tips. This suggests that the tip growth process likely arose in a parallel manner, having evolved independently in each species (Money, 2001; 1999). There are considerable similarities in the biomechanical aspects of tip extension in fungi and oomycetes to suggest the process evolved convergently (Money et al, 2004). Work from a multitude of species has led to the proposal of several theories describing distinct mechanisms for each group, but all of these share commonality. This highlights the fact that by understanding tip growth, we may gain insight into a range of important biological processes in different species.

This thesis investigates the processes underlying tip growth using the oomycete *Achlya bisexualis* as a model organism. Although hyphal tip growth in *A. bisexualis* has been well studied for several decades, very little study has been carried out in growth through solid media in this species, which forms the focus of this thesis. Studies of growth through solid media are also uncommon in the most closely related tip growing organisms, fungi. Pollen tubes however grow through solid media and have been extensively studied. Due to morphological similarities with oomycetes, they provide a useful model to improve understanding of oomycete hyphal extension during invasive growth.

1.1.1 Description of tip growth-a dynamic balance

Tip growth is a form of growth characterized by localised cellular extension at the extreme apex of a tubular cell (Geitmann et. al, 2001). Tip extension can be explained in general terms of plasticity and elasticity, as maintenance of tip shape and growth rates are thought to be a balance of these. Regions of the cell which change shape, and undergo irreversible deformation, are described as plastic. Those which instead absorb and resist the increase, remaining unchanged, are considered elastic. During tip extension, the area at the tip is thought to be far more plastic, and less elastic. A gradient of decreasing plasticity and increasing elasticity develops as distance away from the tip increases. Most growth occurs in the apical 5μm of a growing hyphal tip (Jackson and Heath, 1993) with some variation based on tip size. This is the region of greatest plasticity.

The role of vesicles

A widely held view of tip growth involves incorporation of secretory vesicles into the extreme apex of the cell. In fungi, vesicles aggregate in the apical region in a body known as a Spitzenkörper or apical vesicle cluster, which is thought to act as a vesicle supply centre (Barnicki-García et al., 1999). These vesicles originate from Golgi apparatus or equivalent structures, and fuse with the cell membrane via exocytosis. Exocytosis results in deposition of the nascent cell wall, and incorporation of new cell membrane from the endomembrane system. The cell then becomes compliant at the site of deposition, and expands due to internal cellular pressure. The contents of the vesicles influence the local properties of the cell wall at the site of exocytosis. Spitzenkörper trajectories have been closely associated with the direction of hyphal extension in the fungus *Neurospora crassa* (Riquelme et al, 1998). The location of sites of exocytosis thus likely determines the direction of growth of the cell, a central feature of tip growth. There is no apparent Spitzenkörper in the arrangement of vesicles at the tip in oomycetes. However there is a large population of vesicles near the hyphal apex of *A. bisexualis* (see Fig 1.1). This aggregation has been observed in all tip growing species lacking Spitzenkörper, and is known as the apical vesicle cluster.

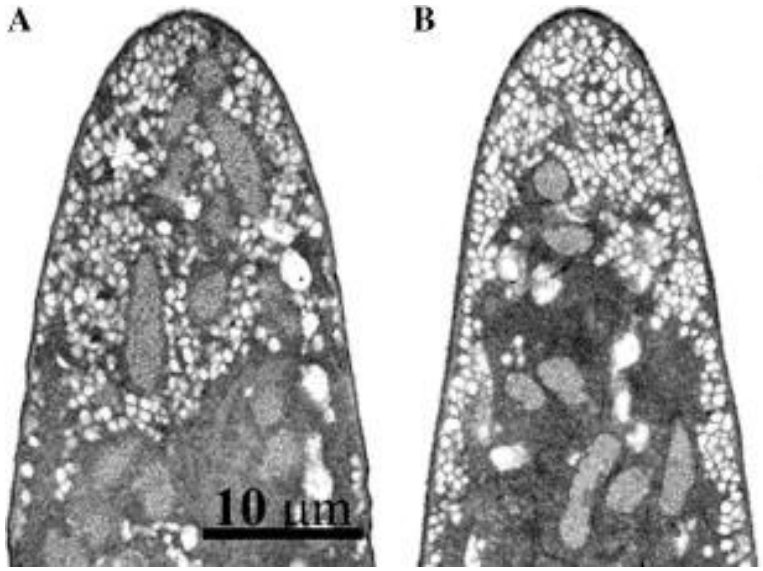


Figure 1.1 TEM image showing the aggregation of secretory vesicles (small white circular inclusions) arranged in an apparent apical vesicle cluster in the oomycete *A. bisexualis* hyphae. The hyphae were A) invasive, and B) non-invasive, and vesicle distribution was consistent in the two growth conditions. Larger inclusions represent other organelles (Yu et al., 2004).

The role of turgor

Turgor is the internal cellular pressure and is maintained by osmotic regulation in most species. The force of turgor acts outwards and is restrained by the cell wall. Original models of tip growth proposed that increases in cellular turgor pressure provided the driving force behind tip extension. However turgor driven growth theories are contentious for a number of reasons. Turgor is ubiquitous and isotropic in cells; this

means the force acts equally in all directions. Thus the force itself cannot be considered the simple underlying mechanism, because although it explains expansion, it does not account for directional growth. Additionally, tip growth has been observed in the presence of little or no turgor (Money and Harold, 1992). In hyphae, turgor reduction is accompanied by a proportional decrease in the tensile strength of the apical hyphal wall (Money and Hill, 1997). These observations forced a reconsideration of the key components involved in the model of turgor driven growth. The roles of the cell wall and also the internal structural support provided by the cytoskeleton have now been investigated in more detail. Both topics will be discussed further in upcoming sections.

The role of the cell wall

During tip growth an equilibrium is maintained between the force of cellular turgor, as detailed above, and the putative restraining forces of the cell wall and cytoskeleton. The cell wall absorbs the turgor pressure generated within the cell. When the turgor exceeds the absorbance capacity of the cell wall, the cell expands. While turgor acts equally throughout the cell, certain areas of the cell wall are more plastic, thus localised expansion will be permitted in these specific areas of plasticity. If this reduced region follows a single axis of growth tip extension will occur. How the axis is maintained is something that has remained a controversial issue despite many decades of study into tip growth in a range of species. Incorporation of more cell wall must occur to allow for expansion. The properties of this cell wall are thought to be regulated in order to control localized extension. The decrease observed in tensile strength with reduction of turgor is viewed as an adaptive response; in which the compliance of the cell wall is continuously

tuned to the magnitude of the pressure within the hypha to maintain extension growth (Money and Hill, 1997).

One possible means of regulation is wall softening via cleavage enzymes, acting to break bonds at specific sites on the cell wall. The strength of the cell wall is a product of the interactions and bonds formed between its constituent polymers, and the strength of the polymers themselves. Enzymatic cleavage of either bonds or polymer molecules would reduce the tensile strength of the wall and create a region of high extensibility (Money and Hill, 1997). Consistent delivery or activation of these cleavage enzymes in one region would result in directional growth along an axis following the highly extensible region.

Regulation of extensibility may be further explained by the regulation of the cytoskeleton. The membrane skeleton is thought to be attached to the cell membrane via integrin-like proteins (Chitcholtan and Garrill, 2005). If the cytoskeleton has a role in structural reinforcement at the tip, localized cytoskeletal rearrangements may influence the ability of the cell membrane to expand in certain areas. The influence of the cytoskeleton will be discussed in further detail in this thesis.

1.1.2 Cell surface expansion

During tip growth, the cell surface expands in an anisotropic manner (Shaw et al, 2000). This means that during cell expansion, any given area of cell wall will not necessarily

expand the same amount as an adjacent region. Furthermore, in order to form the hyphoid characteristic of tip growth, a gradient of expansion capacity (extensibility) is necessary.

Tip growth has also been investigated from a mechanical perspective. Studies revealed that tubular rubber balloons are an excellent model for the heterogeneity and anisotropy of surface expansion in tip growth in the root hair *Medicago trunculata* (Dumais et. al, 2004; Bernal et. al., 2007). Balloons exhibit a gradient favouring expansion at the tip while resisting expansion with increasing distance from the tip. *In vivo* experiments tracking microsphere movement on the growing tip revealed a disparity in the relative rates of cell surface expansion occurring at the tip. Experiments conducted with the same species led to the proposal of a model of expansion anisotropy designating three characteristic zones of expansion, as shown in Figure 1.2 (Shaw et. al., 2000). Remarkably it was not the centre of the extreme apex that exhibited the fastest rate of cell surface expansion, but in fact the annulus immediately adjoining it.

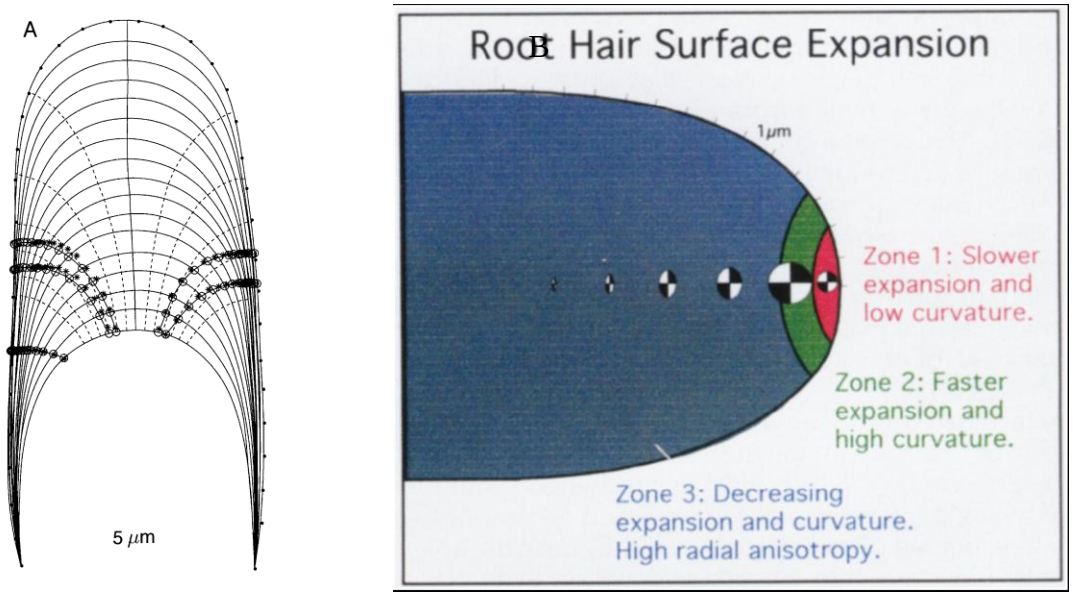


Figure 1.2 Cell surface expansion at the hyphal apex. Figure A shows the course of cell surface expansion determined by microsphere tracking during growth of *Medicago trunculata* root hairs. Figure B displays the 3 distinct zones of expansion characterised by relative rates of radial and circumferential expansion. Note zone 2 shows the fastest expansion rate (Shaw et al., 2000).

Shaw and colleagues present the apical dome separated into a central region of slow expansion and high curvature, a second zone of faster expansion and high curvature distal to the apex, and the remaining zone decreasing in expansion and curvature, adjoining the remainder of the hyphal cylinder. As the distance from the apex increases, the rate of meridional (forward direction) expansion decreases compared to radial expansion until, in the sub-apical region, it becomes negligible. Expansion at this distance is only in the circumference of the tube, and even that only expands a minute amount. Both tubular balloons and root hairs of *M. trunculata* have general functional similarity to oomycete

hyphae extension. Tips of *M. trunculata* and *A. bisexualis* are morphologically similar at the extreme apex thus models of this sort can be used to enhance the general understanding of the surface expansion in *A. bisexualis*.

Interestingly, the shape the tip takes can be very distinct in different species, ranging from rounded tips of the fungal hyphae of, *Neurospora crassa* and *Lilium longiflorum* pollen tubes to the tapering tips of the oomycete *A. bisexualis*. Such dimorphism can even be seen among genera. The oomycetes are a good example of this, highlighted by the true hyphoid, cylindrical hyphoid, and the conoidal hyphoid morphologies of *Saprolegnia parasitica, Aphanomyces astaci, Leptolegnia* sp. *and A. bisexualis,* respectively. (Diéguez-Uribeondo et. al., 2004). While from a mechanical standpoint, it may make sense for the apical dome with a low expansion rate to maintain a sharp 'piercing' shape, evidence from fungal hyphae suggests there are a variety of successful morphologies.

Furthermore, studies in the above oomycetes show that the curvature of the extreme apical region of all species matched the same hyphoid curve. It is not until sub-apical regions that species specific deviations occur (Diéguez-Uribeondo et al, 2004). This suggests the extreme apical region and not the entire tip may play a greater role in tip extension. Thus it seems the processes underlying directional cellular expansion may be more important than tip shape.

1.1.3 Invasive and non-invasive tip growth

Cells extending via tip growth can grow in a range of environments. These environments

can pose some unique difficulties. Invasive growth is the term used to describe tip extension through solid media. This mode of growth likely evolved primarily for the advantage it confers in the ability to obtain nutrients which are inaccessible to cells that are unable to invade substrate (Money, 1999). Invasive growth differs from non-invasive growth in that it requires the ability to generate enough force to overcome the strength of the media. Thus, unless the substrate is liquefied, some mechanical force will be required to penetrate the surroundings (Money, 2001). This mechanical force can be very large, as in the case of appresoria (penetrative hyphae) which achieve penetration through Mylar®, which would require generation of a force sufficient to exceed the estimated yield stress of 50-80 MPa of the material (Howard et al., 1991; Goriely and Tabor, 2006). It is likely that to achieve this, invasively growing tips possess mechanisms to generate the greater force required to penetrate through solid media, relative to non-invasive growing tips.

The mechanisms describing how this greater force is generated are not clear. To achieve cellular extension through solid media, the protrusive force must not only exceed the withholding capacity of the cell wall, but also have sufficient force to penetrate the solid media. Theories have for a long time implicated the cell wall; suggesting the role of localized cell wall softening with a gradient of cell wall extensibility toward the tip (Harold, 1997). Instead of the force of pre-existing turgor pressure being absorbed by the cell wall, the force is allowed to transfer through a softer cell wall. Less of the force of turgor will be absorbed by the wall, and more of the force will be exerted onto the surrounding media.

Study of tip growth thus far has been undertaken of diverse organisms as previously explained. However there is a lack of study in invasive growth in fungi and oomycetes. Investigation has primarily been restricted to pollen tubes, which are a useful model of invasive, but not non-invasive growth. Thus the model is limited in that it does not approach the processes surrounding the change between invasive and non-invasive growth. Pollen tubes grow invasively in nature and thus mechanistic details are directly comparable only to studies of invasive growth in other species.

1.1.4 Substrate and substrate variability

It is also important to consider the heterogeneous nature of substrates, and thus the ever-changing challenge substrates present for invasion. In natural systems, substrates are rarely homogeneous. Penetrating tips will undergo periods of non-invasive and invasive growth, and require the ability to adapt accordingly. This is especially relevant in the case of filamentous species, many of which in nature typically colonise tissue of dead organisms. To colonise this tissue, hyphae must overcome the inherent varied resistance the tissues cells present as a substrate; from the reinforced cell wall to the fluid cytoplasm. It has been hypothesised that some tip growing hyphal species weaken the surrounding substrate by secretion of substrate degrading enzymes; however there is little direct evidence to support this (Money, 2001). Even if this does occur, tip growth occurs through solid media. Thus greater force is required to penetrate the substrate. It is likely that tip growing cells have other mechanisms enabling variation of the penetrative force, and can vary this in response to external challenge.

Despite the difficulties invasive growth poses, disadvantages are outweighed by the advantage it confers the hyphae in the ability to cope with a changing environment. Evidence abounds to demonstrate the success of organisms growing invasively; with such major and widespread effects as seen in the infections of the invasive oomycetes in the diseases of *Pythium insidiosum* in pythiosis and the oomycete *Phytophthora infestans* was the organism responsible for the potato famine of the 1840s.

1.2 Roles of cellular components and models of tip growth

Tip growth is a complex process involving many cellular components. Currently it has not been established which component plays the most important role in cellular extension. The proposed mechanistic models are not mutually exclusive, however components have been separated in this discussion to enhance explanation. Components of particular interest are the cell wall, the cell membrane, and the cytoskeleton, and each will be discussed in detail.

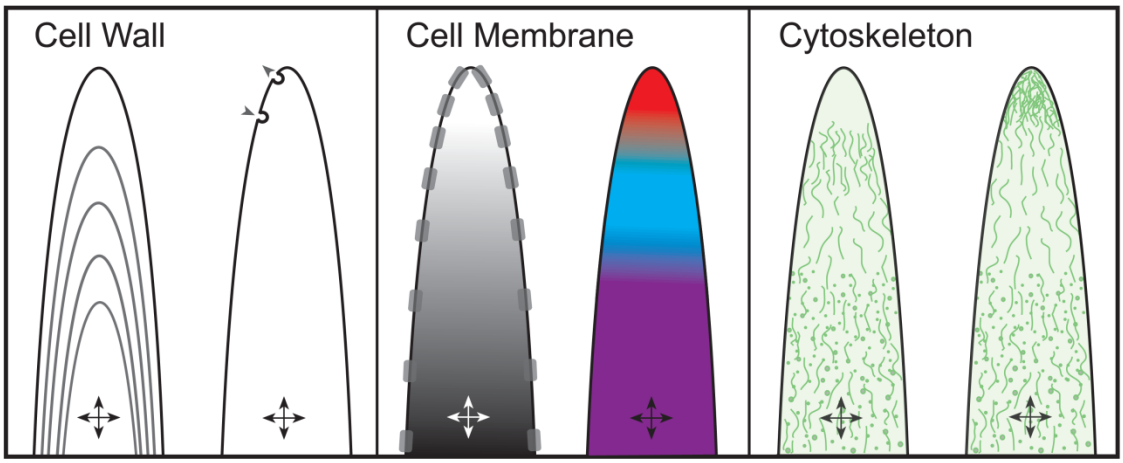


Figure 1.3 Schematic demonstrating cellular components implicated at the extending apex in various models of tip growth. The cell wall favours radial over circumferential expansion, increasing in extensibility toward the apex; and its expansion is influenced by exocytosis and endocytosis of wall polymers via secretory vesicles. Distribution of ion channels on the cell membrane may influence regulation of dynamic tip high Ca^{2+} and pH gradients, there may also be a role of an alkaline band in extension as observed in growing pollen tubes. Cytoskeletal F-actin rearrangement to form an apical depleted zone (ADZ) has been associated with invasive growth; and apical reinforcement of F-actin is associated with non-invasive growth. Turgor is assumed isotropic in all cases.

1.2.1 Turgor driven growth

The widely accepted model of tip growth implicates turgor as the driving force of directional expansion; with the view that the cell wall yields to the force exerted by the internal hydrostatic pressure (Money, 1997). This force can be very large, as in the case

of the aforementioned appressoria, which generate turgor pressures of at least 8MPa (Howard et. al., 1991). Turgor is withheld by the tensile strength of the cell wall. Thus in a turgid cell, the structure of an isotropic cell wall would absorb the force, resulting in no net cellular extension (resistance to deformation).

Cell walls of tip growing species are composed of a variety of interlinking polymers, as explained previously. Mechanical models consider that the interlinked polymers perform work when the cell surface expands. Since turgor acts isotropically, in order to generate the tubular cells characteristic of tip growth, the apical region must continually possess the polymers least capable of resisting deformation. In this model, the bond strength of the cell wall polymers is paramount. Initially, models did not take into account the possibility of internal structural reinforcement of the cell wall; the putative restraining force of the cytoskeleton. This will be discussed in the context of the cytoskeletal contribution to tip extension in upcoming sections.

Whether or not turgor provides the driving force for tip growth has been intensely contested over the decades of study in this field. In earlier years, it was suggested that turgor should not be considered responsible for plant cell expansion (Burström, 1971). However, this appeared to be an academic matter of definition of terms (Money, 1997); and investigation in the field of tip growth continued with the underlying assumption that turgor provided the driving force for cellular extension, but mindful that turgor alone could not generate the controlled morphology and directional cell growth that is observed in tip extension.

Cellular turgor is continually maintained by osmotic adjustment in fungi (Lew et. al, 2004), which appear to adapt to osmotic stress and maintain growth. It is proposed that cellular turgor is likely regenerated via solute accumulation. However this assertion was made with the inherent assumption that turgor provided the driving force for growth, since the inference was made from the observation that growth resumption parallels this buildup (Money and Harold, 1992).

In 1992, an important discovery was made that undermined even the assumption that turgor provided that background force resulting in cellular extension. Experiments revealed the oomycetes *Achlya bisexualis* and *Saprolegnia ferax* were capable of hyphal tip growth yet were incapable of turgor regulation (Money and Harold, 1992). During osmotic stress induced turgor reduction the extension rate of *A. bisexualis* hyphae was maintained until turgor fell to less than a third of its normal value. Hyphal extension rates were also able to recover from this osmotic stress; but without requiring regeneration of cellular turgor. Furthermore, poorly polarized growth still continued when turgor fell so low it was no longer measurable. Due to this remarkable ability, study using *A. bisexualis* and other oomycetes as a model system provides an interesting case study; of an organism where turgor does not underlie growth and other mechanisms are most likely responsible for cellular extension. *A.bisexualis* has since become the focus of many studies investigating a wide range of components suspected to be involved in the process of tip extension. The oomycete is also an excellent model for filamentous growth due to its non-pathogenic nature and large size, enabling higher resolution imaging relative to

other species. Additionally, *A.bisexualis* is coenocytic (non-septate), thus cannot differentially regulate its turgor in a compartmentalised manner, as is postulated to occur in septate hyphae.

Furthermore, cellular growth has been observed to be sustained when up to 97% of hyphal pressure has been eliminated (Money, 2001). Studies in the oomycete *Saprolegnia ferax* also demonstrated growth of hyphae with reduced turgor, although interestingly with somewhat blunter tips than normal hyphae. The cell wall instead becomes increasingly more extensible as turgor is reduced, and extension continues. It was proposed that this evidence negates the requirement for turgor to drive tip extension and morphogenesis (Harold et. al., 1995). It is conceded, however, since hyphae did not successfully penetrate substrate, that turgor may be necessary during invasive growth.

However, the role of turgor increase in non-invasive tip extension has not yet been entirely ruled out. Another consideration is the possibility of cytoplasmic compartmentalization of turgor. This phenomenon has been observed in the form of cellular blebbing in animal cells, but has yet to be seen in fungal or oomycete hyphae (Charras et al., 2005).

1.2.2 Cell Wall

The cell wall is a load bearing structure, and at equilibrium is responsible for the tensile strength of the cell. Tensile strength in localized areas can be manipulated to result in

directional growth of the cell (Money and Hill, 1997). The cell wall decreases in rigidity approaching the apex of a growing tip (Shapiro and Mullins, 2002). There are several potential mechanisms to explain the development of this gradient, implicating wall softening enzymes, cell wall rigidification; and activities of secretory vesicles, as will be discussed in upcoming sections.

Cell wall composition varies hugely amongst species. In *A. bisexualis* the major structural cell wall component is cellulose, cross-linked in a glucan network. The predominant structural cell wall component in fungal hyphae is instead chitin. Pectin is the main cell wall component at the pollen tube apex, and callose also contributes structural reinforcement (Parre and Geitmann, 2005). In root hairs, the primary component is again cellulose, in this case cross-linked with hemicelluloses and pectins (Galway, 2006).

