F-actin and integrin like proteins in *Phytophthora cinnamomi*

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Abstract

Tip growth is the primary form of growth in hyphal organisms and some plant cells. Tip growth in hyphae is highly dependent on F-actin, which acts to regulate and support. growth. One of the models suggested for tip growth, the amebae model of tip growth, suggests that F-actin may also be the primary source of protrusive force for tip growth in some conditions, and that proteins with a similar function to animal integrins would be present an involved in tip growth (Heath and Steinberg 1999). In this thesis we examine the role of F-actin in the growth of the oomycete *Phytophthora cinnamomi* and the effects on growth of the F-actin disrupting compound Latrunculin B. We demonstrate that F-actin plays a critical role in the tip growth of *Phytophthora cinnamom*i with it's disruption causing rapid cessation in directional growth, followed by significant sub-apical swelling. Further more we examine *Phytophthora cinnamomi* for the presence of an B4 integrin like protein that has been previously reported in the oomycete *Achlya bisexualis* (Chitcholtan & Garrill 2005) and show that the B4 integrin like protein is not present in *Phytophthora cinnamomi*.

These experiments help further our understanding of tip growth in *Phytophthora cinnamomi* an economically important plant pathogen.

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Abbreviations

°C	Degree Celsius
dH_20	Distiled H_20
DIC	Differential Interference Contrast
DMSO	Dimethyl sulphoxide
EGTA	Ethylene glycol-bis (β-aminoethyl ether)N, N, N, N,-tetraacetic acid
F-actin	Filamentous actin
G-actin	Globular actin
GIMP	GNU Image Manipulation Program
g/l	Grams per litre
Ilps	Integrin like proteins
kDa	Kilodalton
LMP	Low Melting Point
mM	Millimolar
µg/ml	Micrograms per millilitres
µg	Microgram
µl	Microlitre
µm	Micrometre
PAGE	Polyacrylamide gel electrophoresis
PYG	Peptone, Yeast extract, Glucose

PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
pH	Potential of hydrogen
SDS	Sodium dodecyl sulfate
v/v	Volume/volume
w/v	Weight/Volume

Introduction

Phytophthora cinnamomi

Phytophthora cinnamomi Rands is a member of the genus *Phytophthora* of the *Oomycota* which currently contains around 60 described species (Cooke et al, 2000) (*Phytophthora cinnamomi* Rands; kingdom *Chromista*; phylum *Oomycota*; order *Peronosporales*; family *Peronosporaceae*; genus *Phytophthora*)(Hardman, 2005). The Oomycetes are recognised by their habit of hyphal growth, aseptate hyphae, and production of biflagellate, heterokont zoos pores (Cooke et al, 2000). Due to morphological similarities of oomycete and fungal hyphae (the vegetative growth form of both types of organism) and similar methods of nutrient acquisition they have often been mistakenly included with the true fungi. However, there a significant structural, biochemical and genetic differences between the Oomycetes and true fungi of the Kingdom Fungi (Cavalier-Smith, 1998). Biochemical and structural differences such as the use of cellulose by oomycetes for the cell wall compared to chitin for true fungi, as well genetic differences of which an example is changes to the small subunit ribosomal RNA gene (Forster, 1990). These difference and others clearly place the oomycetes into a different phylogenetic group to the true fungi (1313Figure 1).

Members of the *Phytophthora* genus are best known as the pathogens responsible for a number of devastating plant diseases, which affect a wide range of economically important crops and cause extensive damage to natural plant communities (Hardman, 2005; Cooke et al, 2000). .