Given the variety of cell wall compositions observed amongst tip growing species, it follows that cell wall softening will be species specific, dependent on the major structural constituents. This is especially relevant, for example, in the case of enzymatic cleavage. In *A. bisexualis* the activities of a class of cellulases (endoglucanases) have been investigated (Money and Hill, 1997; Shapiro and Mullins, I; II, 2002). In pollen tubes pectinases have been found to reduce cell stiffness (Parre and Geitmann, 2005(b)). Which bonds are broken during putative enzymatic cell wall softening is unresolved. It has been suggested in *A. bisexualis* that there may be cleavage of cellulose microfibrils themselves, or of the cross-linking glucan molecules which bridge the microfibrils (Reiskind and Mullins I; II, 1981; Shapiro and Mullins I; II, 2002). Regardless of the

underlying mechanism, cell wall structural integrity (tensile strength) reduction is associated with tip extension (Money and Hill, 1997). As progress continues, theories evolve to incorporate new information about the roles of other activities contributing to the properties of the cell wall yielded by advances in technology and fluorescent imaging.

Pulsatile growth

As growth rate measurements became more accurate and time lapse photographic studies of growth were replaced by real time video, evidence began to point to the possibility of a discontinuously growing cell wall (Pierson et al., 1995). One theory attempting to explain this growth rate variability is that of pulsatile growth. The theory of pulsatile growth is based on regular periodical variation in secretory rates at the hyphal apex. It is suggested that this can be further refined to the rate at which cell materials are exocytotically discharged (Lopez-Franco et al., 1994; Pierson et. al., 1995). It is not clear whether this is more likely to lead to periodic cell wall stretching during growth, or accumulation of 'excess' wall polymers at the tip during vesicle discharge phases. Models propose that initial surface expansion is achieved by stretching, and greater expansion involves addition of new stress-bearing polymers (Money, 1997). In either case the wall absorbs the discharged vesicle contents during each pulse, decreasing the mechanical resistance of the wall to cellular turgor and allowing expansion of the tip.

Pulsatile growth appears to be an essential part of the functionality of the pollen tube. Lopez-Franco et al. (1994) suggested the mode of growth was also present in a wide variety of fungal taxonomic groups, including oomycetes, ascomycetes, basidiomycetes,

deuteromycetes and zygomycetes. They observed continuous fluctuation between fast and slow hyphal elongation rates in several species including *Saprolegnia ferax, Trichoderma viride, Rhizoctonia solani and N. crassa*, amongst others. They considered this fluctuation a manifestation of periodic physiological changes during tip growth, suggesting the input of other processes undergoing pulsation in the cell, including flux of cytoplasm, ion concentration change and electrical phenomena (Lopez-Franco et al., 1994).

While pulsatile growth may be a vital part of pollen tube extension, it has been proposed that the observation of pulsatile growth may be an artifact incurred due to non-linear hyphal extension, involving movement in the z plane. Studies investigated the growth rate fluctuation in the oomycetes *Saprolegnia ferax*, and compared it with the apparent growth rate reduction that would be expected should the hypha move up and down during imaging, and revealed a very close match in the growth rates (Jackson, 2001). Additionally, investigations in the oomycete *A. bisexualis* have revealed the apparent motion to be nothing more than an imaging artifact in this organism (Jackson and Garrill, unpublished data).Thus pulsatile growth may not be a common mechanism underlying tip growth in all species. It is therefore necessary to investigate the roles of other cellular components.

Possible mechanistic explanations

One consideration, and that adopted by earlier models (Lockhart, 1965 reviewed in

Money, 1997) is that extensibility is determined by the physical properties which result from the molecular composition of the wall itself. This is determined by the molecular composition of the secretory vesicles supplying nascent cell wall. The primary molecule responsible for reinforcement and strength of the cell wall in *A. bisexualis* is cellulose (Reiskind and Mullins I and II, 1981). Although cellulose microfibril cleavage may be implicated in increasing tip extensibility, importantly cellulose crystals, known to be load bearing structures, are absent entirely from the extreme apex of the growing tip. (Shapiro and Mullins II, 2002). This spatial variation is also seen in callose deposition in pollen tubes. Callose has been indirectly linked to reduction of extensibility, and is again absent at the growing apex (Parre and Geitmann, 2005).

Secondly there is the idea that the nascent cell wall is first deposited, and subsequently modified to determine its physical properties. This model infers the cell wall is released at the apex by secretory vesicles in a compliant state, then rigidifies via cross-link formation as it increases in distance from the point of vesicle delivery. Thus the extensibility of the cell wall could be regulated by controlling the rate of cross-linking occurring in the cell wall.

An alternative proposition is that cross-linked cell wall may be deposited into the apical region and then softened. The softening is proposed to occur via the action of enzymes that degrade the cell wall components (Li et. al, 2001). The identity of the softening enzymes depends on the major structural cell wall components. One class of enzymes that affect the cell wall are expansins. These are found in plants and interfere with the

hydrogen bonding capacity of cell wall polysaccharides under mechanical strain (Sharova, 2007).

Another class of enzymes thought to control cell wall softening are endoglucanases. Endoglucanases have been associated with a reduction in the tensile strength of the cell wall in oomycetes; and similar observations have been made with beta-glucanases in *L.longiflorum* pollen tubes (Money and Hill, 1997; Li et. al, 2001). There are a suite of endoglucanases present at the growing hyphal tips of the oomycete *A. ambisexualis* (Hill; 1996). Endoglucanase is a glucan hydrolase (cellulase) which cleaves at β(1,4)-glucan bonds. Significantly, cellulose microfibrils, but not crystalline cellulose can be hydrolysed by endoglucanases (Money, 1994). These molecules, being invulnerable to cleavage, may be important in maintenance of the boundary of the softened growing apex.

Cellulose molecules are part of a network interlinked with glucans which contain large amounts of β(1,4) and β(1,6) bonds susceptible to cleavage by the enzymes. It is unclear whether the increased extensibility associated with endoglucanases is due to cleavage of cellulose, glucan or a combination of both (Money, 1994). Furthermore, there is experimental evidence that certain endoglucanase enzymes are delivered to the apical cell wall in secretory vesicles as membrane-bound pre-cursors. It is theorised they remain there until the pre-cursor forms undergo proteolysis and activation, releasing glucanolytic polypeptides to cleave bonds in the apical cell wall (Hill et al., 2002). This would decrease the rigidity of the cell wall, allowing tip extension. This is an attractive

proposition to describe regulation, presenting the opportunity for fine tuned and rapid response to change in external stimuli.

A combination of all models may provide the most accurate explanation of the role of the cell wall. In all models, the cell wall is likely to have a major role in determining the localized extensibility of the cell. However the composition and properties of the cell wall are certainly influenced by the contents of the exocytotic secretory vesicles that supply it.

1.2.3 Cell Membrane

The cell membrane acts in synergy with the endomembrane system as part of a system of secretory vesicles. These vesicles are responsible for deposition of the nascent cell wall. The generally accepted view of tip extension involves localized incorporation of new plasma membrane and cell wall deposited by exocytosis of secretory vesicles.

TEM and immunofluorescent imaging has clearly shown formation of a vesicle dense area known as a Spitzenkörper in *N. crassa* hyphae (Roberson and Fuller, 1988). This structure is seen exclusively in the growing apex and at sites where branching occurs (Bartnicki-Garcia, 1989). The Spitzenkörper is viewed as an apical aggregation (body) of secretory vesicles within a cytoskeletal background matrix; in which other organelles are arranged. It is a dynamic entity in a constant state of flux, and appears to have crucial roles in regulating hyphal extension and morphogenesis. The Spitzenkörper is thought to

function in regulation of exocytosis, responsible for receiving, distribution, and organization of secretory vesicles and signalling molecules (Lopez-Franco et al., 1994). Where a Spitzenkörper is absent in other species, an equivalent apical vesicle-dense region occurs in a similar location. This is seen in TEM images of hyphal apices of *A. bisexualis* (Yu et al, 2004). Furthermore, experiments tracking the axis of growth and Spitzenkörper movement have shown this structure has a role in determining the axis of tip growth (Riquelme et al., 1998)

Current models characterising vesicle dynamics also incorporate apical vesicle recycling, although this theory is somewhat controversial (Torralba and Heath, 2002). Apical vesicle recycling theory involves endocytosis of sub-apical cell membrane with associated cell wall. Exocytosis delivers nascent cell wall to the cellular apex. In the area of exocytosis, the cell is plastic. According to the model, the location and relative rates of endocytosis and exocytosis would affect the composition of the cell wall in the specific areas in which they occur.

Endocytosis has been demonstrated using the membrane-selective dyes FM 1-43 and FM 4-64 localised to the extreme apical dome of root hairs of *Arabidopsis thaliana* and *Triticum aestivum*; and rhizoids of the green alga *Chara contraria* (Ovečka et. al., 2005; Limbach et al., 2008). Thus the model fits well with the observation of differential zones of cell surface expansion (Shaw et al, 2000). Genetic evidence also corroborates the existence of endocytosis in growth of filamentous species. *N. crassa* genomes encode a large number of homologues of genes associated with endocytosis in yeast, providing

further support for the importance of endocytosis in tip growth in fungi (Read and Kalkman, 2003).

The apical vesicle recycling model may also contribute to the gradient toward cell wall rigidification with increasing distance from the tip. Constant localized modification of the cell wall composition would enable maintenance of local plasticity and thus directional growth. Altered growth and morphology, and abnormal accumulation of cell wall constituents was observed with impaired endocytic function in the fungus *Aspergillus oryzae* (Higuchi et al, 2009). Pollen tubes with defective membrane recycling also have defects in maintenance of apical cell wall extensibility and polarity (Sousa et. al, 2008). Thus the balance between exocytosis and endocytosis may have a role in controlling the extensibility of the apical region.

Possible mechanistic explanations: role of the cell membrane in maintaining tip high gradients

The spatiotemporal regulation of endocytosis and exocytosis will affect the localized composition of the cell membrane This may link to other putative functions of the cell membrane, which has been implicated in maintenance of gradients of both Ca^{2+} and pH within the cell.

Calcium

One of the undisputed features of calcium (Ca^{2+}) and tip growth is that, in hyphae, and

other tip growing cells studied, is that they maintain a tip high gradient of cytoplasmic Ca^{2+} (Heath and Skalamera, 2001). Both Ca^{2+} influx and a cytoplasmic Ca^{2+}gradient towards the apex are necessary for growth in the pollen tube tip (Jackson and Heath, 1993). Interference with this Ca^{2+} concentration results in inhibition of growth (Cardenas et al., 2008). Ca^{2+} is likely to have a role in transport and fusion of secretory vesicles. It has also been shown to have some control over F-actin restructuring proteins held within their bundle structures (Vidali et. al., 2001).

The distribution of cytoplasmic Ca^{2+} is different from that of membrane associated Ca^{2+}. (Jackson and Heath, 1993). There is evidence to suggest that the mechanical properties of the actin membrane skeleton are affected by the gradient of cytoplasmic Ca^{2+}. Originally it was thought that structural actin filaments may be severed into fragments by Ca^{2+}. Since this time a number of Ca^{2+} regulated actin membrane skeleton-associated proteins eg. calmodulin, villin and calcineurin have been shown to be necessary for growth, and some have differential distribution along the tip axis (Heath and Skalamera; 2001; Vidali et. al, 2001). These proteins have important roles in actin filament organisation as discussed further in chapter 2. Additionally, high Ca^{2+} concentrations inhibit cytoplasmic streaming at the extreme apex. The intracellular Ca^{2+} concentration reaches as high as between 1-10μM and changes in the same periodic manner to the oscillating periods of rapid and slow tip extension in *L. longiflorum* pollen tubes (Messerli and Robinson, 1997; Messerli et. al., 2000). Few studies have been undertaken in *A. bisexualis*, however and, pollen tubes provide only some comparison to hyphae which are also undergoing invasive tip growth, but may not extend via pulsatile growth.

pH

Tip growing cells have also been seen to mainatin a tip high proton (H^+) gradient. This characteristic gradient is also dynamic, and its activity correlates with growth, as seen in the pollen tubes of *L. longiflorum*, where periodic oscillation of an alkaline band occurs in parallel with pulses of extension (Lovy-Wheeler et al., 2006). Disruption of pollen tube growth via inhibition of actin filament polymerisation has recently been associated with abolition of apical acidity (Cardenas et. al., 2008). However recent developments in intracellular pH imaging of actively elongating *Aspergillus niger* revealed a longitudinal cytoplasmic pH gradient was not apparent in the apical 20μm of hyphae in this organism (Bagar et al., 2009). This result implies a pH gradient may not be a feature of all tip growing systems. Further study will be necessary to determine the role of pH in *A. bisexualis* hyphal extension.

Ion channel distribution

How the cell establishes and maintains these Ca^{2+} and putative H^+ gradients is unclear. One possibility is differential ion channel distribution over the length of the cell. By creating a series of protoplasts (of membrane) from intervals along the length of the cell, it was discovered that a greater density of stretch-activated ion channels was found at the tip of the oomycete *Saprolegnia ferax* (Garrill et al., 1992). In studies of *Saprolegnia ferax*, there is a suggested link between the spatial distribution of ion channels and the cytoskeleton. (Levina et. al., 1994). Since Ca^{2+} and pH have a role in filament formation, variable distribution of stretch-activated ion channels may influence the arrangement of

the actin cytoskeleton by modulating the tip high Ca^{2+} and H^{+} concentrations. Furthermore, the changes in cell wall tension during growth may influence the activity of these channels and provide an element of feedback regulation in tip extension (Heath, 1995; Jackson and Heath, 1993).

1.2.4 Cytoskeleton

The cytoskeleton inevitably forms an integral part of every mechanism proposed to describe tip growth. However whether its role is in provision of a protrusive or restraining force is a subject of much debate. Firstly, the former is considered.

Amoeboid movement theory

Some doubts have been expressed regarding whether or not a force withholding turgor is necessary (reviewed in Money, 1995; 2001). These doubts are based primarily on shortcomings in the theory of turgor-driven growth, resulting from the discovery of growth in reduced turgor outlined above. Thus alternatives to turgor in providing the driving force for tip extension have been investigated.

Due to experimental evidence of tip growth in low turgor, it has been argued that, in oomycetes at least, the cytoskeleton may through polymerisation provide the force required for cellular expansion when the cell contents are at or near atmospheric pressure. Based on the dimensions of microfilaments, Money (1997) has calculated that actin polymerisation is unlikely to generate a force that would exceed the internal pressure until turgor drops below 0.02 MPa, which, incidentally was the limitation of the turgor

measurement device used in the turgor reduction experiments noted above. This would be consistent with a role for the F-actin cytoskeleton in providing a protrusive force to sustain growth under conditions of extremely low cellular turgor.

The possibility of a myosin-actin interaction akin to that involved in cytoplasmic streaming has also been considered. This model implicates the contraction of myosin resulting in microfilament sliding, generating a protrusive force at the tip (Money, 1997). There is also the intriguing possibility of generation of a higher particle number in the apical by severing actin microfilament filament bundles to form a larger number of smaller filaments arranged in a network. This would result in a localised increase in gel pressure via hydration of the network of actin and generate force for cell extension (Condeelis, 1993).

It has been proposed that the role of the apical actin configuration in cell elongation should instead be compared to pseudopod extension in animal cells. Discoveries of pseudopod-like formation in other cell mechanisms such as white blood cell crawling (Friedl et al., 2001) have inspired research investigating the potential for implication of actin cytoskeletal arrangements in providing a protrusive force to enable morphogenesis of the cell tip. Such protrusions have been observed in the case of pseudopodia, where the cell extends as sustained polymerization builds actin filaments in a particular direction. A characteristic gradient develops with enhanced F-actin at the tips of pseudopodia (Iwadate and Yumura, 2008; Yumura, 1993).

The amoeboid-like growth form has been observed in hyphae of *A. bisexualis*. Despite the claim that turgor may not be responsible for hyphal morphogenesis of the related species *S. ferax* (Harold, 1996); under reduced turgor, the morphology of *A. bisexualis* was considerably altered. Hyphae displayed apparent cytoskeletal extensions against the cell membrane (Money and Harold, 1993; Money, 2001). This apparent form of cellular extension occurred in the absence of 97% of the cellular turgor.

However, it has been suggested that provision of a protrusive force via apical actin rearrangement is irrelevant in the context of plant and fungal tip growth due to dissimilarities in the nature of cellular extension in the different cell types. Animal cells forming pseudopodia do not increase cell surface where tip growth necessitates this (Geitmann and Emons, 2000). Therefore it is unlikely the mechanisms would align closely, considering the significance of the cell wall in restraining cellular forces. The generation of new cell wall during tip extension may reduce the need for the generation of protrusive force, which is required to extend the boundary of animal cells.

The current view of the cytoskeletal contributions to cell growth implicate filament formation by polymerization, incorporating elements of F-actin extension, in parallel with amoeboid movement theory, but with a major difference. Instead of the generation of a protrusive force to extend the cell boundary, may, in concert with the cell wall resist the pressure of turgor, which provides the protrusive force for tip extension.

With this in mind, it is possible to envisage both a reinforcing and extending function of actin in apical extension as not mutually exclusive and which is occurring might depend on growth conditions. A role for F-actin has been suggested in maintaining structural rigidity (Jackson and Heath, 1990). Significantly, inhibition of actin polymerisation has been found to result in acceleration of growth of cells with normal turgor, and deceleration of growth of those with low turgor (Jackson and Heath, 1990). This finding is consistent with a differential role for turgor under different conditions, and a greater importance of the cytoskeleton in providing protrusive force at low turgor. As such, turgor would remain the driving force behind tip extension, but the regulation of its effect, particularly at low turgor is reliant on the polymerization of the F-actin cytoskeleton. This would overcome concerns with regulatory control of growth being maintained external to the cell (ie. in the cell wall, or substrate); into the cytoplasm, where it is accessible to normal intracellular regulatory mechanisms (Heath, 1995; Kaminskyj and Heath, 1996).

Possible mechanistic explanations

Investigations into the role of the cytoskeleton in tip growth have primarily focused on the study of actin and its polymerization. Microtubules (MTs) have not been well studied in oomycete or fungal tip growth. However there is evidence MTs may play a role in maintenance of direction in root hair growth (Ketelar et al, 2003) and extension rates of *N. crassa* hyphae may be dependent upon the dynamic characteristics of MTs (Uchida et al, 2007). Moreover, recent discoveries have been made, implicating MTs in delivery of

molecules responsible for actin arrangement to the tips of the filamentous fungi *A. nidulans* and *N. crassa* as is seen in S*accharomyces cerevisiae* (reviewed in Fischer et. al, 2008). However, microtubules are not always present in the apical region tip growing cells (Bourett et al., 1998), and their disruption has no effect on the growth of the filamentous fungus *Ashbya gossypii*, and tip growth in the yeast *Candida albicans*. Thus although they may play an important in some tip growing species, they are less likely to form an integral part of the mechanism underpinning tip growth in general. The role of intermediate filaments has not been documented in this context. Therefore, the focus of mechanistic studies instead remains actin, which is present, albeit in different distributions, throughout the apical region of all tip growing cells.

The possible functions of the F-actin cytoskeleton are controversial and have been discussed in great detail (Geitmann and Emons, 2000). In addition to the provision and/or resistance of force, F-actin may also act in the spatial and temporal control of vesicle fusion; and acting as a filter to control movement organelles and vesicles. Many of these processes have previously been described in the context of the roles of other cellular components, highlighting the interconnected nature of tip growth regulation.

Differences in cytoskeletal arrangements complicate the elucidation of the function of actin in tip growth. This is highlighted by Figure 1.4; displaying the different spatial arrangements of F-actin bundles observed in pollen tubes, fungal hyphae, and root hairs (de Ruijter et al., 2001). Even within fungi, and the morphologically parallel oomycetes, great disparities can be observed, in both cytoskeletal and vesicular arrangements. This

suggests there is some flexibility in the role of actin in tip growth, since rearrangements are observed in various species, but the nature of the rearrangements is species specific.

In summary, the exact manner in which the cell wall, cell membrane, and cytoskeleton interact to maintain control of tip growth has yet to be determined. Continuing study into each of these factors independently and in combination is building towards resolving the relative importance and interactions of the various components.

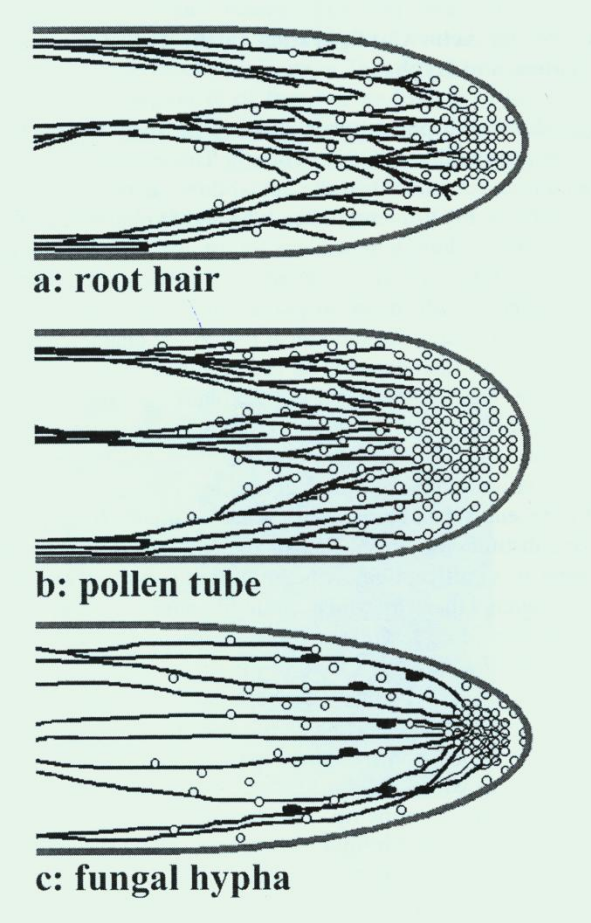


Figure 1.4 Schematic showing typical actin cytoskeleton organisation in three tip growing species, demonstrating distinct species-specificity. Diagrams show a) the highly branched network typical of root hair apices, b) the absence of actin at pollen tube apices, with the subtending dense collar zone and c) longitudinal actin microfilaments leading to the Spitzenkörper at the fungal hyphal apex (de Ruijter et al., 2001).

1.3 Objectives

In this thesis I examine the changes to the cytoskeleton of hyphae of the oomycete *Achlya bisexualis* grown invasively in increasing concentrations of agarose media. To complement this I investigate the nature of the agarose substrate involved, and the effect of concentration on its structure. Earlier work by Yu and colleagues in 2004 reported the presence of an apically actin depleted zone (ADZ) in both fungi and oomycetes, including *A. bisexualis* . Furthermore recent experiments present evidence to support the enhanced presence of ADZ in growing tips during invasive compared with non-invasive growth (Walker et al, 2006 ; Suei et Garrill, 2008)

This thesis aims to extend that work by investigating the possibility that the level of actin depletion during invasive growth correlates with the resistive strength of the media. Additionally, analysis of the agar media is undertaken to examine the physical properties and variation within the structure of the agarose substrate, in order to provide a better understanding of the conditions that *A. bisexualis* hyphae face during invasive growth.

Chapter 2:

F-actin cytoskeleton imaging of *A. bisexualis* hyphae

2.1 Actin Introduction

2.1.1 G-actin

Actin is a 42 kDa monomer complexed with ATP or ADP and Mg^{2+} *in vivo*. This monomeric form of the protein is referred to as globular (G-) actin, and it is predominantly found in the ATP bound form. Without a bound nucleotide, G-actin rapidly denatures (Carlier, 1998). The molecule has a relatively simple structure consisting of 4 sub-domains in two lobes which are separated by a cleft (see Figure 2.1.1). ATP is bound via ionic and hydrogen bonding to amino acids in the ATPase fold of G-actin and provides the energy for microfilament (filament) formation.

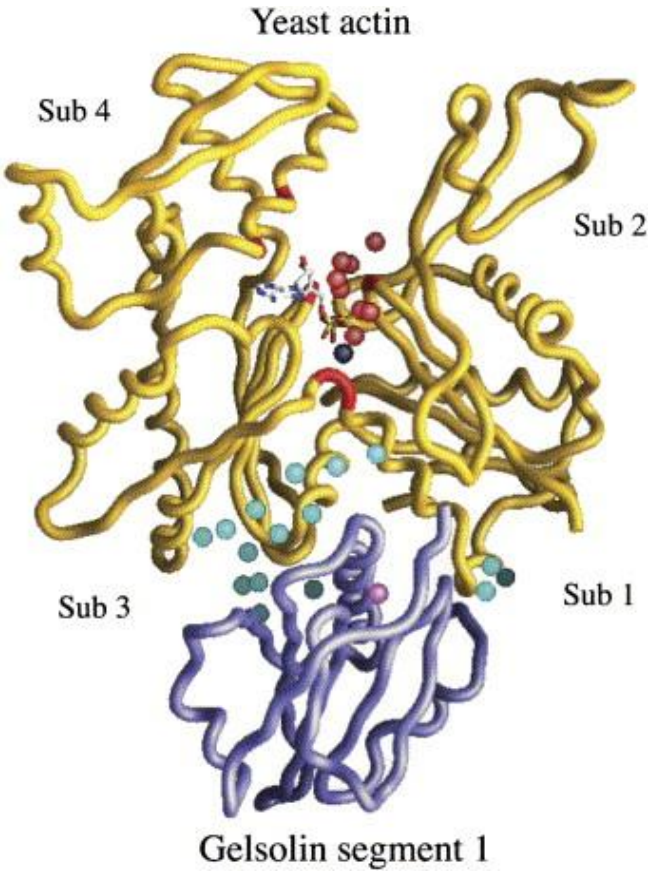


Figure 2.1.1 Structural representation of yeast actin complexed with gelsolin segment-1 (dark blue). G-Actin (yellow) is composed of four sub-domains (Sub 1, 2, 3 and 4), which form a nucleotide binding cleft (red backbone). A binding cleft is located between sub-domains 1 and 2 and sub-domains 3 and 4. Displayed in the cleft is an adenine nucleotide with a divalent cation (black) and associated water molecules (light blue). In the context of a filament (not complexed with gelsolin), the top of the molecule as shown in the diagram would generate the pointed end of the actin filament, and the lower segments the barbed end. (Walker and Garrill, 2006).

2.1.2 Role of actin in other cells

Actin is a ubiquitous protein in eukaryotic cells and performs a number of vital functions. The dynamic actin component of the cytoskeleton is a primary candidate for further study in elucidation of the mechanisms of tip growth. Actin has been associated with a number of processes in other cells. These functions include structural reinforcement; cytoplasmic streaming and organelle translocation; determination of morphology and cellular motility/migration (reviewed in Pellegrin and Mellor, 2007; Dos Remedios et al; 2003).

2.1.3 Structure and Dynamics

Filamentous (F-)actin

G-actin monomers polymerise into paired helical filaments known as filamentous (F-) actin (see Fig 2.1.2) which has a diameter of approximately 7nm (Walker and Garrill, 2006). Polymerisation to form the filaments involves hydrolysis of bound ATP to ADP and phosphate. The ATP binding cleft is oriented identically relative to other monomers in all subunits within a filament. The filament is a homopolymer in which 28 G-actin subunits form 13 turns of the helix. Importantly, the asymmetric structure of the G-actin monomer constrains its polymerisation to form a structurally polarized (asymmetrical) filament. Due to this asymmetry, polymerization and depolymerisation occurs at different rates at each end of the microfilament (Carlier, 1998; Walker and Garrill, 2006). Polarised polymerization thus offers a mechanism by which actin filaments lengthen and shorten.

Filaments associate to form bundles, of F-actin. F-actin cables associate at the cell periphery to form a network known as the membrane skeleton.

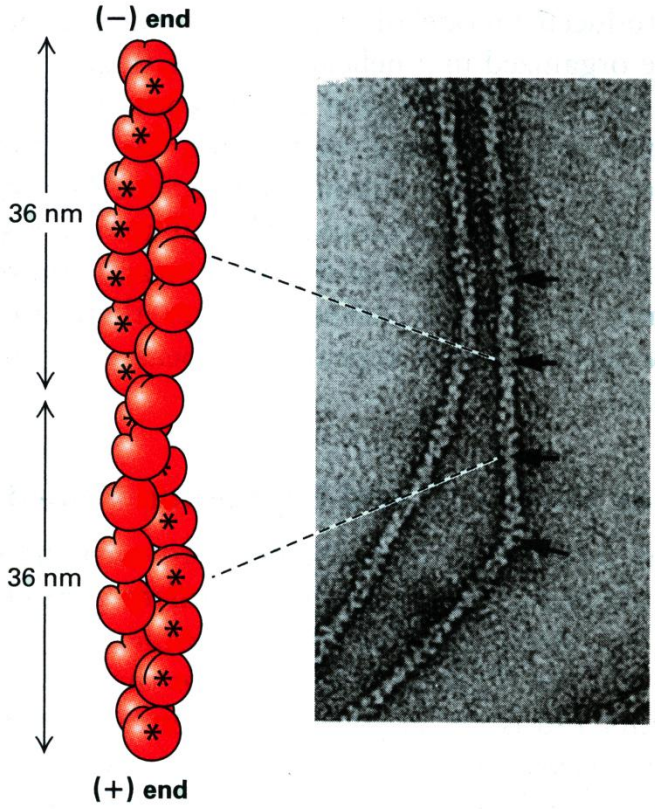


Figure 2.1.2 Schematic and electron micrograph showing actin as a helical filament. G-actin sub-units (red) polymerise to form the polarised microfilament (F-actin). The barbed end is shown at the top ((-)end) and the pointed end at the bottom ((+ end). One full turn (72nm) of the helix is shown (Lodish et al, 2008).

The dynamic nature of actin

The membrane skeleton is dynamic, with constant F-actin assembly and disassembly, and filaments continually being rearranged. Current models propose individual actin filaments are continually in a state of flux. In a single filament, monomers constantly

associate at the ‘barbed’ end, and dissociate from the ‘pointed’ end of the actin filament (see Figure 2.1.3). When this flux is occurring yet the filament length remains unchanged, the process is known as treadmilling. During treadmilling no net change occurs in the intracellular pool of actin monomers participating in the process. Collectively, with all filaments in a state of flux without net change, treadmilling offers a mechanism in which structural integrity is maintained while the cytoskeleton is dynamic. Treadmilling is responsible for the protrusion of lamellipodia in many cell types (Carlier, 1998). This dynamic nature may also contribute to the capacity of the actin cytoskeleton to modulate local structural integrity in response to stimuli. There is a link between the treadmilling process and the potential function of F-actin in provision of protrusive force in hyphae. This ability may have important implications in tip growth under conditions of low turgor, as discussed further in chapter 3.

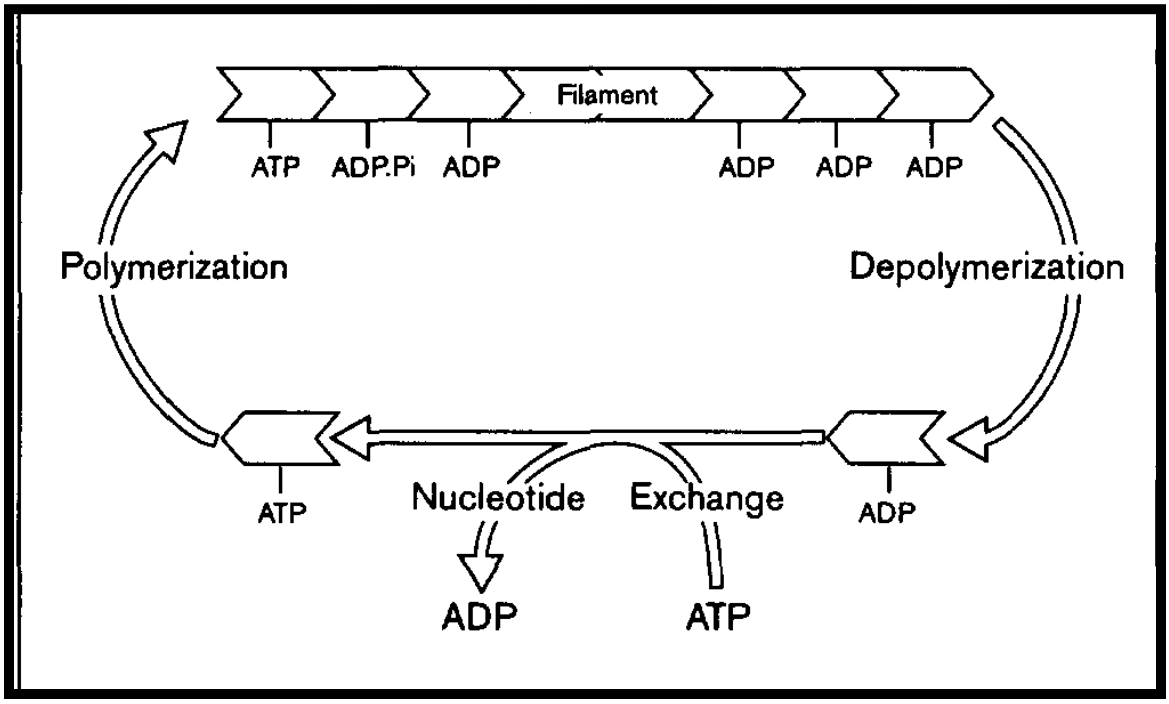


Figure 2.1.3 Treadmilling of an actin filament, showing association of G-actin involving ATP hydrolysis to the barbed end of the filament, and dissociation of de-phosphorylated G-actin from the pointed end of the filament. G-actin monomers in the cellular pool undergo nucleotide exchange to regain ATP, and the process repeats. Treadmilling refers to the steady state of filament formation, where the rate of G-actin association equals the rate of dissociation. This results in no net change in the cellular pool of G-actin, and the polymer (filament) maintains constant length (Carlier, 1998).

Actin arrangement in vivo

The distribution of the actin cytoskeleton varies dependent on cell type and function. Fungal and oomycete hyphae possess a peripheral network (membrane skeleton) of F-

actin attached to the cell membrane and cell wall by putative integrin containing linkages. (Chitcholtan and Garrill, 2005). This network can be further resolved to the components into which it assembles: plaques and bundles.

F-actin filaments and filament bundles align along the sub-apical region of the tip, in an axial orientation. This arrangement has been linked to limitation of cell circumference expansion by providing resistance to circumferential stresses. The distribution of F-actin bundles at the cellular apex varies greatly amongst species studied. In root hairs a similar sub-apical mesh of fine bundled actin is observed (Heath and Skalamera, 2001). In the fungus, *N. crassa,* and others, F-actin is found associated with the Spitzenkörper. (Bartnicki-Garcia, 1989, Suei and Garrill, 2008). In pollen tubes there is a reinforced cortical fringe known as a collar zone bridging the sub-apical and apical zone (reviewed in de Ruijter et al., 2001). In the oomycete *A. bisexualis,* a faint zone of F-actin reinforcement can be observed. The species-specific differences are shown in Figure 1.4; Chapter 1.

The influence of polymerisation on activities occurring at the tip during growth has been well studied. Many studies involving inhibition of actin binding proteins (ABPs) closely associate interrupted actin filament formation with cell growth disruption. (Suei, 2008, PhD thesis.) The movement of F-actin and myosin generate cytoplasmic streaming, responsible for translocation of organelles, including secretory vesicles to the apical tip during growth (Vidali et al, 2001). Importantly, experiments have reported tip growth in the absence of cytoplasmic streaming. This finding suggests that vesicular transport to the

apex is not a rate limiting step in tip extension, and instead polymerization of a subpopulation of actin at the apex, near the Spitzenkörper has a more important role (Bartnicki-García et al., 1989; Heath and Skalamera, 2001). The Spitzenkörper is suggested to be a site of apical polymerization of actin filaments (Braun, 2001, 2004). F-actin re-arrangements in this region are thought to play a role in determining the rate of exocytosis.

Plaques are well characterised in yeast, where they are thought to be 3-D arrangements of short lengths of filaments (Engqvist-Goldstein and Drubin, 2003). They are thought to be held in place by actin binding proteins (ABPs). This adds a second level of dynamic spatial rearrangement, the variable location of aggregation of filaments. The rearrangement of plaques and cables within the cytoplasm may have a role in growth and morphogenesis. Species-specific changes occur at sites of branching and at the rapidly growing tip. In hyphal tips, plaques are relatively evenly spread throughout the sub-apical region of the hypha. In growing *A. bisexualis* and *N. crassa* hyphae they are absent from the extreme tip (Yu et. al, 2004). However, it is thought that in non-growing tips plaques extend over the entire tip. Hyphal growth in the fungus *Aspergillus nidulans* ceases with the migration of plaques into the hyphal apex (Upadhyay and Shaw, 2008). What the plaques represent is an issue of contention. One interpretation is that they are sites of attachment of actin to localized regions of the cell membrane, comparable to focal contacts in animal cells, another suggestion is filasomes. (Heath and Skalamera, 2001). Although the function in fungi and oomycetes remains uncertain, actin patches (plaques)

have been indisputably associated with cell membrane invagination, endocytosis and cell wall morphogenesis. (Upadhyay and Shaw, 2008; Engqvist-Goldstein and Drubin, 2003).

2.1.4 Actin Binding Proteins

It is impossible to consider actin dynamics without acknowledging the importance of actin binding proteins (ABPs). The interaction of actin and actin binding proteins is well studied however not directly addressed in this thesis, thus a brief summary of the roles of ABPs is provided.

Actin binding proteins include ADF/cofilin (actin depolymerising factor); the Arp 2/3 (actin related proteins); formin; cofilin; profilin and capping proteins. The functional roles of these proteins have been divided into seven general categories, which are not mutually exclusive. These categories are filament stabilization preventing depolymerisation; prevention of polymerisation; filament depolymerisation; filament cross-linking which assists bundle and branch formation; filament translocation; capping and filament severing proteins (Dos Remedios et al, 2003; S. K. Walker; MSc thesis). Evidence to elucidate the roles of specific ABPs in tip growth primarily derives from ABP inhibition and immunolocalisation studies of tip growth. These techniques have been used to implicate a large number of proteins with actin in other species including ADF, Arp2/3, formin, villin, profilin, cofilin and others (Dos Remedios et al., 2003; Suei, 2008, PhD thesis).

Recently experiments have been undertaken in fungal and oomycete hyphae, using GFP fusion proteins tracking *in vivo* F-actin responses to further characterize the activities of ABPs (Upadhyay and Shaw, 2008). *N. crassa*, antibody staining studies showed localization of a fungal protein related to the filament-severing protein cofilin found in animal cells, at the tips of invasive but not non-invasive hyphae (Suei and Garrill, 2008). It has yet to be determined which ABPs have essential functions in fungal and oomycete tip growth.

2.1.5 Actin Depleted Zones (ADZ) and tip growth in A. bisexualis.

Rearrangements of filaments relative to one another may play a large part in directional tip growth. In past experiments growing hyphal tips have typically exhibited a reinforced fibrillar actin cap. When the presence of filamentous actin depletion at the extreme apex of growing hyphal tips in the oomycete *Saprolegnia ferax* was first reported in by Jackson and Heath in 1990, the finding was met with initial skepticism due to the concerns associated with fixation and staining efficacy. However almost all experiments in fungi and oomycetes before this time were performed under non-invasive growth conditions. This would explain why the ADZ had not previously observed, given its association with invasive growth. Since the first observation of ADZ, invasive hyphal growth has been further studied and there has been a gathering body of evidence to support the presence of what has since been termed an F-actin depleted zone (ADZ) in fungi and oomycetes.

In 2004, studies staining for F-actin in the oomycetes *Phytophthora cinnamomi* and *A. bisexualis* found that both genera exhibited apical F-actin depletion (Yu et. al, 2004). This ADZ was found to be associated with invasive growth (Walker et. al, 2006, Yu et al, 2004). The mechanism was hypothesized to involve localized F-actin depolymerisation at the tip. The ADZ has been well characterized in pollen tubes, where the polymerization is thought to be a site of dynamic actin microfilament rearrangement (Vidali et al., 2001). Experiments enforcing apical F-actin depletion corroborate the importance of F-actin. When apical F-actin is disrupted using a UV micro-beam, tips become more fragile and prone to bursting (Jackson and Heath, 1992). This disruption simulates the formation of an ADZ, and further implicates F-actin in the structural reinforcement of the tip, although that the extent of apical F-actin depletion would likely be somewhat less severe *in vivo*. The co-localisation of the filament-severing protein cofilin with ADZ in tips of invasive hyphae (Suei and Garrill, 2008) suggests generation of ADZ may occur to create greater protrusive force. Further supporting evidence has recently come from advances with *in vivo* GFP-actin fusion proteins. In these experiments the localization of GFP-Actin fusion proteins was consistent with that observed in immunocolocalisation studies (Upadhyay and Shaw, 2008).

Achlya bisexualis

Early experiments in tip growth focused on increases in turgor driving tip extensibility and growth. However, this notion was challenged when it was discovered oomycetes exhibit growth in the absence of measurable turgor as mentioned previously (Money and

Harold, 1992). The oomycetes from the family Saprolegniaceae, Kingdom Stramenopila, form large fast growing hyphae, making them ideal for use in experiments in which to study this process (Money, 2001). Oomycetes are water moulds with an opportunistic lifestyle, colonizing dead or decaying matter such as animals and faeces. Invasive growth is an essential process in colonization of this type of substrate. The presence of the ADZ has been correlated with invasive growth, but the characteristics of the ADZ have not yet been well defined. Since oomycetes are larger than hyphae of *N. crassa* in which the ADZ has been studied, they allow a greater spatial resolution of F-actin arrangement. Additionally oomycetes are a unique model as by studying tip growth in *A. bisexualis*, experimental results can be interpreted from the knowledge that increasing turgor was not the process underlying hyphal extension.

The contribution of oomycetes to interpretation of results of experiments with other species is however more generalised than specific. Oomycetes are in fact more closely related to chromophyte algae and flagellate protists, however parallels are drawn between oomycetes and fungi due to morphological and biomechanical similarity, despite phylogenetic distance (Money et al, 2004). As such, rather than specific relevance as a model to other species, they provide another comparative model to incorporate into the greater field of tip growth.

2.1.6 Actin Imaging and ADZ analysis in A. bisexualis

In the case of hyphae, imaging experiments have revealed a wealth of information

regarding the nature of F-actin distributions. Studies were conducted using the fluorescent probe Alexa 488 phalloidin (AP) which is a phallotoxin that competitively inhibits F-actin, binding with a similar affinity for both large and small filaments. AP binds actin at a ratio of 1:1 probe: actin and permits visualization of F-actin distribution within fixed cells.

Previous experiments counting ADZ in *A. bisexualis*, and *Phytophthora cinnamomi* found a large disparity in the number of ADZ observed in invasive compared with non-invasive growth. 70% of hyphae exhibited ADZ in invasive hyphae of *A. bisexualis;* and 74% in *P. cinnamomi*. The figures dropped dramatically to 9% and 24% in the respective non-invasive hyphae. (Walker et al., 2006)

These results present a convincing argument for the increased existence of an apical actin depleted in zone in invasive growth. However the model of apical F-actin rearrangement, explained previously, does not explain the presence of hyphae without a cap yet grown in invasive conditions; and conversely the presence of hyphae with ADZ in non-invasive conditions. The experiments in this chapter were undertaken to investigate the possibility that the variability in the number of ADZ are in direct response and proportion to the substrate concentration they are grown in. Furthermore, this chapter focuses on the dimensions of the ADZ in overall analyses of ADZ numbers at increasing agarose concentrations. This brings analysis to a more detailed level than attained in previous experiments which consider only presence or absence of ADZ, or use calculations to provide only a rough estimate of ADZ size

2.2 Materials and Methods

2.2.1 Culture maintenance and agarose preparation

2.2.1.1 Origin and maintenance of cultures

A. bisexualis Coker stock was obtained from zoospore stores in the University of Canterbury culture collection. The strain was originally isolated from NZ *Xenopus laevis* dung. Zoospore stocks were stored at ¯20°C and cultures prepared from zoospores were stored at 4°C. Stock *A. bisexualis* was sub-cultured every 14 days and incubated at a temperature of 21°C. All cultures were grown on a base layer of 2% peptone-yeast-glucose (PYG) media containing 0.12% w/v glucose (BDH, UK); 0.05% w/v bactopeptone (Gibco BRL, UK); 0.05% w/v yeast extract (Gibco BRL, UK) and 2% w/v bacteriological agar made up in nanopure water.

2.2.1.2 Preparation of Invasive and Non-invasive cultures

Invasively growing cultures were prepared according to the method developed by S.K. Walker (2004, MSc thesis). Hallmark brand cellophane was cut into 7mm diameter circles, and boiled three times for 10 minutes with rinsing to remove processing impurities. They were then autoclaved and stored in nanopure water. A cellophane circle was then overlaid onto the base layer of 2% PYG agar and inoculated using a plug from a 0.5mm diameter cork borer from near the growing edge of stock *A. bisexualis*.

Non-invasively growing cultures were prepared according to standard laboratory manner. A Petri dish containing a base layer of 2% PYG agar was inoculated directly with a

0.5cm^2 sub-section of stock culture under sterile conditions.

2.2.1.3 Preparation of Agarose

To make stock agarose, Ultrapure™ low melting point agarose (Invitrogen, NZ) was added to 6mL nanopure water at concentrations of 1%, 2%, 3% and 4% (w/v) and sterilized by autoclaving under wet run conditions at 121°C for 20 minutes at 15 Psi. The agarose stock was then stored at 20°C out of direct sunlight. To re-melt for use in experiments, vials containing stock agarose were microwaved in a water bath on high for one minute. Lids were loosened slightly to prevent pressurization while maintaining humidity. The agarose was then cooled to a temperature which permitted safe handling before use in overlaying samples.

2.2.2 Actin Imaging Methods

2.2.2.1 Culture Preparation

Under sterile conditions, 2 plates of non-invasive *A. bisexualis* were prepared according to previously described protocol, and incubated at 20°C for 4 days.