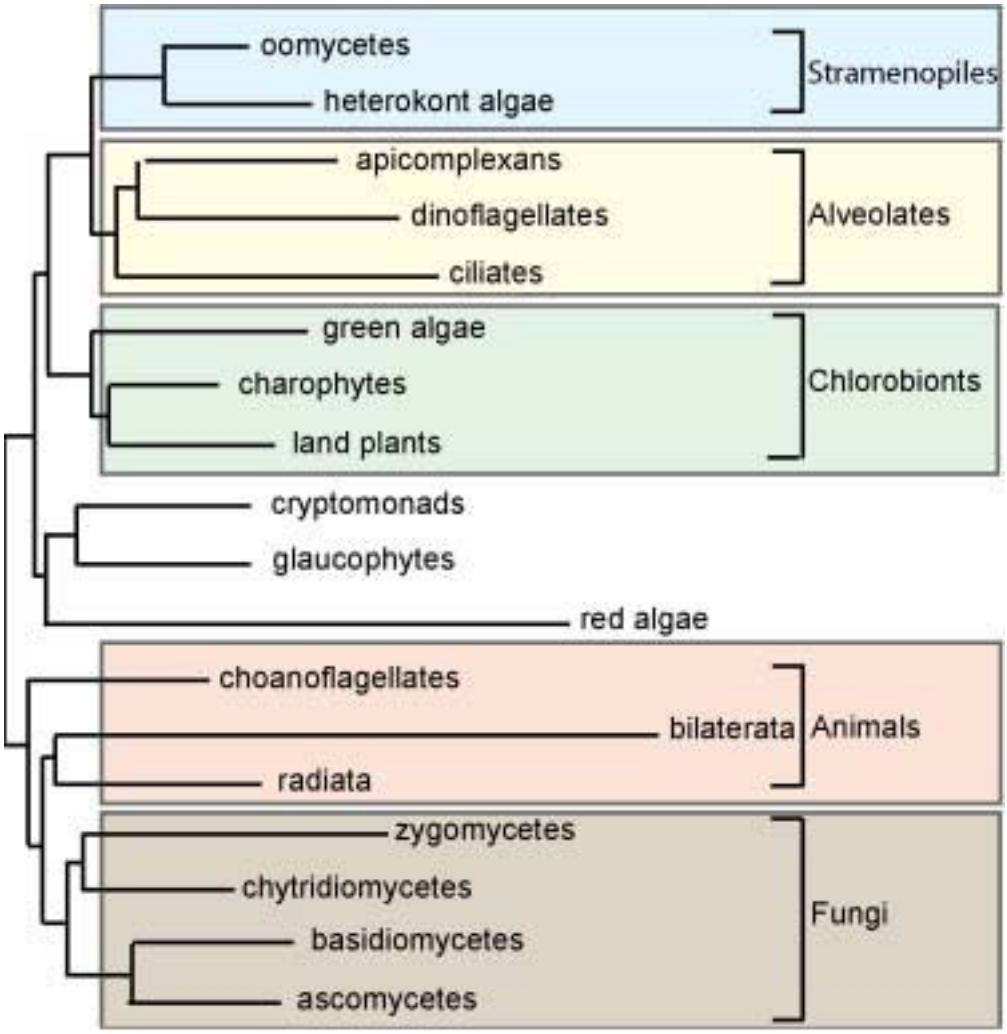

Figure 1: Phylogentic tree showing the evolutionary relationships between that major groups of Eukaroytes. There is a significant evolutionary distance between the True Fungi and the Oomycetes (Hardham, 2005)

All members of the genus are known to infect plants though the host range varies between species (Randall, 2005)A classic example of this being *Phytophthora infestans* which is responsible for the devastating potato Late blight, the disease which caused the Irish potato famines of the 19th century (Randall, 2005). The disease is still problematic today, annually costing over $5 billion worldwide in crop damage and control measures (Tyler, 2001). Due to the differences between Oomycetes and true fungi, most fungicides developed against true fungi have proven to be ineffective against Oomycete diseases such as those caused by *Phytophthora* species. An example of this is the azole fungicides, which target ergosterol biosynthesis however Oomycetes do not synthesise sterols but instead acquire them from their host (Tyler, 2001). In cases where such chemicals have proven effective resistance has rapidly been acquired (Tyler, 2001). Also attempts to develop resistant plant species have found that *Phytophthora* species rapidly overcome such resistance (Tyler, 2001).

Phytophthora cinnamomi Rands was first described from an isolate from cinnamon trees in Sumatra in 1922. In 1980 it occurred thoughout the world and it become clear that it was a significant threat to a wide range of plant species, with around 950 potential host species having been identified (Zentmyer, 1980). This wide range of hosts included important crops such as avocado, pineapple, peach, chestnut and macadamia; as well as a range of horticultural plants and a number of important forest trees such as oak, pine and eucalyptus. In Australia *P. cinnamomi* had become a significant problem, having arrived in the 1920's via contaminated stock; it spread to Jarrah (*Eucalyptus marginata*) where it caused the disease Jarrah Dieback, which rapidly spread following the construction of roads though native forest (Podger et al, 1965; Podger, 1972). *Phytophthora cinnamomi* was not identified as the causative agent until the 1960s.