2.2.2.2 Sample Preparation Fixation

After 4 days growth hyphae were cut 0.75cm behind the growing edge of the non-invasive culture mycelium and placed in welled slides. Underlying cellophane was removed, and sufficient PYG was broth added to cover the hyphae (~50µL) to prevent dehydration during preparation. PYG broth was then removed and the sample overlaid

with 200µL of the appropriate concentration (0 to 4%) low melting point agarose. A second 200µL layer was overlaid to ensure hyphae were fully contained within the agarose. The agar was poured immediately prior to reaching setting temperature, in order to minimize the potential for heat damage to hyphae. A minimum distance of 2-3mm was reserved between the growing edge and the edge of the well to allow for hyphal growth. The hyphae were covered again with PYG broth and left in darkness for 2 hours at room temperature to allow growth recovery. The broth was then removed from samples and 200µl fixative solution (containing 0.5% v/v methylglyoxal (Sigma); 4% paraformaldehyde (Pro-Sci Tech, Australia) made up in 50mM PIPES pH 6.8) was applied and left for 1 hour to ensure quality of fixation. All steps involving fixative were performed with ventilation. Fixative solution was then removed and samples were carefully rinsed twice with 0.75ml washing solution (50mM PIPES, pH 6.8). This was followed by washing twice for 30 minutes on an orbital shaker in 50mL of washing solution.

2.2.2.3 Staining

After removal of the washing solution, 20µL of the actin specific fluorescent probe Alexa 488 conjugated phalloidin (Molecular Probes, Invitrogen, NZ) was added to the sample and left for 1hour in darkness to stain the hyphae. Hereafter samples remained either in darkness or under tinfoil to minimize photobleaching of the fluorophore. Following staining, the fluorescent probe solution was removed and samples were rinsed and washed twice in an identical manner to the protocol established earlier for fixative removal. Control samples were incubated in pH 6.8 PIPES in place of fluorescent probe.

After rinsing and washing, excess solution was removed and 100µL of the antifading agent Citifluor® was applied to the sample surface. Additionally, a block of 2% agar was placed on top of 0% agarose samples in order to reduce hyphal movement and ensure the mycelium remained in the focal plane for imaging

2.2.2.4 Staining method development

A new method of fixation and staining of *A. bisexualis* hyphae was also developed where samples were stained immediately after removal of fixative solution, without the two rinsing and washing steps. The experiment was otherwise identical to the previous protocol.

2.2.2.5 Imaging

Samples were viewed with a laser scanning confocal microscope (Leica SP5, Wetzlar, Germany) using a 63x glycerol immersion objective lens with a numerical aperture of 1.3 at 4x magnification. Hyphae with the tapering tip characteristic of growing *A. bisexualis* were preferentially chosen from the transmitted light images as these were considered to have been growing when fixed. A mercury lamp was used in each experimental replicate to make an initial assessment of quality of sample staining. Sufficiently well stained fluorescent hyphae were then imaged. Images were taken with the focal plane progressing through the hypha, producing a stack of serial optical cross sections for each individual hypha. A step width of 0.5µm between optical sections and 3 times line averaging was reached as a compromise between image quality and reducing hyphal drift in images. Excitation light was provided by an argon laser set at 20% illuminating the

Alexa 488 Phalloidin fluorophore. Emission spectra were collected over the spectral range specified (498nm-600nm) for the fluorophore by Invitrogen. Concomitant transmitted light (TL) images were captured and used for assessment of hyphal condition. A number of differential interference contrast (DIC) images were also captured to allow for comparison with earlier work which was limited to this mode of imaging. Using Leica software, serial optical sections were overlaid to form *maximum projection* images. Files were retained in Leica format and exported as TIFF images to be used in subsequent ADZ assessment and fluorescence intensity analysis.

2.2.2.6 Image Processing and Data Analysis

The presence of the ADZ at increasing agarose concentration was assessed using two approaches. Firstly images were visually assessed for the presence or absence of a depleted zone using maximum projection images and individual serial optical sections. For each percentage agarose the total number of ADZ present over all experimental replicates was counted. This figure was used to calculate the total percentage of ADZ present in samples from each of the 0-4% agarose concentrations.

Secondly, using ImagePro Plus®™ image analysis software, the area of each individual ADZ was calculated. The calculated area for each hypha was then divided by its sub-apical width at a point where width becomes consistent (this was done to correct for variable hyphal size). This distance was typically between 20-30µm back from the apex. This produced data of ADZ size relative to the diameter for each individual hypha. These ADZ area: hyphal diameter data were obtained for all hyphae with ADZ for each of the 0-4% agarose concentrations and used for comparative analyses. Images for presentation

were prepared using Adobe Photoshop 7.0.

2.2.2.7 Comparative Data Analysis

A histogram was plotted of the ADZ area:hyphal diameter ratio for each agarose concentration. These data were used to observe the variation in range and distribution of ADZ dimensions of hyphae grown in increasing agarose concentrations. Using the image processing software associated with the Leica program, fluorescence intensity line profiles (line profiles) were created for a sub-sample of randomly selected hyphae from each 0-4% agarose concentration. The line profiles were normalised to provide comparative data from which composite graph of the 5 hyphal line profiles was created for each agarose concentration using Excel. Each of the 5 hyphal line profiles represents an average profile calculated from five line profiles taken from the central axis of a single hypha.

2.2.2.8 Statistical Analyses

ADZ prevalence and dimension data were analysed using one way analysis of variance (ANOVA). Data for ADZ prevalence were transformed using an arc sine square root transformation. Both analyses were performed using Minitab statistical analysis package.

2.3 Results

2.3.1 Detailed description of hyphal staining and apical actin depleted zone.

A large number of *A. bisexualis* hyphae from cultures grown in 0-4% agarose were imaged. Samples from all growth conditions stained successfully for actin and a number of common features were observed, including a brighter sub-apical zone of around 10μm; plaques, cables and zones of either depleted or enhanced fluorescence at the hyphal apex. Diminished apical fluorescence, representing the previously reported ADZ was observed in hyphae from all agarose concentrations. ADZ had both variable dimension and location relative to the centre of the tip, as detailed below. Additionally, brighter apical fluorescence, representing the F-actin (fibrillar) cap was observed in a large proportion of hyphae, particularly in the non-invasive 0% agarose sample. Unstained control samples exhibited negligible auto fluorescence. Figures 3.2.1A and 3.2.1B show examples of both the ADZ and actin cap.

Hyphal tips were typically of a shape approximating an ellipsical paraboloid, with an average diameter of 9.98μm (standard deviation of 3.3 μm; n=297) across all agarose concentrations. DIC images showed hyphae to be in excellent condition, indicating successful preservation of hyphal integrity during fixation and staining. Fluorescence was largely limited to the periphery of the hyphae and was relatively constant throughout the length of the hypha, with the notable exception of hyphae displaying ADZ or a fibrillar cap as shown in Figures 3.2.1.A and 3.2.1B.

F-actin bundles

Ribbon-like lengths of actin fluorescence, known as bundles of F-actin filaments, were present throughout the length of the hyphae. Bundles typically extended over 3-4μm, although distinguishing one cable from another is often impossible, due to alignment of bundles in close proximity. Bundles aligned longitudinally along the growing axis, and were present in two different spatial arrangements: occurring evenly within a network, or surrounding apparent pores in the actin cytoskeleton, as shown in Figure 3.2.1 A and 3.2.1B.

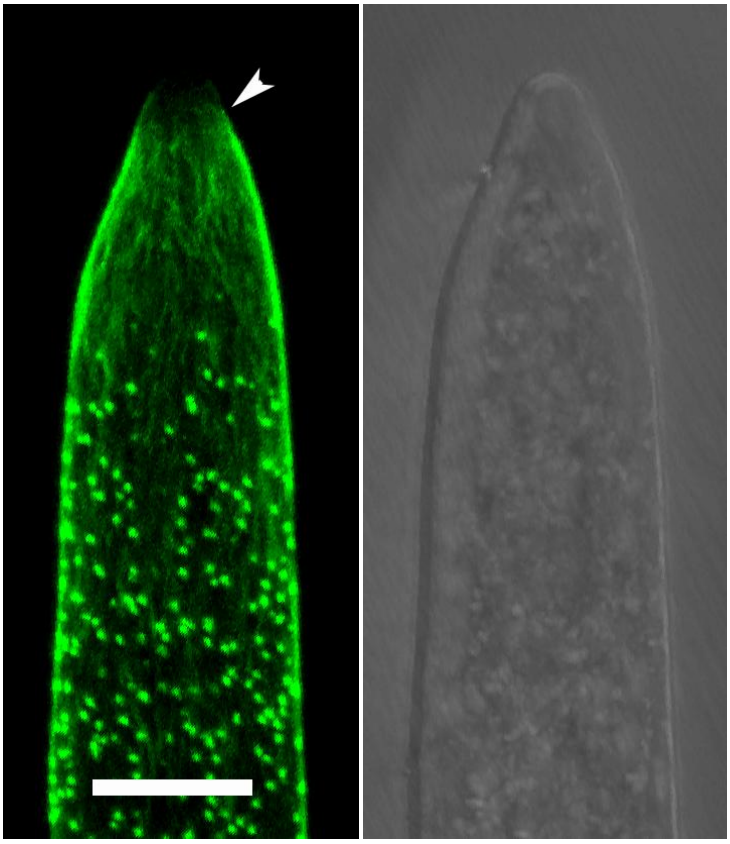
A bright sub-apical zone of cables was frequently present, as shown in Figure 3.2.1C. This zone extended over approximately 10-15μm and was observed at a distance of around 10μm back from the hyphal tip. This was however not observed consistently in all hyphae.

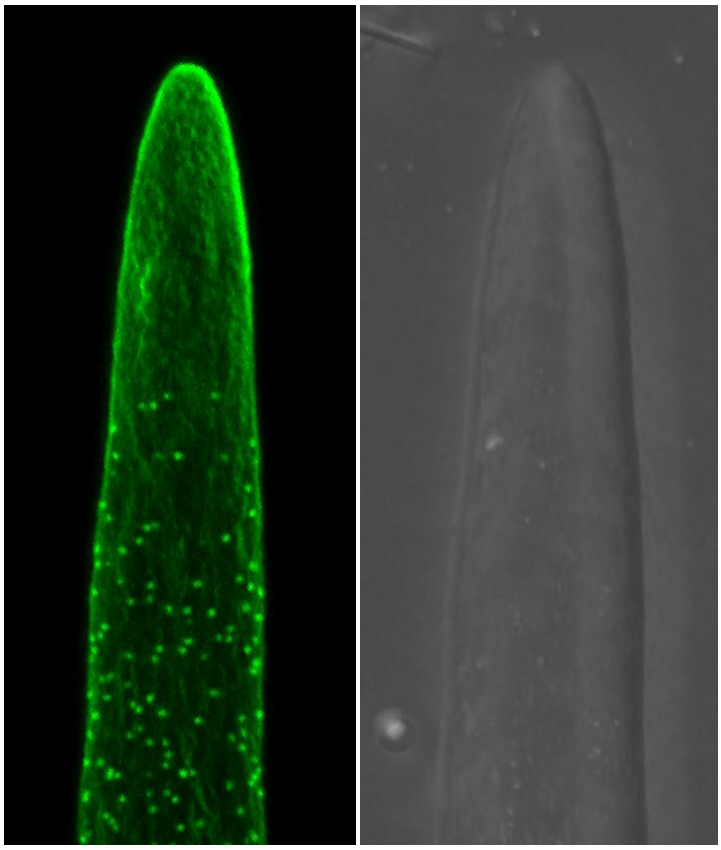
Pores

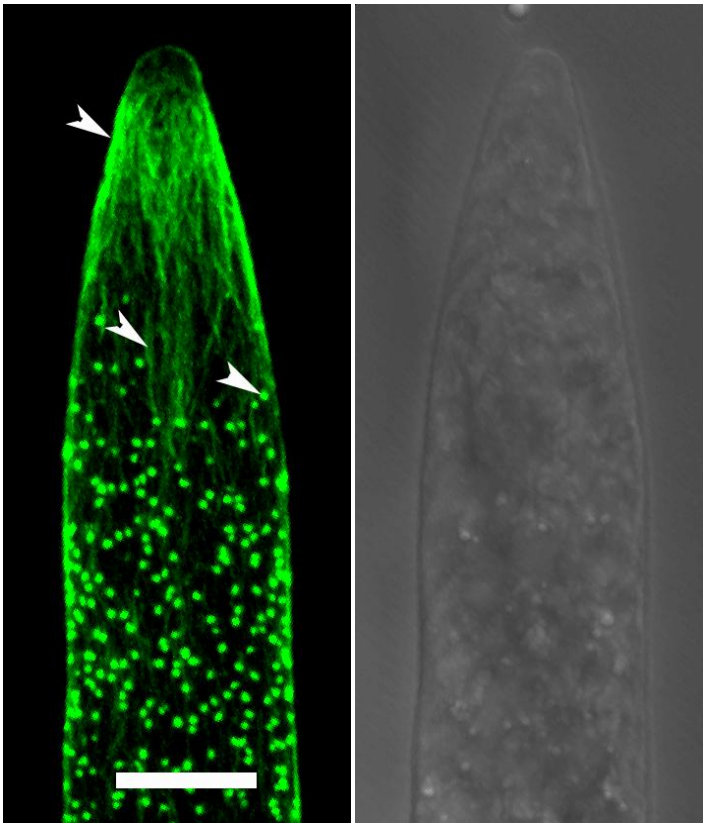
F-actin staining revealed an apparent network of pores within the F-actin membrane skeleton of *A. bisexualis* hyphae, accentuated in the 30-40μm distal to the apical region (see Fig 2.3.1B). The pores are not specific to invasive or non-invasive growth, and are present in hyphae grown in all agarose concentrations**.** The pores have an approximately oval shape of consistent size, and appear in a regular honeycomb-like arrangement. Careful analysis of cross sections reveals the pores may be closed in toward the interior of the hyphae.

Plaques

Plaques appear in images as the brightest spots of approximately 0.5μm^2 as shown in Figure 2.3.1C. These are observed throughout the sub-apical zone ending abruptly around 15-20μm from the apex. All plaques within a single hypha possess similar size and fluorescence, however individual plaques were not necessarily of exactly equal x, y and z dimensions. Many plaques were of extended length with fluorescence often spanning the 5μm step width in confocal imaging.







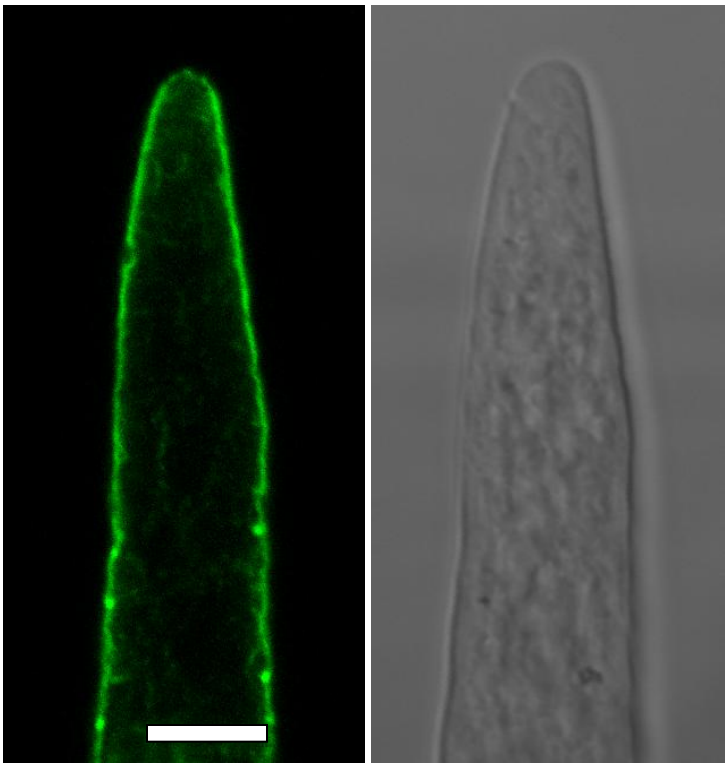


Figure 2.3.1 Images A-D. Fluorescent and concomitant TL confocal images of example hyphae stained with the F-actin specific probe Alexa 488 phalloidin. showing the features frequently observed during imaging. A: hypha grown in 4% agarose with an apical actin depleted zone. B: hypha grown in 0% agarose with a reinforced apical actin cap and apparent pores. C: hypha grown in 4% agarose showing the collar zone, plaques and bundles as indicated by arrows. D: hypha grown in 0% agarose showing localization of F-actin to the periphery of the hypha. Images A1-D1 are the simultaneously captured transmitted light (TL) images showing cytoplasm devoid of discontinuities which indicates quality of fixation. Images A-C are maximum projection images (overlaid stacks of serial optical sections); image D is a single meridional optical section. Scale bars =10μm.

2.3.2 Number of apical actin depleted zones (ADZ)

There were no statistically significant differences in the number of ADZ amongst non-invasive or invasive samples as shown in Table 2.3.1 below, ($P = 0.287$; $r^2 = 13.34\%$; 95% CI). The number of actin depleted zones appeared lower at 0% agarose concentration compared with all other concentrations, however the difference was non-significant ($P = 0.066$; $r^2 = 8.84\%$; 95% CI). In the 0% agarose (non-invasive) sample 20.7% of hyphae exhibited ADZ, and the percentage of hyphae with ADZ in the 0-4% agarose (invasive) samples ranged between 40.9% and 56.9%. Sample sizes were 87, 51, 41, 22 and 96 hyphae for the 0-4% samples respectively.

Agarose Concentration (%)	0	1	2	3	4
Proportion displaying ADZ (%)	20.7	56.9	48.8	40.9	54.2

Table 2.3.1 Percentage of apical actin depleted zones (ADZ) observed in hyphae from cultures grown in media ranging in concentration from 0 to 4% agarose (w/v).

2.3.3 Dimensions of apical actin depleted zones (ADZ)

The average ADZ area: hyphal diameter value ranged between 0.430 and 1.49 and no trend was observed with increasing agarose concentration. Statistical analyses reveal no significant difference between samples of any agarose concentration ($P=0.055$; $r^2=6.35\%$; 95% CI)

Agarose concentration (%)	0	1	2	3	4
Per ADZ only ($\mu^2/\mu m$)	0.634	0.526	0.430	1.09	0.654

Table 2.3.2 Average ADZ area: hyphal diameter ratio for hyphae grown in 0-4% agarose media. The value is expressed per ADZ, where the dimensions (the ADZ area: hyphal diameter) are averaged over the number of hyphae exhibiting ADZ.

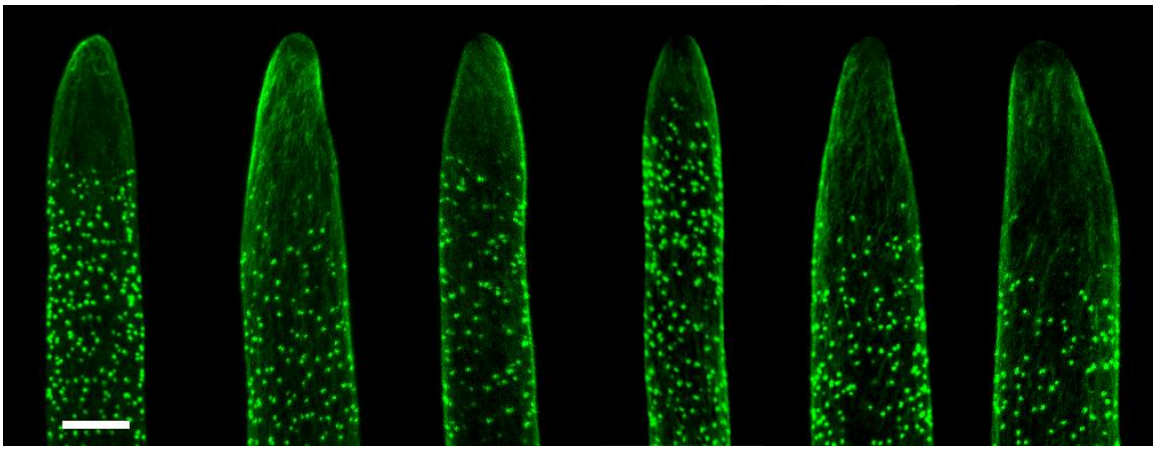


Figure 2.3.2 F-actin stained *A. bisexualis* hyphae showing examples of different ADZ dimensions present in hyphae grown at 1% agarose concentration. Hyphae are arranged displaying a trend towards increasing the extent of apical F-actin depletion. Scale bar ~10μm.

2.3.4 Distribution of ADZ dimensions

ADZ dimensions varied considerably amongst hyphae from cultures grown in identical conditions (see Figure 2.3.2 above). This broad range of ADZ dimensions was observed within experimental replicates in adjacent hyphae within the same mycelium. There was some indication however of differential distribution of area: hyphal diameter ratio, With

pronounced skewing of the 0%, 1% and 2% agarose sample towards a smaller ratio, indicating a higher proportion of relatively small depleted zones. In contrast the 3% agarose sample is distinctly skewed in the opposite direction (see Figure 2.3.3). This distribution favouring a larger ratio indicates a higher proportion of relatively large depleted zones. The 4% sample however has variable distribution of ADZ dimensions and does not align with the pattern suggested in the 0%-3% samples.