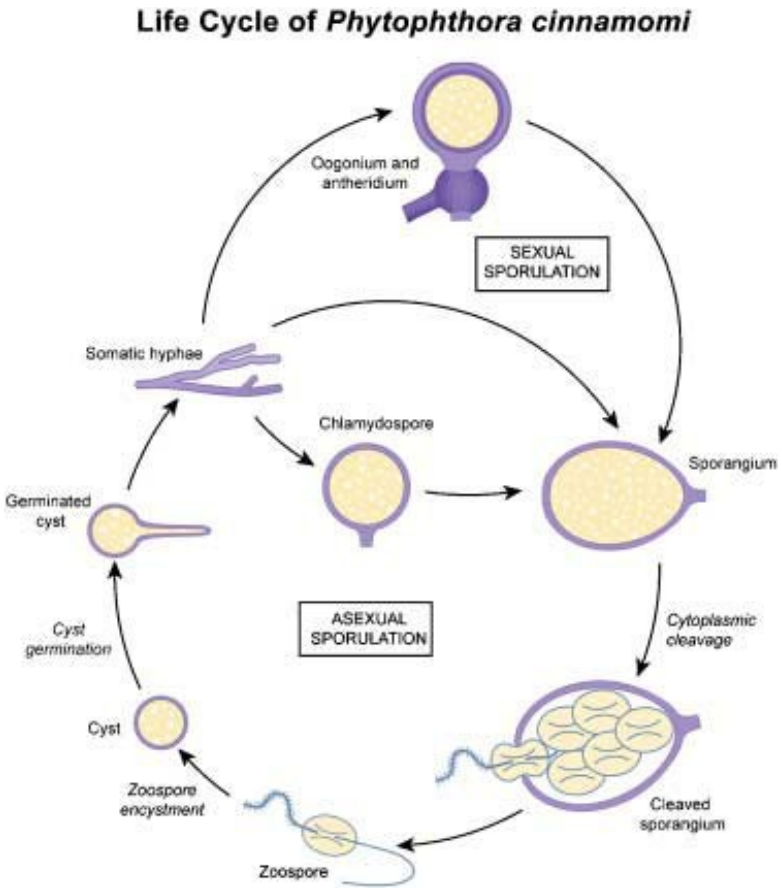

Figure 2: The Lifecycle of Phytophthora cinnamomi (Hardham A, 2005)

Many of the understorey species also proved to be susceptible to the disease resulting in epidemics as it spread through native forests.

With the continued problems with *P. cinnamomi* in Australia further research has been done to examine the host range of the organism among the local species. Studies in Western Australia (Wills, 1992), Tasmania (Brown et al, 2002) and Victoria (Weste, 2003) have suggested that as many as 2000 species may be at risk from *P. cinnamomi (*Shearer et al, 2004). Thus infection by *P. cinnamomi* leads to a significant change in the composition of species in an area as susceptible species are killed off and replaced by other resistant species often herbaceous perennials, rushes, sedges and introduced weed species.

Examination of the conditions associated with the various epidemics has suggested that epidemics occur when conditions favour asexual sporulation and zoospore production. Such conditions include heavy rain and an increase in soil temperature. Further supporting this link are recent surveys that have shown the re-establishment of susceptible species in regions they had previously disappeared from, after 5 consecutive years of the driest conditions on record (Hardham, 2005). The existence of this link between environmental conditions and disease epidemics has led to the examination of the possible effects of global warming on diseases caused by *P. cinnamomi.* Studies suggest that an increase of rainfall and temperature could lead to an increase in the severity of *P. cinnamomi* oak diseases in Europe and an increase in it's range (Chakraborty et al, 1998; Bergot et al, 2004).

With *P. cinnamomi* diseases resulting in significant economic losses for various crop

species such as pineapple (Zentmyer, 1980), avocado (Erwin & Ribeiro, 1996 as cited by Hardham, 2005) and forestry species such as pine (Zentmyer, 1980) as well as serious damage to biodiversity *P. cinnamomi* is a species that is of considerable interest to both commercial and non-commercial groups and thus an important target for research. A better understanding of it's lifecycle (Figure 2), biochemistry, genetics and molecular biology will help in the development of methods of control as well as providing insight that will be of use in understanding and controlling other members of it's genus. While significant research into the related species *Phytophthora infestans* has been underway for some time, *P. cinnamomi* has significant differences in it's primary habitat and methodology of infection compared to *Phytophthora* species such as *Phytophthora infestans. Phytophthora infestans* can be considered as an example of *Phytophthora* species which follow a lifestyle of aerial dispersion and infection involving caducous papillate sporangia, while *Phytophthora cinnamomi* is an example of *Phytophthora* species which tend towards a lifestyle as soil-inhabiting, root infecting parasites with noncaducous sporangia (Cooke et al, 2000). This difference in lifestyle would suggest that exact methods of control or management are likely to vary between the species even though closely related. Thus it is important to study a variety of *Phytophthora* species to better understand the genus, with *Phytophthora cinnamomi,* due to it's great importance as a disease organism, being a prime candidate for study.

Actin

Actin is one of the two primary proteins that make up the cytoskeleton in most organisms, the other being tubulin, which forms microtubules. It has a molecular weight of approximately 42kDa, and can exist as either a monomer known as G-actin or as a polymer of repeating monomer subunits. When two polymers link up they form a left-handed helical structure referred to as a microfilament, which is also known as F-actin. Microfilaments are capable of joining up to form cables and dynamic 3-dimensional networks. These actin-based structures, in combination with microtubules, form the basis of the cytoskeleton. Actin monomers consist of a single polypeptide with two domains, a “large” and a “small”, each domain featuring two sub-domains (Figure 3) (Vorobiev et al, 2003). There are two features of the actin monomers that are particularly important in defining the characteristics of actin microfilaments. The first is an ATP and cation binding site, the hydrolysis of ATP affects the dynamics of the microfilament. The second is the longitudinally asymmetric nature of the monomer, which affects the rates at which monomers can associate and disassociate from a microfilament. As a result of the biochemical differences in the structure the association of monomers occurs primarily at one end, the “barbed end”, and dissociation occurs primarily at the “pointed end”. The dynamics of F-actin are affected by cellular conditions, actin monomer concentrations, and the presence or absence of a large variety of actin binding proteins (ABPs) (Walker and Garrill, 2006). Specific ABPs are associated with various F-actin arrangements and structures, and ABPs may stabilise or destabilise of F-actin microfilament and the more complex F-actin structures derived from the microfilaments, these interactions allow F-actin structures to be highly