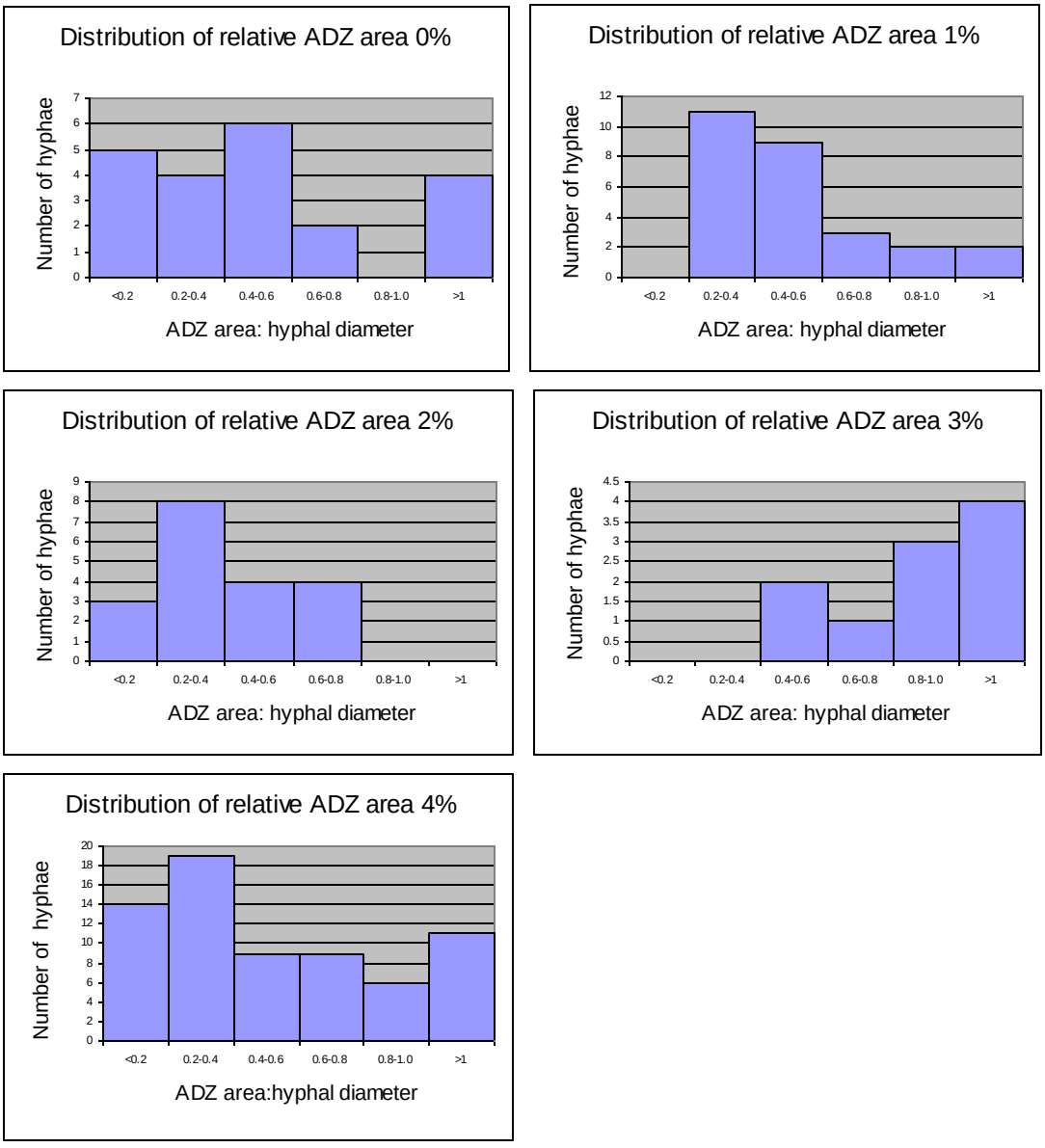
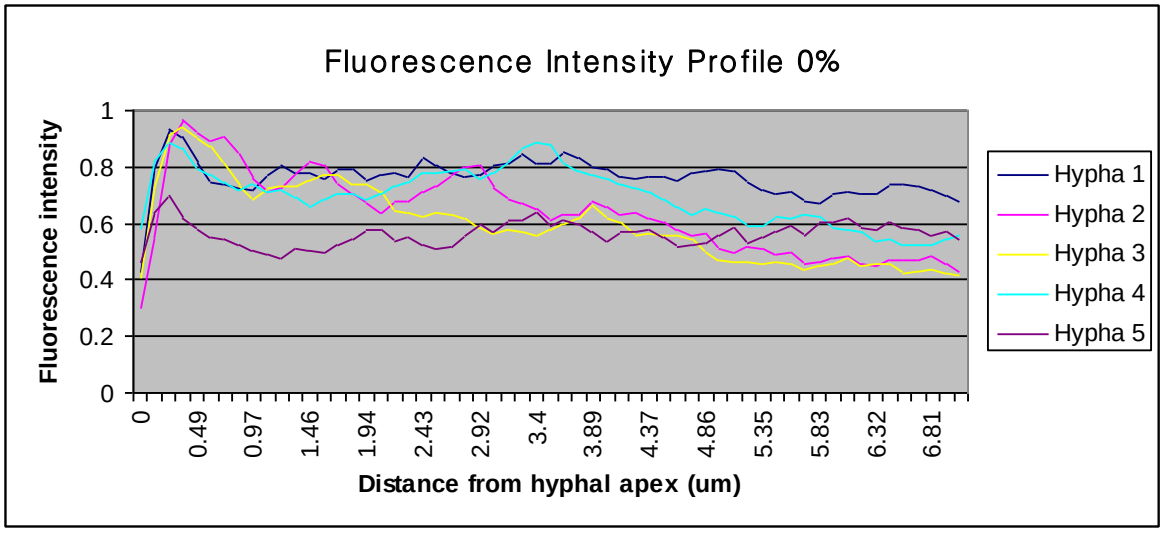
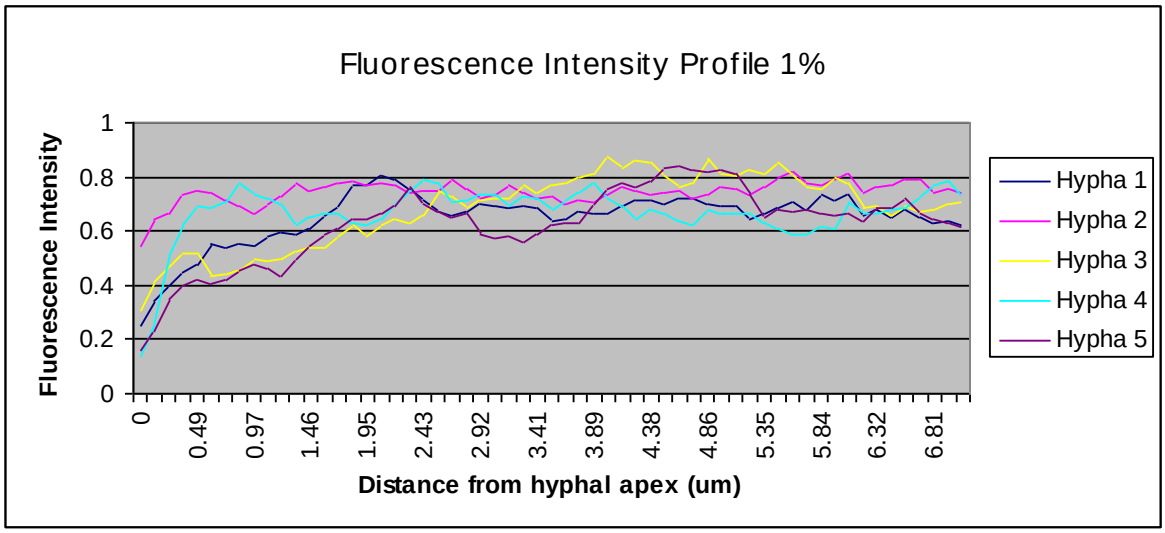


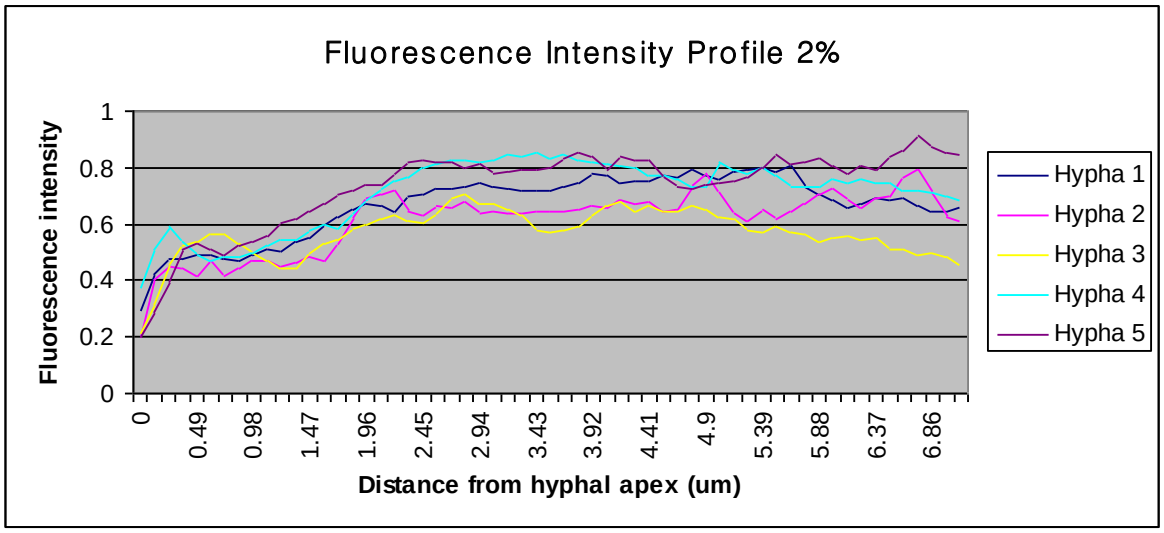
Figure 2.3.3 Distribution of ADZ dimensions represented by ADZ area: hyphal diameter ratio for each percentage agarose concentration. Results suggest a general progression from a higher proportion of small depleted zones toward a higher proportion of large depleted zones in 0% to 3% samples. The 4% sample shows a wide distribution of depleted zone dimensions.

2.3.5 Line fluorescence intensity profiles

Line fluorescence intensity profiles (line profiles) from ADZ-exhibiting hyphae grown in 1-4% agarose concentrations initially spike then gradually increase until reaching a fluorescence intensity where they become approximately level. There is an abrupt peak at the beginning of all line profiles which is particularly enhanced in the 0% compared with the 1, 2, 3 and 4% agarose concentrations. Estimations of the ADZ length of all five samples are approximately 1; 2.5; 2.4; 3.6 and 2.9μm for the 0-4% agarose concentrations respectively. The end point was defined as the distance at which the graph levels off to become approximately static. The endpoint of the gradual increase becomes less defined as agarose concentration increases; and the gradient of line profiles become less steep indicating the fluorescence increases less sharply. There is a large amount of localized fluorescence variation (noise) due to the inherent fluorescence variability in the images (presence of cables) within and between individual hyphae. The ADZ in the 0% samples is barely detectable. The ADZ length is much smaller in the 0% sample than the 1-4% samples, and the general profile shape in the 0% sample differs significantly in that it does not gradually increase toward a static level as seen 1-4% agarose concentrations, instead reaching the static level almost immediately.


Fluorescence Intensity Profile 0%
Fluorescence intensity
1
0.8
0.6
0.4
0.2
0
0
0.49
0.97
1.46
1.94
2.43
2.92
3.4
3.89
4.37
4.86
5.35
5.83
6.32
6.81
Distance from hyphal apex (um)
Hypha 1
Hypha 2
Hypha 3
Hypha 4
Hypha 5


Fluorescence Intensity Profile 1%
Fluorescence Intensity
1
0.8
0.6
0.4
0.2
0
0
0.49
0.97
1.46
1.95
2.43
2.92
3.41
3.89
4.38
4.86
5.35
5.84
6.32
6.81
Distance from hyphal apex (um)
Hypha 1
Hypha 2
Hypha 3
Hypha 4
Hypha 5


Fluorescence Intensity Profile 2%
Fluorescence intensity
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0.8
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0.4
0.2
0
0
0.49
0.98
1.47
1.96
2.45
2.94
3.43
3.92
4.41
4.9
5.39
5.88
6.37
6.86
Distance from hyphal apex (um)
Hypha 1
Hypha 2
Hypha 3
Hypha 4
Hypha 5

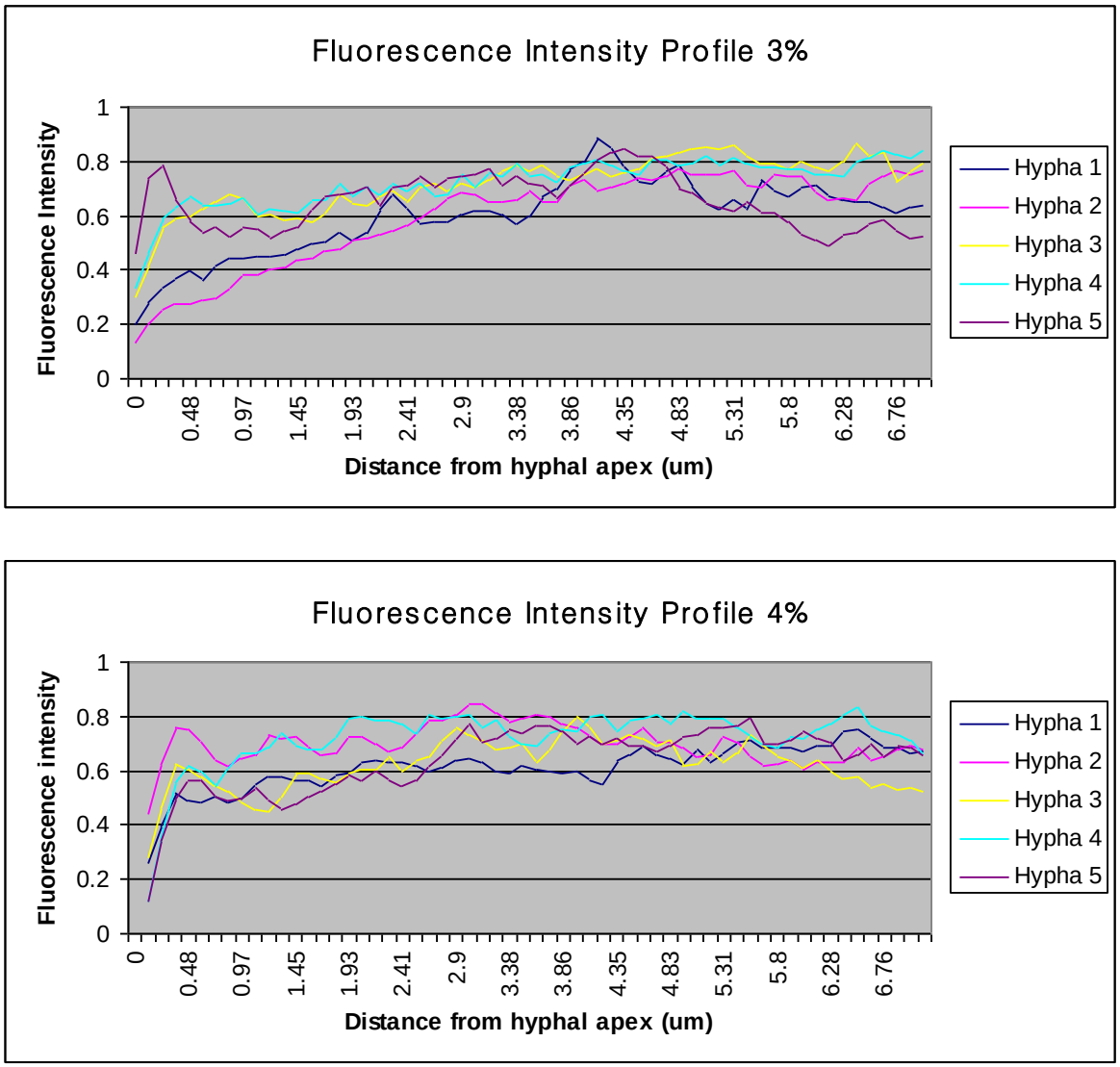

Figure 2.3.4 Composite line fluorescence intensity profiles for 5 hyphae from each of the 0-4% agarose concentrations. Line profiles reflect the level of F-actin fluorescence at sample points taken along a meridional axis. Profiles reveal a shift from an abrupt increase in fluorescence intensity at the hyphal tip in the 0% sample, to a more gradual increase in hyphae at increasing (1-4%) agarose concentrations. The lines were longitudinal to the axis of the hypha, extending from the extreme apex to 7µm into the sub-apical region.

2.3.6 Staining method development

The new method, employing immediate staining following fixative removal, yielded hyphae with enhanced fluorescence, and produced confocal images of higher quality than previous protocol generate. Higher resolution images were obtained of cables and plaques and, and pores were more easily distinguishable. F-actin depleted zones were clearly defined. Additionally, a greater proportion of hyphae within a sample exhibited quality fixation and staining simultaneously. It would also allow for reduction of the laser power, slowing the rate of photo-bleaching of the fluorophore.

2.4 Actin Imaging Discussion

2.4.1 Description of F-actin stained A. bisexualis hyphae

F-actin bundles

Actin microfilament bundles (F-actin bundles) were observed throughout the apical and sub-apical regions of hyphae. Staining revealed two main distributions in hyphal tips. Bundles were either enhanced forming a fibrillar cap, or diminished forming an apical F-actin depleted zone (ADZ). Results are in agreement with other studies in fungi and oomycetes, where the fibrillar cap is frequently observed in non-invasive growth (Kaminskyj and Heath, 1996; Heath and Harold, 1992). In agreement with this tendency, during these experiments the fibrillar cap was predominantly present in non-invasive hyphae. Conversely, the F-actin depleted zone was found to be associated with invasive growth, as has been observed previously (Walker and Garrill, 2006). The putative role of filaments in cell extensibility will be discussed in further detail in upcoming discussion of the ADZ.

A reinforced zone of F-actin was observed, similar to the dense cortical fringe known as a collar zone present in pollen tubes (Lovy-Wheeler et al., 2005). As previously mentioned, F-actin has a potential role in structural rigidity (Jackson and Heath, 1990). The reinforced F-actin zone occurs at the zone where tapering toward the tip begins. This is the point at which the hypha begins to expand radially in addition to circumferential expansion. Microfilament bundles are primarily aligned longitudinally in tips consistent with a role in resisting radial stresses. The tensile strength of the cell wall increases

toward hyphal apices (Money, 1997). It could be speculated this zone provides reinforcement to counter the reduced tensile strength in the cell wall in this region similar to the proposed role of fibrillar caps detailed below. This may provide structural support in a region where perpendicular stresses from turgor are at a maximum, and the cell wall is no longer yielding to these stresses. Perpendicular stresses would also be maximal at the extreme apex. However at the extreme apex the cell wall yields to the stresses and deforms resulting in cell extension. The collar zone of pollen tubes likely represents a fragile but rapidly turning over zone of actin associated with the plasma membrane, with a potentially pivotal role in defining growth rate and polarity (Lovy-Wheeler et al, 2005). The reinforced zone in hyphae occurs at approximately the same distance relative to the tip, therefore possibly has a similar role in hyphal growth. However the zone is a less prominent feature in *A. bisexualis* than pollen tubes, and is not observed in all hyphae, therefore may have a similar but less important role in *A. bisexualis*.

Plaques

The function of plaques remains disputable in hyphae, but is well characterized in yeast. Plaques, known as actin patches in yeast, have been indisputably associated with cell membrane invagination, endocytosis and cell wall morphogenesis. (Upadhyay and Shaw, 2008; Engqvist-Goldstein and Drubin, 2003).

The distribution of plaques is limited to sub-apical regions of *A. bisexualis* hyphae. Since it is completely absent from the apex of growing tips it is tempting to speculate it could be associated with localized tip extensibility. However, in yeast, actin patches are not

observed in fully grown cells, they are instead localized to sites of growth in dividing cells, thus they are correlated with extension (Engqvist-Goldstein and Drubin, 2003). Since in *A. bisexualis* they are present throughout the non-elongating region of hyphae, the speculation above can only be applied to this specific scenario, and cannot be extended to suggest the functions of plaques in general.

Studies of actin patches in yeast have led to the theory that patches in this species are comprised of short filaments arranged in a cross-linked scaffold rising out from the cell membrane that surrounds a membrane core. It is proposed the barbed ends of actin filaments are oriented toward the membrane and filament polymerisation generates protrusive force for extension (Rodal et al, 2005). However this is unlikely to be the case in hyphae, unless assertions thus far that movement of plaques toward the hyphal apex occurs with cessation of growth (Upadhyay and Shaw, 2008); are not entirely correct. In this case, the above function in yeast suggests plaques in hyphae may be linked to growth under conditions of low turgor by provision of protrusive force. This would be consistent with experimental observations of a rounded shape in apparently non-growing tips, associated with elimination of the plaque free region characteristic of growing tips (Riquelme et. al., 1998).These tips may in fact have still been growing, although more slowly, and have possibly changed to utilisation of actin polymerisation as a protrusive force.

An alternative is the proposition of a mechanical role in regulation of growth in *A. bisexualis*. Careful analysis of spatial arrangement of plaques in *A. bisexualis* reveals an

apparent helical arrangement around the periphery of the hyphal tube conceptually comparable to the arrangement of chlorophyll in algal rhizoids (Yoshida and Schimmen, 2009). Bundles, however are aligned longitudinally, providing resistance to radial stress as described earlier. Due to this arrangement, it is tempting to speculate that plaques may be interlinked and act as a restraint against circumferential stresses. Thus plaques could be a contributor to maintenance of elasticity in the sub-apical regions of the hypha, and their absence at the tip may be a means of reducing the force resisting the radial stresses imposed by turgor. However, since actin staining does not reveal interlinking at this magnification, further study at a higher resolution would be required to support this theory.

Pores

The consistent size and regular, honeycomb-like arrangement of F-actin bundles into pores throughout the apical and sub-apical regions in *A. bisexualis* suggest a role of F-actin in the structural reinforcement of the hypha at a greater scale than filament bundles themselves. Pores appear, when stained with AP, to be voids surrounded by aggregates of filament bundles arranged in a network. Similar actin microfilament arrangements suggestive of pores as observed in Figure 2.3.1B have been apparent in previously published data (Kaminskyj and Heath, 1996) but have not been well described.

The observation that pores close inwards toward the interior of hyphae is suggestive of a role surrounding organelles. However, observation of the pores in a living system would be necessary to provide further evidence the arrangement is representative of the cytoskeleton of a growing hypha and not an artifact of fixation. Further investigations

utilizing recent advances in GFP-Actin fusion proteins may assist this (Upadhyay et. al., 2008).

Furthermore, the absence of fluorescence at the hyphal tips of *A. bisexualis* may not preclude the presence of F-actin. It is speculated that apparent apical ADZ may represent areas of delicate, unstable F-actin, as has been found in pollen tubes and algal rhizoids (Yu et. al., 2004). Additionally there is some concern that the localization of actin to the periphery of the cell in close contact with the membrane may be an artefact of fixation. The concern is that during fixation the cytoskeleton may draw out toward the sides where covalent cross-linking by the fixatives immediately occurs during membrane permeation. However, recently advanced fixation techniques developed using *A. bisexualis* and *N. crassa* mitigate this concern and improve confidence in interpretation of results. Higher quality preservation of the initial hyphal morphology is observed using the optimum combination of 0.5% v/v methylglyoxal and 4% paraformaldehyde in 50mM PIPES pH6.8 (Yu et al, 2004). Hyphae fixed in this solution maintain their initial morphology despite no longer being able to actively maintain turgor when fixed. Thus it is inferred structural elements of hyphae have been well preserved.

2.4.2 ADZ prevalence

Results suggested a difference in the number of ADZ observed in non-invasive (20.7%) and invasive samples (56.9, 48.8, 40.9 and 54.2% ADZ in 1, 2, 3 and 4% agarose respectively). The differences between all values were statistically insignificant. However, with a P-value approaching significance (0.055), the difference between 0%

and 1-4% would likely become statistically significant difference with an increased number of experimental replicates. This would be in agreement with previous work finding that apical F-actin depletion is associated with invasive growth in the oomycetes *A. bisexualis* and *P. cinnamomi* (Walker et. al, 2006).

It is important to consider that in previous experiments hyphae were scored in a binary manner of either i) having or ii) not having an ADZ. This introduces limitations in the interpretation of data as discussed further in this chapter. Many small and large depleted zones are reduced to simply 'ADZ present', and fibrillar caps to 'ADZ absent'. Processing in this manner was however included in this experiment in order to make informative comparisons with previous data.

Invasive growth

During invasive growth hyphae need to maximize pressure exerted on their surrounding media. F-actin rearrangements in hyphae are likely to play an important role in controlling the amount of turgor pressure that is exerted onto surroundings (Suei and Garrill, 2008). It is thought that the presence of F-actin provides structural rigidity at the hyphal tip (Jackson and Heath, 1990). Thus it follows that F-actin depletion in invasive hyphae may reduce structural reinforcement at the tip, increasing tip extensibility and allowing the tip to more readily yield to turgor pressure (Yu et al., 2004). This would allow more of the force of turgor to be exerted onto the surrounding media to enable invasive growth. However, the net balance of turgor and reduced resistance to yielding during invasive growth will likely be influenced by the substrate heterogeneity as

described in further detail in agarose experiments.