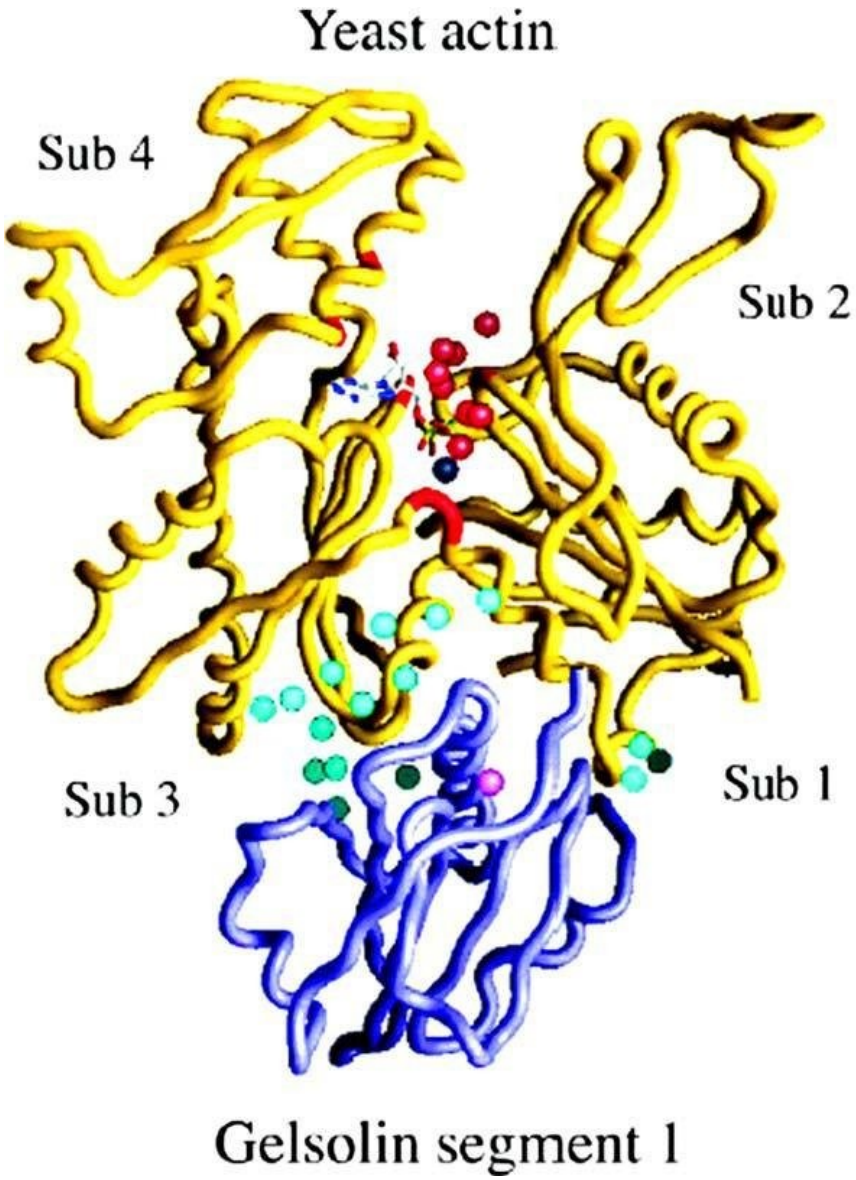

Figure 3: A yeast actin monomer (yellow), in compelex with a gelsolin segment (blue). The 4 actin subdomains can be seen along, as can the nucleotide binding site (red) with a nucleotide present. The cation binding site can be seen with the presence of a Cation (black) and it's associated water molecules (aqua). (Vorobiev et al, 2003)

dynamic. Many actin binding proteins appear to share a conserved actin binding domain which targets a hydrophobic cleft in the monomers structure (Dominguez, 2004). A large number of actin binding proteins have been described, Dos Remedios et al (2002) provide a detailed review of such proteins.

In animal cells F-actin is links to integrin containing focal adhesions on the plasma membrane of the cell (Blystone, 2004). These focal adhesions are aggregates of transmembrane signalling proteins, and also act to link the cytoskeleton to the external environment be that the extra cellular matrix or the substrate the cell is being grown on. This link to the external environment helps anchor cytoskeleton, providing it with a solid base against which force and be generated for use with in the cell, F-actin filaments in conjunction with motor proteins such as myosin are thought to be among the major sources of force generated in this manner. Such force can be used to move other microfilaments, organelles, vesicles and other cellular elements, as well manipulate the shape of the cell. Due to its importance as one of the two primary cytoskeleton proteins and the large number of proteins that bind to it, F-actin has been studied in some depth, with a wide range of methods developed to visualise it in hyphal organisms (Kaminskyj & Heath 1994). As a result it has been found that the arrangement of F-actin in cells varies significantly from species to species. None the less several recurring arrangements of F-actin have been noted. One of the more common patterns in hyphal organisms are patches or plaques of F-actin, usually found in the cortex or periphery near the plasma membrane of the cell (hence these are typically described as cortical or peripheral populations). In hyphae these tend to accumulate near the apex of the hyphae or in areas of growth. Such plaques are dynamic being able to rapidly assemble, disassemble and reassemble in a period of minutes (Heath et al, 2003; Ayscough et al,

1997). In yeast plaques have been partially purified (Young et al, 2004), with each plaque consisting of on average, 85 filaments with an average length of 50nm (20 actin subunits). Associated with the filaments was a Actin Related Protein complex (ARP2/3), containing the actin related proteins Arp 2 and Arp 3, which act as a template for plaque assembly and motility (Winter et al, 1997). There is some evidence for plaques being involved in endocytosis (Engqvist-Goldstein & Drubin, 2003) and cell wall deposition (Utsugi et al, 2002). F-actin is also known to form cables. These are formed when numerous filaments are bundled together by the ABP fimbrin/sac6. Such cables tend to form in a polarised manner, in hyphae aligning parallel to the longitudinal axis. These cables, in conjunction with myosin, are thought to provide routes for the transportation of organelles and vesicles (Adams & Pringle, 1984). This supposed role is supported by sub-apical swelling caused by treatment with Latrunculin B, which inhibits the formation of F-actin. Loss of the F-actin derived cables would lead to the delocalised delivery of vesicles, as the lack of longitudinally aligned cables removes the guide and likely motive source for vesicles. Another interesting pattern of F-actin distribution is that which is found in oomycetes. Oomycetes typically contain a peripheral cap of microfilaments at the hyphal tip. The cap can either be complete or contain an actin depleted zone at apex (Yu et al, 2004; Walker et al, 2006). The actin depleted zone may allow for a localised increase in protrusive force with out change in turgor, or serve as an area for the accumulation of vesicles containing cell wall components (Walker et al, 2006). In the sub apical region the cap changes to a diffuse network of plaques and fibrils. The F-actin cap may play a role in the regulating the movement of organelles and vesicles into and out of the sub-apical region of the hyphae, acting as a filter of sorts (Geitmann & Emons, 2000). It may also play a role in the regulating turgor driven expansion of the cell wall.

F-actin is involved in a wide range of cellular activities in a variety of species. Controversially it has been suggested as a provider of a protrusive force in ameboidal movement in hyphal organisms (Heath & Steinberg, 1999). In yeast the polymerisation of a microfilament with the actin binding protein formin has been shown to generate a force of 1pN (Kovar & Pollard, 2004), as well as having roles in morphogenesis, polarisation, mitosis, meiosis, cytokinesis, septation, and the movement and positioning of organelles (Walker & Garrill, 2006).

Actin Inhibitors

Of great importance in the study actin has been the ability to disrupt the formation of its microfilaments. Two families of inhibitors are of regular use in actin related studies the Cytochalasins and Latrunculins both inhibit F-actin formation although their methods of action vary. Some members of the cytochalasin family are thought to bind to the barbed end of F-actin filaments destabilising them and displacing them from their sites of anchorage (Wakatsuki et al, 2000), however there is some suggestion that the cytochalasins may not be entirely actin specific (Lin & Spudich, 1974; Treves et al, 1987). While the Latrunculins (A, and B) have been shown to bind to G-actin, preventing the formation of F-actin, this combined with the dynamic disassembly of F-actin results in a loss of G-actin preventing the assembly or reassembly of F-actin (Spector et al, 1989).

Hyphal Growth.

Hyphal growth or tip growth is a common highly polarised form of growth that occurs in a wide range of organisms including fungi, oomycetes, and plants. Due to its involvement in the growth of both plants and hyphal organisms it is important that the mechanism of growth is well studied and understood, as this would provide a valuable insight into how such organisms function, and would be of great use in the continued effort to control tip growing pathogens. Currently there is no standard accepted model of tip growth, but a number of hypothesises having been put forward. Most agree on the basic activities that occur during growth but differ in certain areas including what the source of the protrusive force is. In hyphae most of the critical processes are thought to occur at the apex. At the apex, vesicles containing plasma membrane and cell wall components along with agents (ie endoglucases) which soften and weaken the cell wall, are deposited, leading to localised expansion at that area. The endogulcases are thought to weaken the cell wall or increase its malleability, allowing the protrusive force to push it outwards causing the hyphae to grow. The vesicles are thought to be guided to the tip by the cytoskeleton, predominately by actin microfilaments. Various experiments have shown that actin microfilaments are dominant compared to microtubules in this area and the disruption of the actin microfilaments leads to cessation or a significant reduction of the rate of growth. While disruption of the microtubules causes a change in the morphology of the hyphae but generally not, a cessation of growth or as a significant a reduction in the rate of growth compared to actin disruption (Heath et al, 2000). While the mechanisms for the transport and deposition of materials needed for growth may be agreed on, the source of the force needed to extend the hyphae in both invasive and non-invasive conditions is an area of