Given current theories in tip growth as discussed thus far, the presence of reinforced fibrillar actin caps in invasive hyphae; and ADZ in non-invasive hyphae remains unexplained. Fibrillar caps in invasive tips would likely reduce the amount of tip yielding, thus reduce the pressure exerted on substrate. Conversely, ADZ in non-invasive samples would reduce structural integrity, increasing yielding at the tip, and potentially forgo some regulatory control of tip extension. Both cases have been observed in these and other experiments (Walker et. al. 2006; Yu et. al., 2004; Suei and Garrill, 2008). It is not clear whether this is due to underlying processes and conditions occurring in growing hyphae, or experimental error. One possible explanation is the infiltration of the invasive samples by non-invasive hyphae, between the agarose and coverslip as seen in Figure 2.4.1. The hyphae may also be growing invasively, but passing through a pore, microvoid, or region of lower structural integrity at the time of fixation, as explained in detail in chapter 3. In non-invasive samples hyphae may be momentarily in contact with a solid object, such as another hypha, or the coverslip. These conditions may invoke a response in the hyphae that induces a regulatory change and rearrangement of the cytoskeleton to adjust to the perceived change. Some hyphae may take longer than others in responding to the conditions, or be in a period of rest during growth when fixative was applied and fluorescent images show this transitional phase.

Secondly, a theory has been proposed describing a state of constant flux of apical F-actin arrangement during phases of active growth and rest (Lopez-Franco et. al, 1994). Two

variations on cytoplasmic arrangement have been observed at the tips of growing *A. bisexualis in vivo,* and the hyphae were observed switching between the two arrangements. In one arrangement a clear zone appears at the extreme apex, and in the alternative arrangement no distinction is made at any point between the sub-apical and extreme apical regions (Yu et al., 2004). F-actin acting in conjunction with the motor protein myosin is widely considered to be primarily responsible for cytoplasmic movements. These experiments have yielded images of a continuum of the extent of F-actin depletion in growing hyphal apices. Therefore there is a possibility F-actin is also switching between these arrangements dynamic rearrangement of apical F-actin may provide an explanation for both observations.

Additionally, there is a vast amount of evidence demonstrating the role of the cell wall in providing a resistive (restrictive) force as explained earlier (Lockhart; 1965 in Money, 1997; Kaminskyj and Heath, 1996; Money and Hill, 1997). It is generally accepted that the cell wall has the capacity to regulate its extensibility in response to external stimuli. This has important implications considering the role of the cytoskeleton in invasive growth. It is tempting to create a model whereby in order to maximize the force of turgor upon the substrate, both cell wall and apical membrane skeleton could be simultaneously weakened. However this model would apply only to growth through spatially homogenous media or momentary situations, and fails to incorporate the variability of the external environment. This would leave the tip vulnerable to such dangers as osmotic stress and sudden decrease in substrate resistance, with potential for bursting unless instantaneous restructuring is possible. Both natural substrates and specialized growth

media have spatial variability in substrate resistance. The influence of this aspect on tip growth will be investigated in a detailed study of agarose in Chapter 3. An alternate scenario of a dynamic balance of the internal structure of the cytoskeleton and external cell wall structure working in synergy may mitigate shortcomings of this theory.

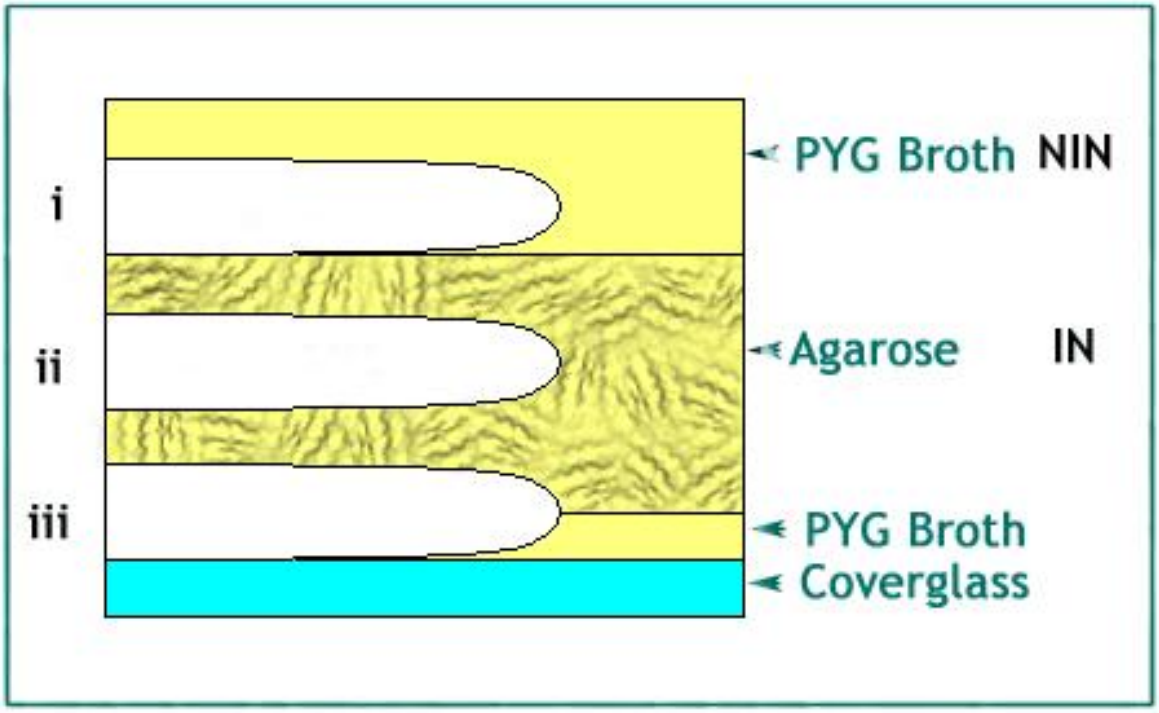

Figure 2.4.1 Schematic of environment surrounding invasive hyphae of cultures grown in agarose according to F-actin imaging experiment protocol (see Chapter 2; Methods 2.2). i) shows a non-invasive hypha on the surface of the agarose growing in PYG broth. ii) shows a fully invasive hypha, these were sought during imaging. iii) shows a partially invasive hypha displaying the suspected growth of hyphae out of the agarose into the underlying PYG broth. Image not to scale.

Non invasive growth

Conversely, non-invasive tip growth is a form of growth where application of maximal force onto surroundings may neither be necessary nor advantageous to the hyphae. Maintenance of morphology, growth rate and growth direction is more likely to be a priority in non-invasive growth. The focus is thus on maintaining structural integrity at the hyphal apex, where the greatest amount of shear stress (tension) would be placed on the cell wall. The cell wall itself will only absorb the force of turgor to a certain threshold. The polymers in the cell wall can only become a set distance apart before they are no longer spatially close enough to maintain any bonding interaction. Reduced polymer interaction reduces the structural integrity of the cell wall. If the force exceeds that level, and the cell does not have solid media surrounding to absorb any force which exceeds its absorbance capacity, the polymers would be forced to completely dissociate resulting in bursting of the cell. In order to resist bursting yet maintain cell wall compliability, the cell would require additional reinforcement to the cell wall. This may explain the presence of the fibrillar actin cap in non-invasive samples. The F-actin cytoskeleton may be reinforced at the apex in order to provide a secondary level of structural reinforcement, additional to the cell wall. This would maintain control of cell morphology while the cell wall is in a compliant state of reduced structural integrity in order to achieve growth. Thus the cell wall may be compliant to *permit* non-invasive growth, yet the actin cytoskeleton may be reinforced in order to *control* the growth.

Experiments investigating the role of the cell wall softening enzymes endoglucanases, were attempted but were unable to provide data due to difficulties involved in purification

of the protein from *A. bisexualis*, which is poorly characterized in this organism.

2.4.3 ADZ Dimensions

As previously mentioned, the presence of the ADZ has previously been correlated with invasive hyphal growth. These experiments characterize the ADZ further, revealing this correlation is not in proportion to agarose media concentration. There were no statistically significant differences in the dimensions of the ADZ of hyphae grown at any of the 0-4% agarose concentrations. The average dimensions of ADZ observed in the 0, 1, 2 and 4% samples are comparable, while the 3% sample reported a considerably larger relative value. This disparity was possibly due to stochastic variation magnified by the smaller sample size obtained for the 3% agarose concentration. Time constraints meant that an increase in sample size was not possible. However, care must be taken in interpreting this result, since although there was an apparent disparity between the 20.7% ADZ in non-invasive and range of 40.9-56.9% in the invasive samples; the result was not statistically significant, although it was approaching statistical significance (P= 0.066).

Thus there was no apparent change in the extent of apical F-actin depletion (ADZ dimensions), in response to increased media resistance. This is contrary to what models of F-actin depletion would predict to be advantageous for hyphae in this situation, as described. Increased media concentration is presumed to present a greater barrier to invasive growth. Reduced apical F-actin would allow hyphae to generate a greater protrusive force to overcome the increased substrate resistance. This did not appear to be the case, since no increase in apical F-actin reduction (ADZ dimensions) was observed in

these experiments. However, it is important to remember that the substrate resistance itself may be variable, influencing results as discussed in detail in chapter 3.

Methodological considerations

The ADZ area was calculated using the trace tool in image analysis package Image Pro Plus™, as it gave a high level of resolution. This was particularly useful for those hyphae displaying an off centre ADZ, which was a prevalent feature of hyphae grown in all agarose concentrations. This feature has also been observed previously in root hairs of *M. trunculata* (Shaw et. al, 2000). This feature will be discussed in further detail momentarily. This method of area calculation was a significant improvement on previous approximations, which incorporated width data into an ellipsical paraboloid equation in order to estimate hyphal tip volume. This method paid no regard to variable shape, or the relative sizes of hyphae in relation to ADZ size.

However this area measurement presented a problem in that standing alone it could not provide a useful value without further manipulation. In order to overcome the variability in the sizes of the hyphae sampled, the area was expressed *relative to the individual hypha*. This was done by dividing the ADZ area by the width of the hypha, at the sub-apical point by which it had become a constant width (~20μm sub-apically). This reduces the influence of an ADZ that was big in a small hypha appearing as though it were a small ADZ, when in fact it was a large area of depletion relative to the tip size, and vice versa. Once the ADZ area of each individual hypha was expressed as a *relative* value, it could be considered in conjunction with all other hyphae grown under the same

conditions. Expressed in this format, the values can be more confidently compared, and the effect of increasing agarose concentration on ADZ area can be assessed. Each individual hypha was represented by a ratio in comparative assessments. This mitigated issues with such as variability of hyphal size and shape, and the number of optical sections overlaid in fluorescent imaging. The method also allowed accurate comparative without the influence of the level of fluorescent probe incorporation. This meant that any relative fluorescence depletion was determined within the individual image, before being compared with other images. In contrast to studies undertaken using less advanced confocal technology, the serial optical sectioning provides a lot more information to interpret and is therefore a major improvement on previous work (see figure 2.4.2).

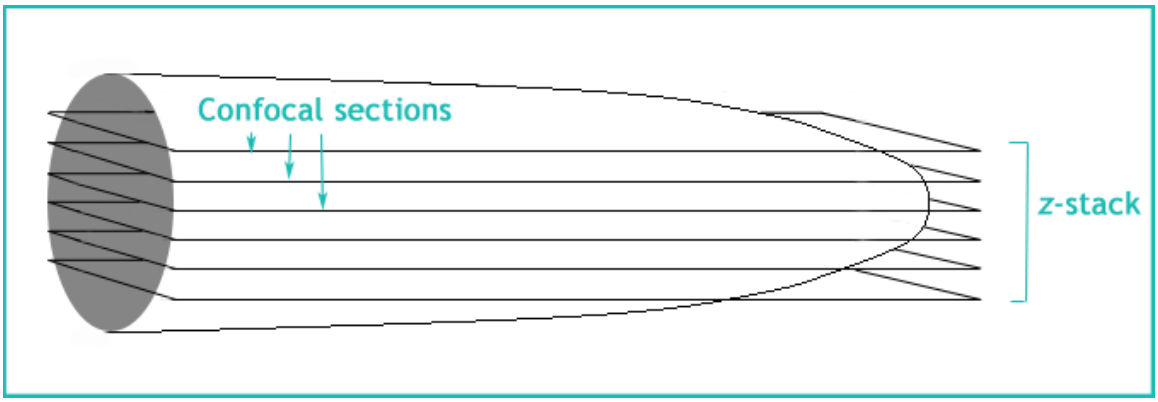

Figure 2.4.2. Diagram showing F-actin imaging via collection of fluorescence emission of the AP fluorophore from serial optical sections. Images are collected moving through the z-stack, and can be overlaid to form a maximum projection image. Six optical sections are shown for simplicity, however around 25 optical sections per hypha were taken during imaging of *A. bisexualis* hyphae.

Off-centre ADZ

The presence of an off centre ADZ highlights the potential for the role of the F-actin cytoskeleton in directional growth. This finding is an interesting finding in light of F-actin imaging in live hyphae which found an area devoid of F-actin that appeared to predict the direction of growth (Jackson and Heath, 1990). The result in these prior experiments was considered somewhat inconclusive however, as not all populations of actin appeared to stain, raising questions with respect to the methodology used. Notwithstanding, the result is in keeping with the theory that the ADZ may function in permitting the exertion of turgor on a specific region of the cell thus a localized region of substrate. The observation of off-centre ADZ in both live cell and fixed cell imaging increases evidence for its existence in hyphae.

2.4.4 Distribution of ADZ dimensions

Graphs of distributions invite speculation that a greater proportion of large ADZ appear to be present in hyphae grown in 1, 2 and 3% agarose than in 0% agarose. This would be in agreement with the theory of constant adjustment of apical F-actin arrangements. However there is no statistically significant trend in the distribution of relative ADZ dimensions. Statistical indications are likely to reflect a large amount of variability in ADZ size in each sample. This may be explained by variability in the substrates, including pores, microvoids and fibrous heterogeneity of the media, as further explained in chapter 3, agarose analysis.

The proportion of hyphae displaying each size of ADZ would reflect the average ADZ size needed to provide the force required for invasive growth at each agarose concentration. The increase in force for agarose compression suggests the force required is likely to increase with agarose concentration. However hyphae grown in 4% agarose exhibited a high proportion of both caps and ADZ. This may be due to errors in selection of hyphae for imaging. Imaging of the 4% sample may have included non-invasive hyphae. This increasingly becomes an issue as agarose concentration increases and hyphae seek a path of least resistance for growth, out of the media (see Figure 2.4.1; above). To some extent these hyphae can be detected and avoided in imaging, by avoiding areas where multiple hyphae are present and lie flat in one focal plane, however it is possible some hyphae may not have been growing invasively as they appeared.

Results cannot be compared to previous work, which has not mapped the distribution of ADZ dimensions.

2.4.5 Line fluorescence intensity profiles

Line fluorescence intensity profiles (line profiles) provide F-actin staining image data in a graphical format, however with two important advantages. Line profiles provide greater insight into regions of low fluorescence intensity; and normalised line fluorescence intensity profiles can be overlaid in a single composite graph to provide a comparison amongst samples grown at different concentrations.

The abrupt peak at the beginning of all line profiles represents the very edge of the

hypha, where line intensity profiles began. Alignment from this point, without including background, overcame alignment issues associated with tapered tip shape. Data from the 5 line profiles could then be combined to create an average line profile for an individual hypha.

The abrupt peak at the beginning of line profiles was likely due to the fluorescence variability from sections at the very edges of the hyphae, the 'top and bottom' of the z-stack of sections imaged perpendicular to the laser beam, where there is a higher chance that the highly fluorescent membrane skeleton section is not imaged, while sections are taken either side of it (see Figure 2.4.2). This is of particular concern in smaller hyphae. This effect was mitigated by the small step width of 0.5μm, and the larger number of sections imaged, and by accurate alignment of the line profiling tool with the extreme hyphal apex.

Line profiles show a gradual increase in F-actin with a less punctuated endpoint with increased agarose concentration. However this difference is subtle, and taken in the context of other data is likely best interpreted as experimental variation, since composite profiles from all agarose concentrations are similar, representing the same statistical variation seen in other image analyses. As previously explained, this variation may be due to fixation of hyphae at different stages during ongoing apical F-actin rearrangement. Few experiments have presented data in this format, but results are in agreement with the model of the ADZ as being a gradual decrease in F-actin toward the tip (Yu, 2005, PhD thesis).

2.4.6 Staining method development

Alteration of the staining method resulted in enhanced imaging, possibly due to reduced physical damage done to cells during multiple washing steps. It is not certain whether the enhanced staining occurred due to reduction of physical damage or due to better preservation of F-actin, but the former case is more likely since bright staining often occurs without any indication of better F-actin preservation than usual. Interestingly, the excellent condition of the fluorescent cytoskeleton may also be attributed to the Alexa Fluor 488 Phalloidin stain itself. The stain comprises a fluorophore conjugated to a phallotoxin that irreversibly inhibits actin. Phallotoxins disrupt actin function by hyperstabilising actin filaments and preventing depolymerisation (Wulf et al; 1979). The addition of the stain immediately after removal of the paraformaldehyde/methylglyoxal combination fixative may have had an additive effect, and allowed better stabilization of the cross-links formed during fixation. This method provides an improvement on the staining and fixation procedures employed in previous actin imaging protocol.

Chapter 3:

Agarose Media Analysis

3.3 Agarose Media Analysis Introduction

Agar has remained the most critical component of solid culture media since its discovery nearly a century ago (Bridson, E, 1994). The physical characteristics gels of its primary constituent polymer agarose are studied in detail in this thesis.

3.1.1 Environmental Fluctuations

Growing tips are controlled by their immediate environment. In their natural context, these environments tend to be inconsistent, yet tips overcome this variability and achieve relatively consistent rates of growth. The oomycete studied, *A. bisexualis*, is a water mould, and colonises organisms such as decaying sea fauna and seaweeds. These substrata would present differing levels of difficulty for penetration and subsequent colonization. Cultures growing through agar face these same challenges. The experiments described in this chapter were designed in consideration of this, investigating the effects of LMP agarose concentration on gel strength and gel structure at a scale relevant to hyphal tip growth.

3.1.2 Agar media

Polymer gels have inhomogenous structures and are a well studied area, usually in an attempt to relate the microscale (local) to the macroscale (bulk) properties of the gels. (Nitta et. al., 2003). Experiments performed using the atomic force microscope have yielded information on the surface structure of electrophoretic agar gels, and some of the

factors that influence the surface structure. Results revealed increasing homogeneity of the media with increasing polymer concentration. Pore size decreased and the distribution of pore size narrowed with increasing agar concentration (Pernodet and Maaloum, 1997). The use of the AFM has also been used to simultaneously image the distribution of viscoelasticity revealing that the local elastic modulus increased with increasing agar concentration and showing a networked arrangement of agar fibres (Nitta et. al., 2003). Localised heterogeneity was also observed within samples of a given agar concentration in viscoelasticity imaging (Haga et al., 1998).

However there is some difficulty in creating a definitive picture of the general structure of agar due to the specific preparation protocol employed. Aspects such as cooling speed, polymer concentration, ionic strength of the buffer and charge of the polymer can influence the final structure of agarose gels (Maaloum and Pernodet 1997; 1998). Maaloum and colleagues endeavoured to observe unperturbed gels, and examine only the surface of the agar. Since this is stronger than internal agar (Nitta et al, 2000) results cannot be applied to the internal structure. Gels used in their experiments are also boiled and imaged under TBE buffer (liquid). Later experiments reveal that ionic strength of the gel buffer affects pore size and distribution. Pore diameters increase and their distribution broadens as buffer ionic strength increases (Maaloum and Pernodet, 1998). Samples for SEM imaging in experiments by Nitta and colleagues in 1999 were dehydrated in acetone and Pt-Pd coated to preserve the agar in preparation for imaging.

3.1.3 Relevance to invasive growth

As mentioned above, local changes in media conditions will have relevance to hyphae growing through that media. This will have important implications in interpretation of results of ADZ distributions in hyphae grown in different media, as discussed further in this chapter. Substrate density is an important factor during growth. Certain nutrients within agar gels become less available at higher agar concentrations. (Debergh, 1983). Molecular activities can also be affected. A decrease in deposition of callose, a molecule with a putative role in load bearing and stress resistance in pollen tubes, was found when the concentration of growth media was increased (Parre and Geitmann, 2005a). Although some studies have examined the nature of agar gels, the method used in this experiment is unique and was developed specifically to facilitate microscopy of invasively growing hyphae. Due to the process and constituents involved in gel preparation, the results of earlier work may not necessarily apply to these experiments. The media used differs, and the layer of formed is very thin, and overlaid with a second layer after taking a period of time to set. Therefore, the nature of the media through which our hyphae grow cannot be inferred from previous work. Since the nature of the gel is crucial in accurate interpretation of results this uncharacterised parameter was investigated in detail.

3.1.4 Molecular characterization and properties of LMP agarose

The media investigated in this thesis is Ultrapure LMP agarose. This substance is free of the charged agaropectin molecules present in standard laboratory agar, and is composed purely of neutral agarose molecules. Polymers are polysaccharides composed of galactose monomers and typically have a molecular weight of 120kDa (see Figure 3.3.1).

Hydrogen bonding between the 3,6-anhydrogalactose monomers constrain the polymer to form an α-helix. Helix interaction causes gel formation. During setting molecules shift from random coil conformation to form an extensively cross-linked network (see Figure 3.1.2). The gel forms and becomes opaque when sufficient cross-linking occurs to form a continuous network. The most important behaviour of agar is its characteristic behaviour of hysteresis. This means agar gels will only become liquid at 85°C; but not return to a solid gel until 39°C. This lag of 40-50 °C is the reason it is so versatile and commonly used in science.

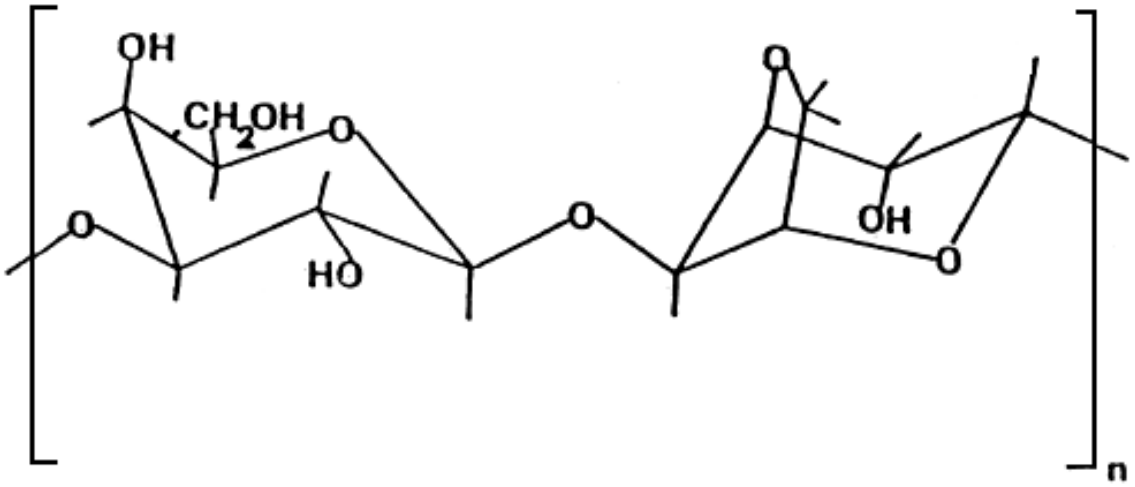

Figure 3.1.1 Structural representation of 3,6-anhydrogalactose monomers, the repeating unit of an agarose polymer.