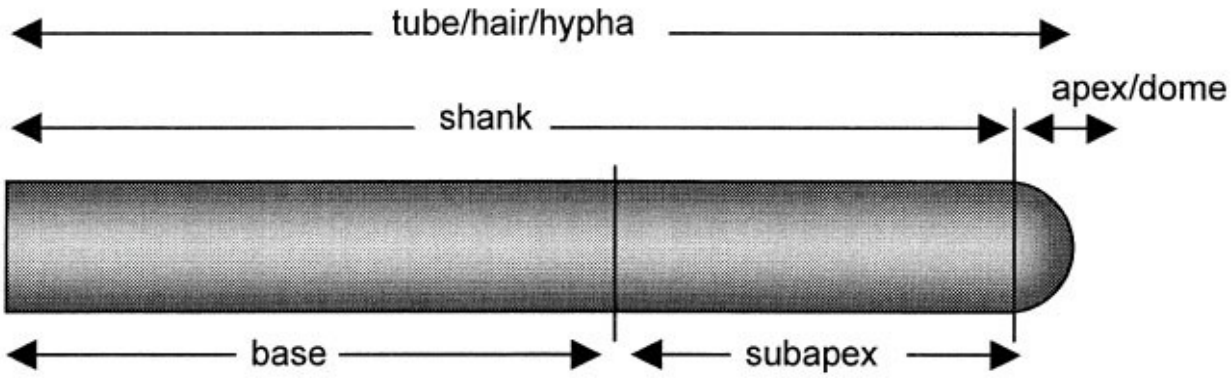

Figure 4: This figure shows the structure of a hyphae and the terms associated with each region. Through out this document these terms will be used, subapex/subapical, apex/apical, and base/basal (Geitmann & Emons 2000)

debate.

Two major hypothesises have been put forward, the turgor pressure model and tube growing ambae model. The earlier of the two to gain significant support, was the hypothesis that the turgor pressure in a hypha drives growth and is the source of the protrusive force required for tip growth in walled cells. Hyphal organisms for the most part have varying degrees of turgor pressure in their hyphae and are able to regulate it and adjust it with regards to the external environment. In the turgor pressure model, the hydrostatic pressure in the hyphae drives the growth. As the cell wall at the hyphal tip is weakened by the action of endogucanases or similar enzymes and the addition of materials to the cell wall, the resistance of the cell wall decreases until it is less than that of the turgor at the tip. The turgor pressure then forces the cell wall outward, expanding the cell (Kropf et al, 1998), while additional compounds then strengthen that particular region of cell wall as the hyphae tip moves further away and that region of wall becomes a lateral wall (Bastmeyer et al, 2002). The model however isn't without it's problems, with experimental data at times failing to show a link between turgor pressure and growth rate (Money, 1997). Also a number of oomycetes have been found that are unable to regulate turgor pressure under changing conditions (Lew et al, 2004), and some hyphal organisms have been found that undergo tip growth in the absence of any detectable turgor pressure (Harold et al, 1996). Such occurrences cannot be explained by the current turgor pressure based models leading some to suggest that while turgor pressure may play a role in tip growth that it may not be the primary or sole source of the protrusive force needed for tip growth or that alternate mechanisms may be present.