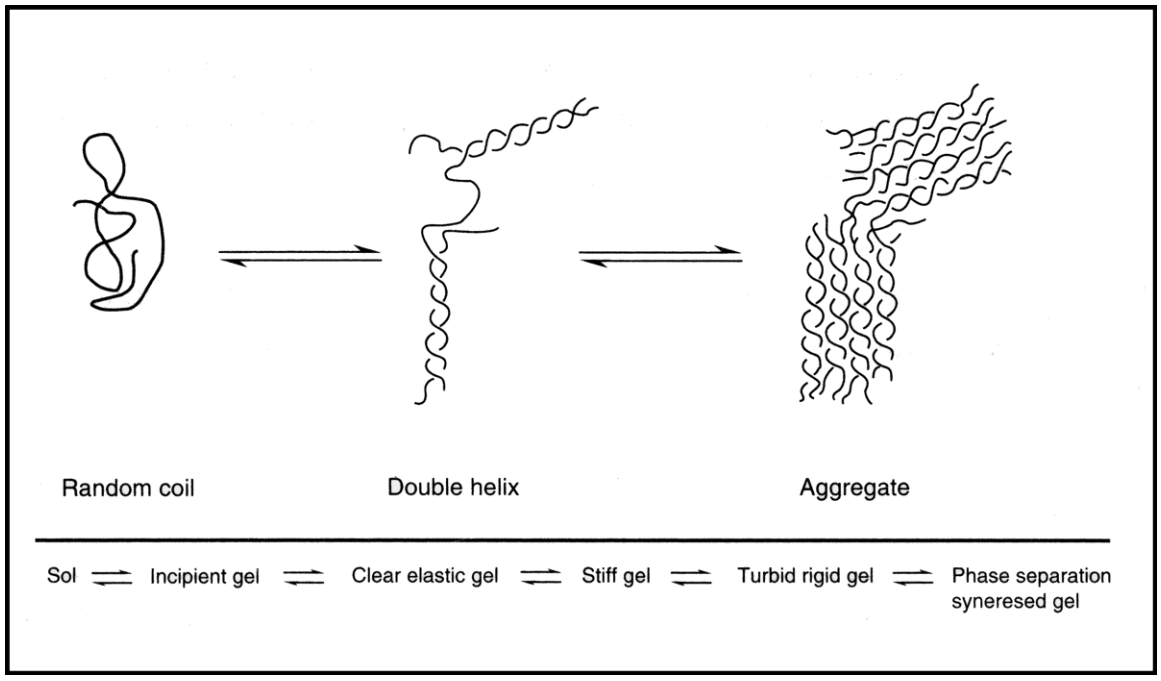

Figure 3.1.2 Agarose polymer arrangement to form a gel structure. Agarose polymers first adopt a double helical conformation. Helices then associate to become aggregated in a solid gel (Bridson, 1994).

The core of this thesis is examining the issue of possible adaptation to growth through solid media. Therefore it is logical to examine in more detail the physical characteristics of the media, at a scale relevant to hyphal tip growth. In order to do this I have approached the matter from two different angles: coarse scale penetrometer measurements of the force required to penetrate agarose gels, and finer scale SEM imaging of preserved agarose samples. The protocol used permits an internal view of the agarose. Experiments focus on defining the nature of the agarose by identifying any differences in gel strength and structure as agarose concentration increases.

3.2 Materials and Methods

3.2.1 Agarose Compression

Stock of 1-4% low melting point (LMP) agarose and welled slides according to general protocol for other experiments. A penetrometer (Fruit firmness tester; 2Lb x 0.020Lb; QA Supplies, USA) with a tapered tip of 11.1 mm width was set up vertically attached to a stand. The prepared LMP agarose was microwaved for 1 minute on high then removed and allowed to cool slightly before pouring into welled slides with cover glass attached to the underside. Agarose was microwaved in a water bath to improve homogeneity of heating. A single layer was poured to above the level of the welled slide and left for 10 minutes at room temperature to set, indicated by opacification. Once set, a razor blade was used to remove the top layer of the agarose from the welled slide, providing a surface that represented the interior of the agarose. The penetrometer tip was then lowered into the sample until the agarose yielded to the pressure applied. The weight required to achieve yielding (compression value) was recorded. After being subjected to force the condition of the sample was carefully noted, and any situations where the agarose split instead of compressing were discarded.

Compression values were obtained for 1-4% agarose media concentrations. Eight sample replicates and three experimental replicates were employed for each media concentration, with attention to cleaning and resetting the penetrometer between each measurement.

The raw compression value obtained (in grams) was converted to imperial units and used to calculate the average pressure required to cause compression of the agarose gel at each agarose concentration using equation 1.1:

Equation 1.1

$$CP = P / A$$

Where CP = Compression Pressure (Psi); P = Raw pressure value; A=Area of tip in square inches

3.2.2 SEM Agarose Imaging

3.2.2.1 Sample preservation

Agarose samples were prepared with 1-4% w/v agarose concentration as described previously in the actin imaging protocol. Hyphal handling, agarose temperature and volume, and time allowed for layers to set between pouring were kept consistent in order to optimize uniformity between experiments. Controls were prepared according to the same protocol, except with the exclusion of hyphae.

Block controls differed in preparation by definition. A larger volume of agarose was poured as a single, thicker layer, and once set, a smaller block section was cut from the large sample and imaged in an identical manner to experimental samples. This provided a measure of difference between samples prepared according to the actin imaging protocol; and samples that represent standard laboratory agar preparation.

3.2.2.2 Freeze Drying and SEM Preparation

Agar samples were cut and indented to facilitate orientation of upper and lower surfaces and location of the growing front once the agar has opacified during freezing. Samples were then immersed in liquid nitrogen followed by overnight freeze drying in a vacuum at an initial temperature of ¯195°C.

Each sample was mounted for imaging on an adhesive carbon fibre plate. A second plate was used to adhere to the top of the agarose momentarily. Tilting the second plate away resulted in tearing of the samples through the plane containing the hyphae. This was a very successful way of revealing the growing front and substrate immediately surrounding the hyphal tips allowing good access for imaging. Where the tear failed to form along the hyphal plane, a fine needle was use to remove layers of agarose to improve access and reveal hyphae and substrate. Samples were then Au coated in a sputterer (Emitech K550X) for 2 minutes at 1.2kV and 20mA to create a layer of 100Å Au in preparation for electron microscopy.

3.2.2.3 Imaging

Agar was imaged using a scanning electron microscope (Leica S440; Cambridge England). Working distance ranged from 10-20μm, magnification 300-5000x with 5kV EHT). Samples of each percentage agarose were imaged in the same manner. Images of agarose in hyphae-containing samples were obtained of 1) the internal surface of *in situ* hyphae, and cross section view of *in situ* hyphae; and 2) the internal surface and cross section views of agarose immediately adjacent to the hyphae. Images of control samples and block controls (lacking hyphae) were obtained from internal surface and cross section views. Images for block control samples were obtained from internal surfaces and cross section views.

3.2.2.4 Image analysis

For pore size analysis, images were taken of a porous region of the adjacent surface as described above, at a magnification of approximately 5000x. Twenty pores from each were measured using image analysis software Image-Pro Plus®, and were used to

determine an average pore size for each of the 1-4% agarose samples and control, and block control samples. Images were prepared for presentation using Adobe Photoshop 7.0.

3.2.2.5 Statistical analysis

Compression force data were statistically analysed using linear regression. Pore measurements were statistically analysed using analysis of co-variance (ANCOVA) in the general linear model. Both analyses were performed using the Minitab statistical analysis package.

3.3 Results

3.3.1 Agarose compression pressures

The compression pressure (threshold at which agarose failed to resist pressure applied by the penetrometer) increased in an approximately linear manner with increasing agarose concentration (see Figure 3.3.1 below). There were statistically significant differences in the pressure that was required to compress each agarose concentration sample from that required to compress all other samples. The regression statistics indicate compression force increases with increasing agarose concentration. The regression equation is -0.277 + 0.502 agarose concentration (r^2 = 74.2%; P = 0.001).

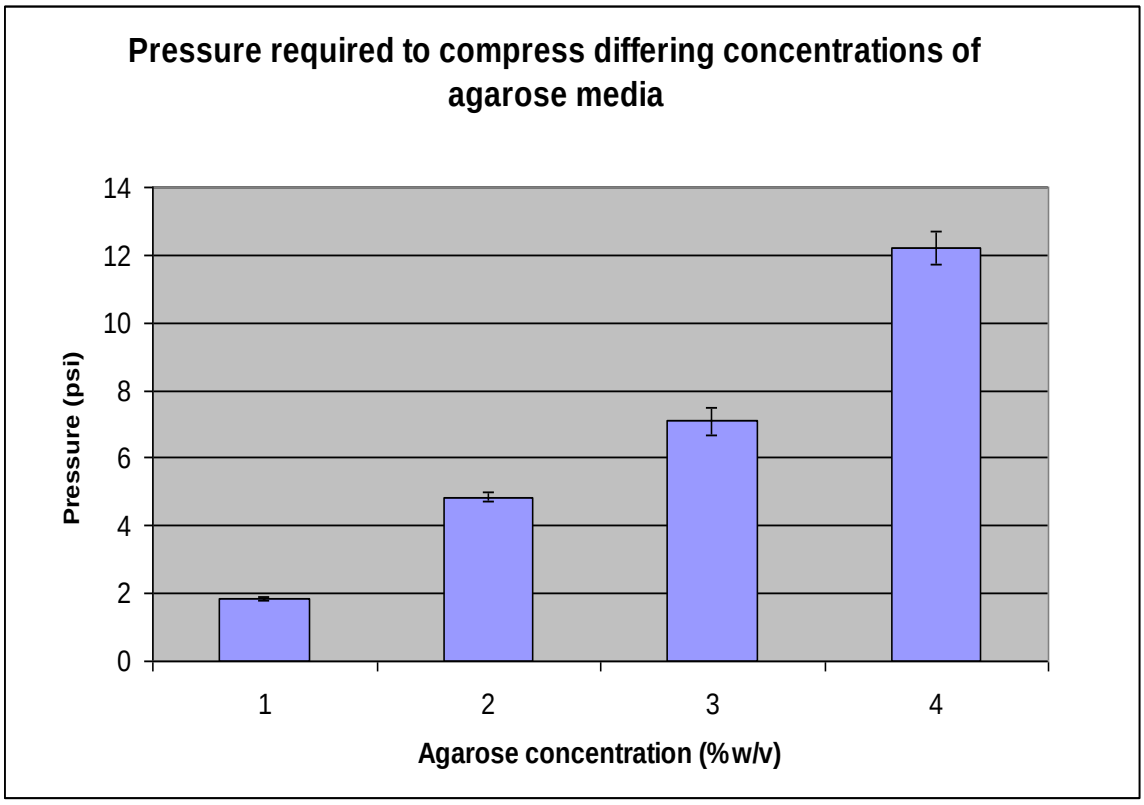

Figure 3.3.1 Increase in average compression pressure value (Psi) with increasing 1-4% w/v agarose gel concentration. Values for compression pressure in 1% ≠ 2% ≠ 3% ≠ 4% as indicated by error bars indicative of standard deviations of sample replicates.

3.3.2 SEM agarose imaging

3.3.2.1 Spatial heterogeneity

Agarose

Imaging reveals significant spatial heterogeneity in agarose from all concentrations. Views of the interior of agarose revealed an interconnected fibrous network structure. Stratification of agarose was observed in SEM micrographs of the interior of preserved agarose gels. Layering occurred in both cross sectional and surface views. Layers varied in width from approximately 10-200μm (see Figure 3.3.2 below). Surface views of single layers revealed some regions that were smoother, or maintained a more consistent fibrous texture. These surface views reveal porous regions with large (relatively irregular) pores, and small (regular) pores (See Figure 3.3.6). The spatial distribution of these pores is highly variable in all samples of 1-4% agarose concentration.

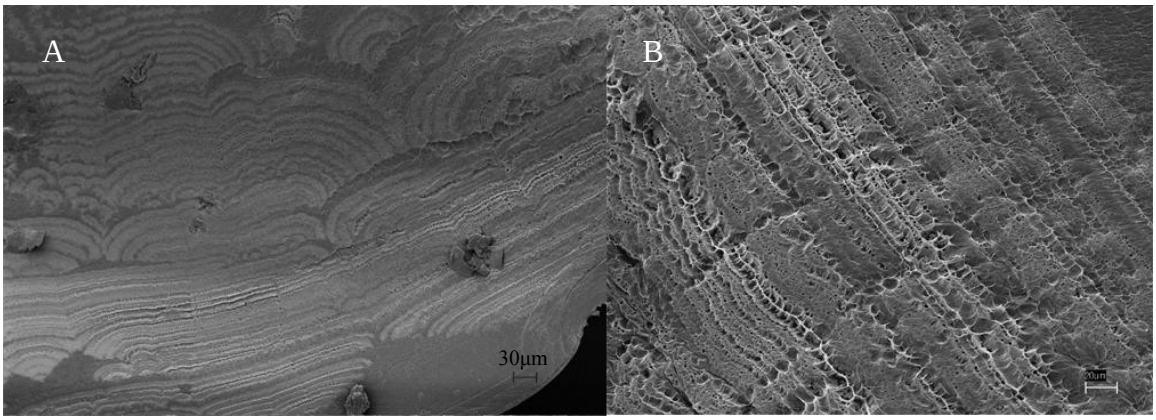

Figure 3.3.2 SEM micrographs of agarose displaying layering within the gel. Layers observed in images likely represent heterogeneity within the gel structure introduced by pouring the agarose during gel preparation. Scale bars A = 30μm and B= 20 μm.

Agarose with in situ hyphae

Agarose surrounding hyphae was heterogeneous, stratified and consisted of an

interconnected fibrous structure. Regions of smoother or more fibrous texture were observed, and small and larger pores were observed, with variable distribution in 1-4% agarose concentrations. Samples of agarose adjacent to *in situ* hyphae were indistinguishable from all samples of agarose alone. However agarose very near the coverslip and surrounding hyphae was very fibrous and there was evidence of half invasive/half non-invasive hyphae (see Figures 3.3.3 and 3.3.4).

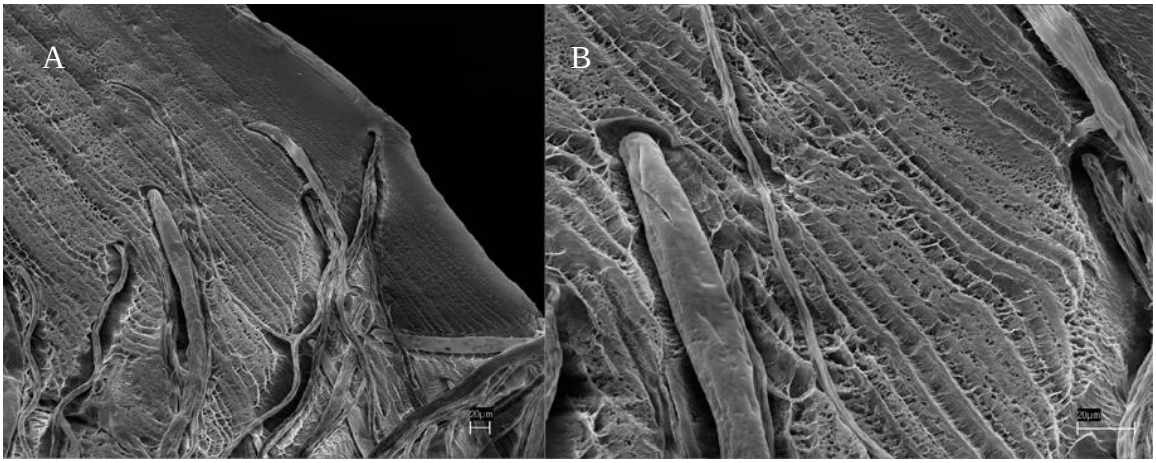

Figure 3.3.3 SEM micrographs of agarose with *in situ* hyphae showing the size of layers within the gel relative to hyphae. B shows a higher magnification of the central left region of A. Scale bars A and B= 20 μm.

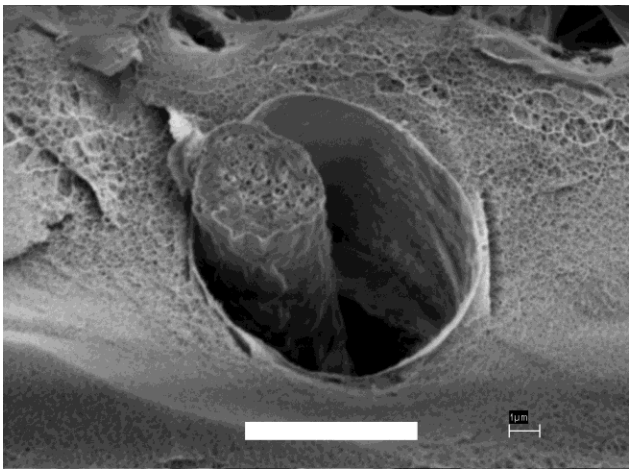

Figure 3.3.4 SEM micrograph of agarose with *in situ* hypha showing a cross section of an individual hypha with fibrous surrounding media. Large white scale bar = 5.5μm.

Agarose prepared from a larger block

Agarose gels prepared from a larger block (block controls) were heterogeneous, stratified and consisted of an interconnected fibrous structure. Regions of smoother or more fibrous texture were observed, and small and larger pores were observed, with variable distribution in 1-4% agarose concentrations (see Figures 3.3.5 and 3.3.6). Samples of agarose block controls were indistinguishable from samples of agarose as prepared for actin imaging; and agarose with *in situ* hyphae.

3.3.2.2 Average agarose pore diameter analysis

Comparing agarose concentrations

Statistics suggest significant differences in relationships between pore size and agarose concentration in the three samples. The average pore diameter increased with increasing agarose concentration in both the agarose with *in situ* hyphae; and agarose control cut

from a larger block (slightly less than agarose with *in situ* hyphae). The average pore diameter from agarose prepared according to actin imaging methodologies was not correlated with agarose concentration (see Figure 3.3.7 below).

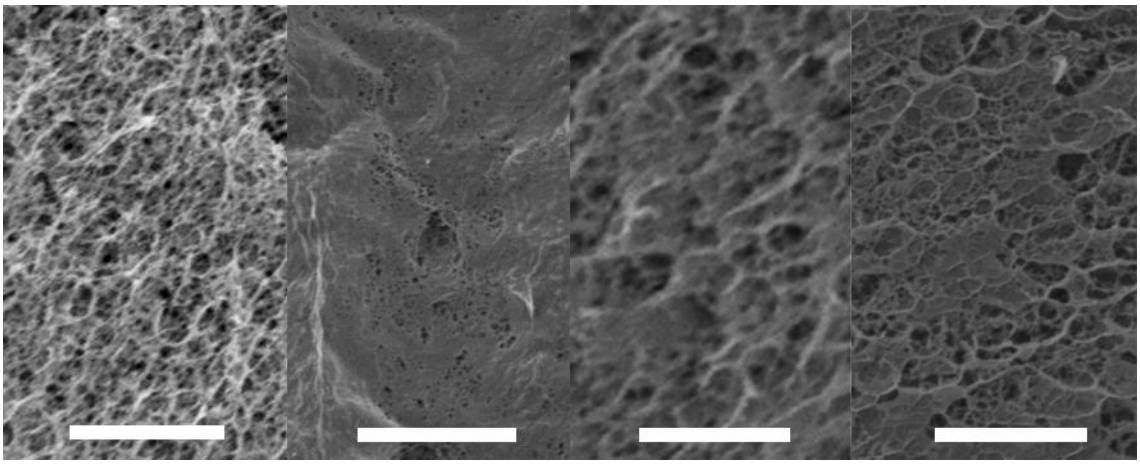

Figure 3.3.5 SEM micrographs showing typical samples of preserved agarose substrate from 1, 2, 3 and 4% agarose w/v (left to right) prepared according to actin imaging protocol. Images display the apparent fibrous interconnected network, and inherent porosity of the gels. Scale bar = 10μm.

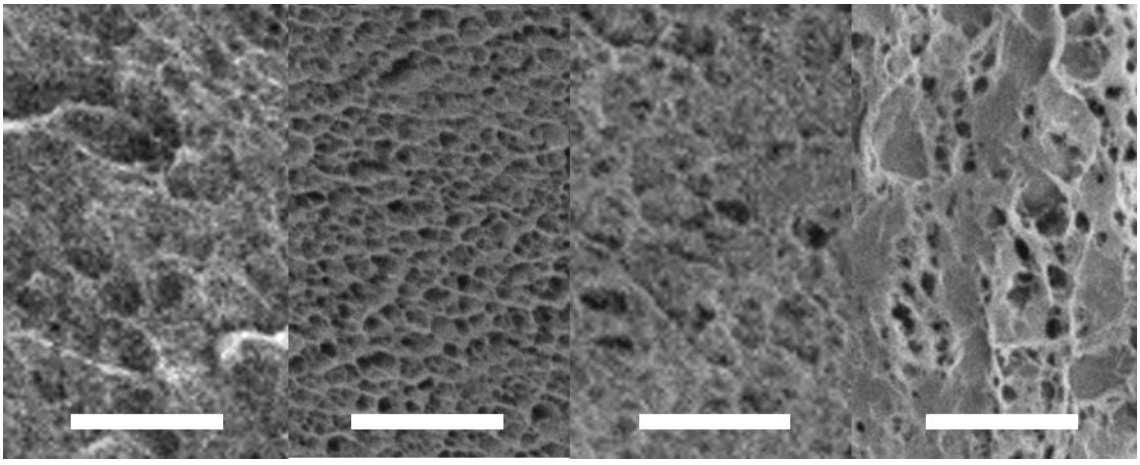

Figure 3.3.6 SEM micrographs showing typical samples of preserved agarose substrate

from 1, 2, 3 and 4% agarose w/v (left to right) prepared as a control from a larger block of agarose. Images display the apparent fibrous interconnected network, and inherent porosity of the gels. Scale bar = 10μm.

Large standard deviations reflect the wide distribution of pore diameter within each agarose concentration. A wide range of pore diameters were observed within all agarose samples with and without hyphae. Images from some agarose concentrations showed pores of relatively constant diameter, other images showed large variation in pore diameter (see Figures 3.3.5 and 3.3.6).

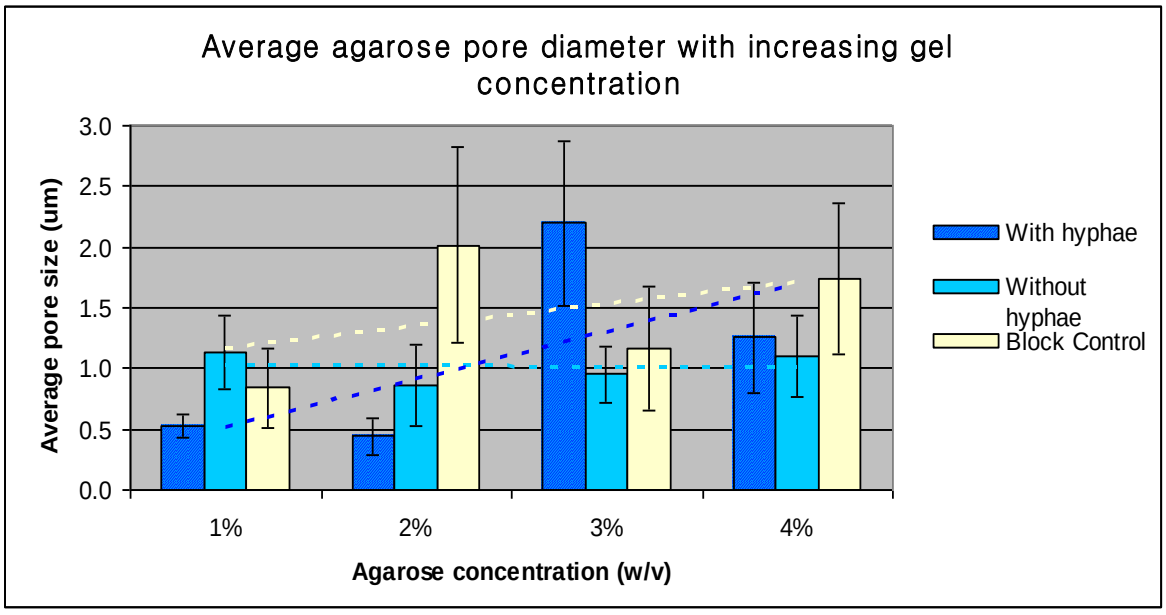

Figure 3.3.7 Variation in average pore diameter (μm) at each agar concentration investigated in actin imaging analysis. All agarose samples with and without hyphae were prepared in an identical manner to those used for actin imaging. Block controls were prepared as detailed in methods. Trend lines show the apparent positive correlation

between pore diameter in agarose with hyphae and block controls; and lack of correlation in agarose without hyphae. Error bars indicate standard deviations of pore diameters from each agarose concentration.

3.4 Discussion

When using agarose as growth media in experiments, gels are often assumed to be uniform and thus expected to introduce little uncertainty in replication. However, as previously mentioned aspects such as cooling speed, polymer concentration, ionic strength of the buffer and charge of the polymer can influence the final structure of agarose gels (Maaloum and Pernodet 1997; 1998). These factors are particularly relevant to the experiments in this thesis involving actin imaging in *A. bisexualis* hyphae grown at different concentrations. Results describe the condition of the agarose gel surrounding the hyphal tips.

3.4.1 Agarose Compression Force

Due to the density of the cross linking polymer, and behaviour of hysteresis, at higher concentrations (in excess of 3% (w/v)) agarose solutions may separate during setting, into a dense lower layer with formation of a lighter gel above it. Practical difficulties ensue in re-melting the solution to homogeneity before use, thus there is uncertainty over the final concentration of the gel used in experiments. The increase found in the pressure required to compress agarose gels was extremely consistent over 1-4% agarose concentrations. The high r^2 value of 74.2% obtained in linear regression reflects this low variation. This indicates that practical issues during agarose preparation had little impact on gel strength. This provides evidence to support the assumption made when examining *A. bisexualis* hyphae grown in media of increasing agarose concentration; that the hyphae are in fact encountering a physical environment that represents increased resistive challenge. Results

are in agreement with work using the atomic force microscope (AFM), which produced results implying that the gelation process of agar gels has a common mechanism over different agar concentrations, despite its complexity (Nitta et. al., 2003).

During agarose preparation, conditions were designed to generate samples that would provide data most relevant to invasive hyphal growth. A single layer of agarose was poured instead of the double layer used in agarose and actin imaging experiments. During setting, the surface of the agarose exposed to the air forms a tougher gel (Nitta et. al., 2000). The first layer in double layer also forms this tough exterior layer, however only hyphae which grew within the first layer of agarose were imaged. The second layer was added to provide the entire mycelium with substrate during growth recovery, and to reinforce samples to cope with physical shear forces during rinsing and washing steps. Leaving the tougher layer in the gel would introduce a force that is not encountered by imaged hyphae. Thus a compromise was reached where the agarose was prepared as a single layer, and the exposed surface removed before compressing, to provide data that most closely approximate the conditions hyphae face *in situ*.

The coverglass and underlying penetrometer base were used in preference to a block of agar to provide a background force. This method avoided any issues of sliding and differential force absorption by background material, as the coverglass/base stand combination was invariable throughout the experiments, thus preferable for comparative analyses.

The results of these experiments are in agreement with previous compression tests of agarose media. In other experiments, Youngs modulus, a descriptor of elasticity, and the

stress at failure (compression pressure) increased linearly with agar concentration of 1-8% (Ellis and Jacquier, 2009). This was interpreted as an indication of a proportional strengthening of the gel network with the amount of polymer strands in the medium, as was also found in these experiments.

However, care must be taken in overall interpretation of what was designed to be a coarse scale measurement of compression force, and in direct extrapolation of the force any given hypha might encounter. Different factors may play more important roles at this smaller scale. Instead the focus remains on the relative properties and structure of the agarose gels at increasing concentrations. Evidence supported the assumption that increasing the agarose gel concentration was generating an increasingly challenging physical environment for invasive hyphae.

3.4.2 SEM Agarose Imaging

3.4.2.1 Heterogeneity- varied local elasticity

SEM micrographs reveal spatial variation of preserved agarose gels in the form of a fibrous interconnected network. This is interpreted to be a three dimensional matrix of polysaccharide fibres of an unpreserved agarose gel in aqueous solution. This matrix would have localized areas of greater structural integrity (thicker fibres), and localized areas of structural weakness (more sparse fibrous network). Regions of the samples were also porous, which would likely represent areas with no structural reinforcement in

unpreserved agarose. Occasional extended regions of constantly smooth or textured surface were observed within a single plane that may represent regions of different strength of agarose polymer interactions. The nature of the fibrous network was indistinguishable across all agarose concentrations. The diameter of fibres varied but was in the range of 0.2 to 2μm. This result was similar to, but slightly larger than, the stiffer grains of around 200nm observed in viscoelasticity images of agar (Haga et al., 1998). Therefore, although increasing agarose concentration of the media increases overall resistance to penetration, this may not be relevant at the scale of hyphal growth. At this scale it may be the role of substrate heterogeneity that best explains the results of F-actin imaging in *A. bisexualis* hyphae.

Heterogeneity in the agarose media would represent a spatially varied challenge to an invasively growing hypha. Imaging of hyphae relative to pores and layers *in situ* provides evidence to support this (see Figure 3.3.6). Furthermore, this heterogeneity is in not correlated with agarose concentration, despite correlation of agarose concentration and the force required to compress the gel.

It is therefore necessary to consider the conditions faced by the hyphae growing invasively. The amount of penetrative force required for hyphae to grow invasively does not necessarily increase with an increase in force required to compress the agarose. At this scale, the constant fluctuations between non-invasive and invasive growth through the heterogeneous media may more accurately reflect the amount of penetrative force required for hyphal growth. These results contribute to understanding of the results of F-

actin imaging experiments, both in this thesis, and in studies to date.

Although in this case the relationship was statistically insignificant, the occurrence of the ADZ has been correlated with invasive growth in previous experiments (Walker et al, 2006; Yu et al, 2004). These results indicate the localized heterogeneity of agarose media; and its resultant fluctuating non-invasive and invasive conditions may play a determining role in the amount of penetrative force required for hyphae to grow invasively. This would align with actin imaging experiments in which a considerable number of hyphae appeared to successfully extend through the media without F-actin depletion. These hyphae may have been passing through a region of little or no substrate resistance and were therefore growing non-invasively at the time they were fixed and imaged.

Furthermore this may contribute to explaining the presence of hyphae with apical actin arrangements ranging from fully depleted ADZ to fully reinforced fibrillar caps within the same culture. An increase in substrate strength is likely to invoke a response to increase the amount of force the hypha exerts on the substrate. Since F-actin is likely to have a role in withholding the amount of force exerted on substrates, this response may take the form of an apical F-actin reduction forming an ADZ in order to reduce any forces restraining turgor. Heterogeneity of the agarose may provide an alternative explanation to those previously outlined in chapter 2. If the force being encountered by a hyphal tip is continually changing, the dimensions of the ADZ may be in constant flux in order to respond to this change. Images may have been captured of hyphae that were fixed at different states during this flux. Additionally, F-actin rearrangements may act in synergy

with cell wall softening to maintain control over the rate of tip growth. However further research in this area would be required to understand this relationship.

3.4.2.2 Pore diameter and distribution

Pore diameter

Pore diameter showed an apparent increase with increased gel concentration in both agarose with *in situ* hyphae and controls prepared from a larger block of agarose. Pore diameter from agarose alone showed no correlation with gel concentration. This is not in agreement with previous findings, which report an increase in average pore size proportional to (agarose concentration)$^{-0.64}$. Pore diameter measurements, however, were similar to previous findings. In these experiments pore diameters ranged between 0.42 and 2.7μm. This is comparable to previous experiments in which the average pore diameter spanned between approximately 247 and 500nm, in agar gels of a range of concentrations in 0.1M TBE buffer (Maaloum and Pernodet, 1997). The differences in pore diameter may be attributable to a number of variables including the mode of microscopy used, gel type, and inclusion of microvoids, as will be considered momentarily.

Except for the inclusion of hyphae, samples with hyphae *in situ* were prepared in an identical manner to samples of agarose prepared according to actin imaging protocol. Therefore it is likely that the difference in average pore size is attributable to unavoidable stochastic variation from sample imaging. Imaging necessitated searching for a well preserved region of agarose. Locating such regions posed a particular challenge in the

case of hyphae-containing samples, as images were taken of agarose immediately adjacent to hyphae in order to provide information most relevant to hyphal tip growth. With a more restricted search area it was more difficult to locate well preserved regions. Additionally, many of these regions appeared to have pores of considerably larger diameter, which have been previously characterised and termed microvoids. The range of pore diameters specified is from 10 to 100 nm, while microvoids are 100–600nm (Gutenwik et al., 2004). Samples also display voids in excess of this size. Thus samples may not necessarily reflect the overall porosity of the agarose and may present regions with a different number of pores and microvoids than observed in other localized areas.

Thus statistics may also detect significantly different distributions due to under-representation of agarose variation in the data. Due to the difficulty in locating an area of agarose that was both well preserved and porous, only one experimental replicate (image) was obtained of each agarose concentration for each of the three agarose types (with and without hyphae; control). In some samples an image was obtained of pores displaying uniform size distribution, where a second image would have displayed a very different pore size distribution. Additionally, the pores were imaged at a resolution in which the pore diameter was frequently less than 10 pixels wide. This generated a large number of identical pore diameter values, which under higher resolution would have been distinct. As a result the apparent variation within sample replicates was very small. This may account for the disparity between these experiments and published literature.

In block samples and agarose with hyphae *in situ*, the increase observed in pore diameter

with agarose concentration was in direct contrast to previous findings, where pore diameter decreased at higher agarose concentrations (Maaloum and Pernodet, 1997). Block control samples were designed to align more closely with the methods of agar preparation in standard laboratory procedures, yet results are in disagreement. However a number of differences remain that may explain this disparity. Primarily, the media investigated in this study was low melting point agarose, which differed from the agar (of electrophoretic gels) studied previously using the SEM (Nitta et. al., 2000). Agar also has a large number of additional constituents and modified polysaccharides that affect properties other than melting point, and may also affect the structure and diameter of pores formed within the gel (Bridson, 1994). Furthermore previous experiments differed in that they imaged the surface of agar that had formed against glass, while these experiments viewed the interior of the agarose, most relevant to invasive hyphal growth.

Pore size distribution

The variability in pore diameter values increased with agarose concentration, as indicated by the increasing standard deviations (see Figures 3.3.2 to 3.3.4). This finding was contradictory to AFM analyses which report narrowing of the distribution, interpreted as homogeneity of the gel, with increasing agar concentration (Pernodet and Maaloum, 1998).

It is important to note that results may not be comparable with SEM and AFM results of others due to difficulty in imaging soft and hydrated samples as (reviewed in Radmacher, 1992). The use of SEM has an advantage over AFM; which is best suited to viewing the

surface, providing only a topological view of samples, as traces of splitting or cutting can obscure the internal view. Most experiments investigating agar have used the AFM, and observe only the gel surface. Previous experiments were also conducted in a PBS buffer, which affects the structure of the gel. (Pernodet and Maaloum, 1998)

Relevance to hyphal growth

Pores and microvoids in agarose would present regions with no structural reinforcement, and as discussed previously, substrate variability of this variety would likely induce a growth response in hyphae characteristic of non-invasive growth. Since F-actin reinforcement is associated with non-invasive growth, an increase in agarose pore diameter at higher gel concentrations would then lead to a corresponding increase in the proportion of hyphae exhibiting F-actin caps. This has not been observed in these experiments or others, as outlined in chapter 2. Therefore results fit neither with current models of invasive hyphal growth nor with results of previous experiments. For these reasons, considerably more experimental replicates would be required before any conclusions are drawn regarding the effect of agarose concentration on pore diameter.

3.4.2.3 Agarose Layering

Layering occurred from the outside surface toward the interior where the layers in the agarose gel became thinner. Artifacts such as layering appear similar to those documented in previously observed in images prepared for electron microscopy (Goldstein et al., 1994). Initial concerns regarding whether they were introduced during the freezing process were allayed by altering the plunge direction during freezing and comparison of

upper and lower surfaces with clean cut edges. Clean cut edges did not have the layering effect from outside towards the interior, but instead retained the layers from upper to lower surfaces as observed in uncut samples. Layering was also observed in block controls. Therefore, while some types of layers were very likely introduced by freeze-drying procedures, at least some of the layering observed is interpreted to be an artifact introduced while pouring the agarose. The agarose is very close to setting point when poured, in order to minimize heat damage to hyphae. When the edges of agarose meet the cold (20°C) surface, instantaneous setting will occur. As pouring continues, the outermost region will be continually setting, and a rippled effect will develop, as observed in Figure 3.3.7. During pouring the lower layers of poured agarose on the coverslip will also be cooler than the agarose being poured. This will likely result in layering as observed in Figure 3.3.6. This is a novel finding, and specific to these experiments. It is also informative as hyphae in actin imaging experiments were included from edge regions which would exhibit this layering pattern.

The parallel tracts within layers in the agarose are interpreted as vestiges of the paths taken by air bubbles. Layering occurs perpendicular, not vertically to the source of poured agar, suggesting the heterogeneity in agarose bonding strength varies in layers, as air does not leave via the shortest path (i.e. to the nearest surface). This pattern may indicate differential strength in the fibrous network, where air bubbles instead move more easily amongst planes of agarose offering less resistance. Layers would thus present areas of varied structure in the agarose, and thus hyphae would be presented with variation in substrate resistance during invasive growth through layers. Any F-actin rearrangement

response to substrate would occur when hyphae traverse layers during growth through the agarose.

3.4.2.4 In situ hyphae

SEM imaging of hyphae *in situ* was a novel experiment. Layering of agarose surrounding hyphae was observed in a rippled pattern as described previously. Hyphae from the surface near the coverslip appeared to be non-invasive, as has been postulated in imaging experiments, and provides evidence corroborating the infiltration of 1-4% agarose samples with non-invasive hyphae that may appear invasive. This emphasizes the importance of determining whether or not hyphae are invasive during imaging to provide the most accurate results.

3.4.2.5 Comparison with block controls

The mode of preparation employed in actin protocol was unique, where the agarose was prepared as an initial overlay with a second layer, and therefore required a customized experimental design. Block prepared agarose was included to control for this, and to provide results that are applicable to standard situations of hyphal growth through laboratory media. However, no differences between the images obtained for block controls, and agarose as prepared for actin imaging were discerned by SEM analysis. This indicates that the mode of gel preparation may not influence the structure of the layering or fibrous nature of the agarose gel. Variations within a region of agarose may have a more pertinent role than the mode of preparation during invasive hyphal growth.

In summary, SEM imaging experiments reveal the nature of the 1-4% agarose gel overlays in which hyphae are grown during actin imaging experiments. Firstly, some significant variation but no directional trend in pore diameter was observed over different agarose concentrations. Secondly spatial heterogeneity was observed in all agarose preparations. The gels comprise a fibrous interconnected network with layering, which is likely to be, to some extent, an artifact of pouring. Neither layering, nor the nature of the fibrous network, were associated with agarose gel strength. This heterogeneity exists at a scale relevant to hyphal growth and assists in the interpretation of actin imaging data. Hyphae growing in agarose prepared in this way will face regions of tougher and softer media, which may require invasive and non-invasive growth. This will likely invoke an F-actin restructuring response at the hyphal apex, and may explain the wide distribution of ADZ number and dimensions found in samples grown in 1-4% agarose media.

Overall, these data provide evidence to support a previous assumption that the resistive force of agarose increases with increasing concentration of agarose gels prepared according to protocol employed in F-actin imaging. However, finer scale SEM experiments suggest a further layer of complexity in the agarose gels, with spatial heterogeneity of density, and extensive porosity within the substrate, at all media concentrations. The results of agarose gel analysis were complementary to F-actin imaging of hyphae grown in 0-4% agarose media. Results indicate that during invasive growth hyphae grow through a fibrous environment with spatial variability in the form of pores, microvoids, and fibrous arrangements of different strength. This may induce an F-actin rearrangement response in hyphae to the fluctuating invasive and non-invasive

growth conditions. Combined results indicate that the influence of local heterogeneity and strength of fibrous agarose structures may in fact play a greater role in determining the resistive challenge invasive hyphae encounter in agarose media.

Conclusions

Tip growth is a form of growth involving localized cellular extension at the extreme apex of a tubular cell. Filamentous fungi and oomycetes utilize this form of growth as their primary mode of colony expansion. Hyphae form extended cells of regular diameter with constant rates of growth, through both solid (invasive growth) and liquid media (non-invasive growth).

Until recently, little was known about invasive growth of fungal and oomycete hyphae. Recent experiments investigating invasive hyphal growth revealed an area of F-actin depletion at the hyphal apex. The prevalence of this ADZ has been associated with invasive growth. Prior to the present study it was unclear however whether ADZ prevalence would be correlated with substrate concentration, and very little data existed describing the dimensions of the ADZ. My experiments addressed this issue by undertaking a statistical study of the prevalence and dimensions of the ADZ in the hyphae of the oomycete *Achlya bisexualis* grown in 1-4% w/v agarose.

Fluorescent F-actin imaging analyses revealed that the presence of the ADZ in hyphal tips is associated with invasive growth, but is not directly correlated with the concentration of the agarose media in which they grow. The average dimensions of the ADZ present were not significantly different in hyphae grown in any agarose concentration. The distribution of dimensions however may suggest a general trend toward larger ADZ at increased agarose concentrations, however further replication would be required to determine whether this is statistically significant. F-actin line

intensity profiles reveal a significant difference between the abrupt decrease in F-actin towards the tips of ADZ-exhibiting hyphae grown in 0% agarose and the gradual decrease in those grown in 1-4% agarose concentrations. The gradient of F-actin depletion toward the hyphal tip may become less steep with increasing agarose concentration.

An assumption inherent to actin imaging experiments was that preparation of increasing agarose concentrations would present growing hyphae with an increasing barrier to invasive growth. Thus additional experiments were conducted investigating the agarose media. Compression force needed to penetrate agarose gels increases at a constant rate with increasing agar concentration. In contrast, scanning electron microscopy revealed spatial heterogeneity within the gels, existing as a layered fibrous interconnected network of agarose comprising dense fibrous regions and pores and microvoids offering little or no resistance. Pore diameter measurements revealed an increase in pore size with agarose concentration in agarose with hyphae and controls, but not in the agarose sample itself. However, this result may be due to experimental bias. Thus the assumption of increased barrier to growth with increased gel concentration may hold at a greater scale, but the differences may be irrelevant at the scale of individual hyphal tip growth.

These results provide new insights into the conditions of the agarose substrate that *A. bisexualis* hyphae will encounter during invasive growth through agarose prepared for actin imaging protocols. Compression force measurements suggest growing colonies would be presented with greater resistance to invasive growth to overcome at higher

agarose concentrations. However heterogeneity within the media introduces periods of invasive and non-invasive growth at a scale experienced by an individual hypha. This small scale change would induce any hyphal response to this change in conditions. Since apical F-actin rearrangement to form ADZ is thought to be associated with invasive growth, it is likely there is ongoing formation and breakdown of ADZ. Thus although agarose media strength does not correlate directly with the prevalence of the ADZ in *A. bisexualis*, it is likely the media still has an important influence on F-actin rearrangements responsible for the depletion.

In combination, results may explain the variation seen in apical actin distribution in hyphae grown under the same conditions, a feature of all studies of hyphal tip growth to date. Localised heterogeneity observed in the agarose gels may explain the presence of ADZ in non-invasive samples, and the presence of reinforced fibrillar actin caps in invasively grown hyphae of the oomycete *A. bisexualis*. Future studies can now be conducted with a broader understanding of conditions hyphae face, and can incorporate the nature of the media into the interpretation of experimental results.

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Appendix

A1.1 ANOVA statistical analysis for ADZ prevalence

One-way ANOVA: ADZ Number versus Agarose Concn (0;1;2,3;4%)

Source	DF	SS	MS	F	P
Agarose Concn	4	5165	1291	1.31	0.287
Error	34	33554	987		
Total	38	38719			

S = 31.41 R-Sq = 13.34% R-Sq(adj) = 3.15%

One-way ANOVA: ADZ Number versus Agarose Concn (NIN(0%); IN(1-4%))

Source	DF	SS	MS	F	P
Agarose Concn	1	3422	3422	3.59	0.066
Error	37	35297	954		
Total	38	38719			

S = 30.89 R-Sq = 8.84% R-Sq(adj) = 6.37%

A1.2 ANCOVA statistical analysis for ADZ dimensions

One-way ANOVA: ADZ area: hyphal diameter versus Agarose Concn

Source	DF	SS	MS	F	P
Agarose Concn	4	3.243	0.811	2.37	0.055
Error	140	47.821	0.342		
Total	144	51.063			

S = 0.5844 R-Sq = 6.35% R-Sq(adj) = 3.67%

A2.1 Regression statistical analysis for agarose compression

Regression Analysis: Compression force versus Agarose Concn

```
The regression equation is
Compression Force = - 0.277 + 0.502 Agarose Concn

Predictor           Coef  SE Coef      T      P
Constant        -0.27696  0.08360  -3.31  0.001
Agarose Concn    0.50178  0.03053  16.44  0.000
S = 0.334412   R-Sq = 74.2%   R-Sq(adj) = 73.9%

Analysis of Variance
Source          DF      SS      MS       F      P
Regression       1  30.214  30.214  270.18  0.000
Residual Error  94  10.512   0.112
Total           95  40.726
```

A2.2 ANCOVA statistical analysis for agarose pore diameter

General Linear Model (ANCOVA): Pore size versus Conditions

```
Factor      Type   Levels  Values
Conditions  fixed       3  Agarose, Agarose + hyphae, Control

Analysis of Variance for Pore size, using Adjusted SS for Tests
Source             DF    Seq SS   Adj SS   Adj MS      F      P
Concn               1   10.9980  10.9980  10.9980  29.91  0.000
Conditions          2    8.0427   6.9017   3.4508   9.39  0.000
Conditions*Concn    2    7.8752   7.8752   3.9376  10.71  0.000
Error             234   86.0363  86.0363   0.3677
Total             239  112.9523
S = 0.606364   R-Sq = 23.83%   R-Sq(adj) = 22.20%

Term                       Coef  SE Coef      T      P
Constant                0.70713  0.09587   7.38  0.000
Concn                   0.19147  0.03501   5.47  0.000
Concn*Conditions
      Agarose          -0.19409  0.04951  -3.92  0.000
      Agarose + hyphae   0.20251  0.04951   4.09  0.000
```