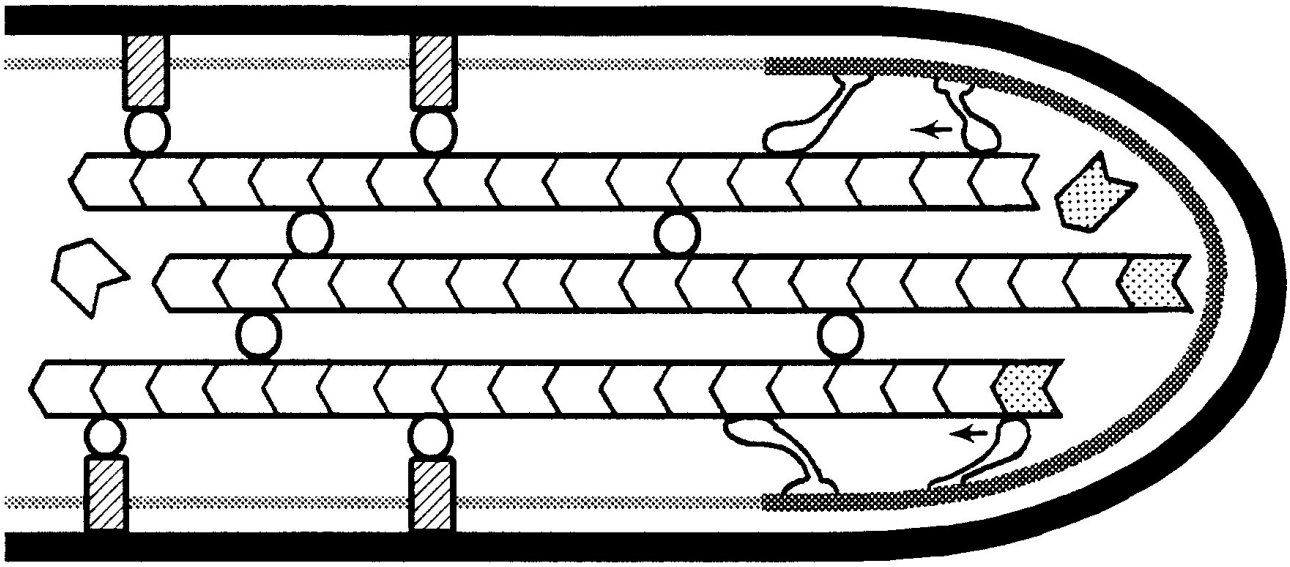

Figure 5: Illustrates how microfilaments attached to the cell wall could provide protrusive force via dynamic rearrangement and by the use of "motors" attached to the plasma membrane (Figure from Money 1997)

Another major hypothesis for explaining hyphal tip growth is that rather than utilizing turgor pressure that it may instead be more readily compared to ameboid movement and that hyphae as such can be modelled as tube dwelling Amebae. In this model growth occurs as the apical cytoplasm is protruded by the action of actin microfilaments in a manner similar to a pseudopodium, Studies have shown that the extension of a microfilament generates a force of at least 1pN (Kovar & Pollard, 2004). Thus large bundles of microfilaments attached to the cytoskeleton and the cell wall might provide sufficient force to drive growth (Money, 1997).

In support of such a hypothesis are various similarities that can be observed between hyphae tips and pseudopodia. With the observations of the critical importance of dynamic F-actin remodelling in both hyphal tip growth and pseudopodia, the discovery of Integrin like proteins in a number of hyphal organisms, and the migration of cytoplasm to fill the enlarged cell wall. Further similarities between hyphal organism and amebae can be seen in the zoospores of a number of hyphal organisms, which move in a manner comparable to ameboid movement and a slime mutant of *Neurospora,* which lacking a cell wall moves across solid substrates like amebae, via the extension of tubular pseudopodia. An in-depth review of all such observations and support evidence for the model has been produced by Heath and Steinberg (1999).

Overall, though various hypotheses have been proposed none have yet acquired sufficient evidence to be fully supported. It is quite possible that the actual mechanism of tip growth may be an amalgamation of the various theories or that differing groups such as the Oomycota may utilise different mechanisms of growth from the Eumycota. Either way, further research into such matters is needed to further our understanding of the growth of hyphal organisms.

Integrins

Integrins are a large family of heterodimeric transmembrane receptors (Xiong et al, 2001; Xiong et al, 2002; , Giancotti et al 1999), consisting of an α and β subunit. They have primarily been studied in animals though recent studies have found evidence of epitopically related proteins that have been called the Integrin like proteins (Ilps) in both plants and Fungi (Chitcholtan & Garrill, 2005; Heath & Steinberg, 1999). However for the moment, such Ilps have not been characterised in depth, thus our understanding of their possible roles and importance must be based largely on correlations made with studies of integrins in animals, in which the proteins have been extensively examined.

In mammals there are 18 α subunits split into two categories, the 9 A domain containing α subunits which have an A domain homologous to the A domain of the von Willebrand factor and 9 α subunit that lack the A domain (Humphries et al, 2003). There are 8 β

subunits which combine with the 18 α units to form 24 different receptors (Xiong et al, 2001, 2002; Giancotti et al, 1999; Humphries et al, 2003) which are then named after the subunits they're made up of. Integrins act to transfer chemical and mechanical signals between the extra cellular matrix (ECM) and the cytoskeleton of the cells. Of relevance to the current study they are also critical in the migration of cells via an amebiodal mechanism.

Animal cells grow, move, and reproduce in tissue specific environments know as the extra cellular matrix, an intracellular system of proteins, sugars, connective fibres, and other biologically active molecules. The ECM helps to define the various tissues, providing the cells that grow in it the necessary information to determine which tissue they are in. This regulates the cell types they can differentiate into, as well as providing

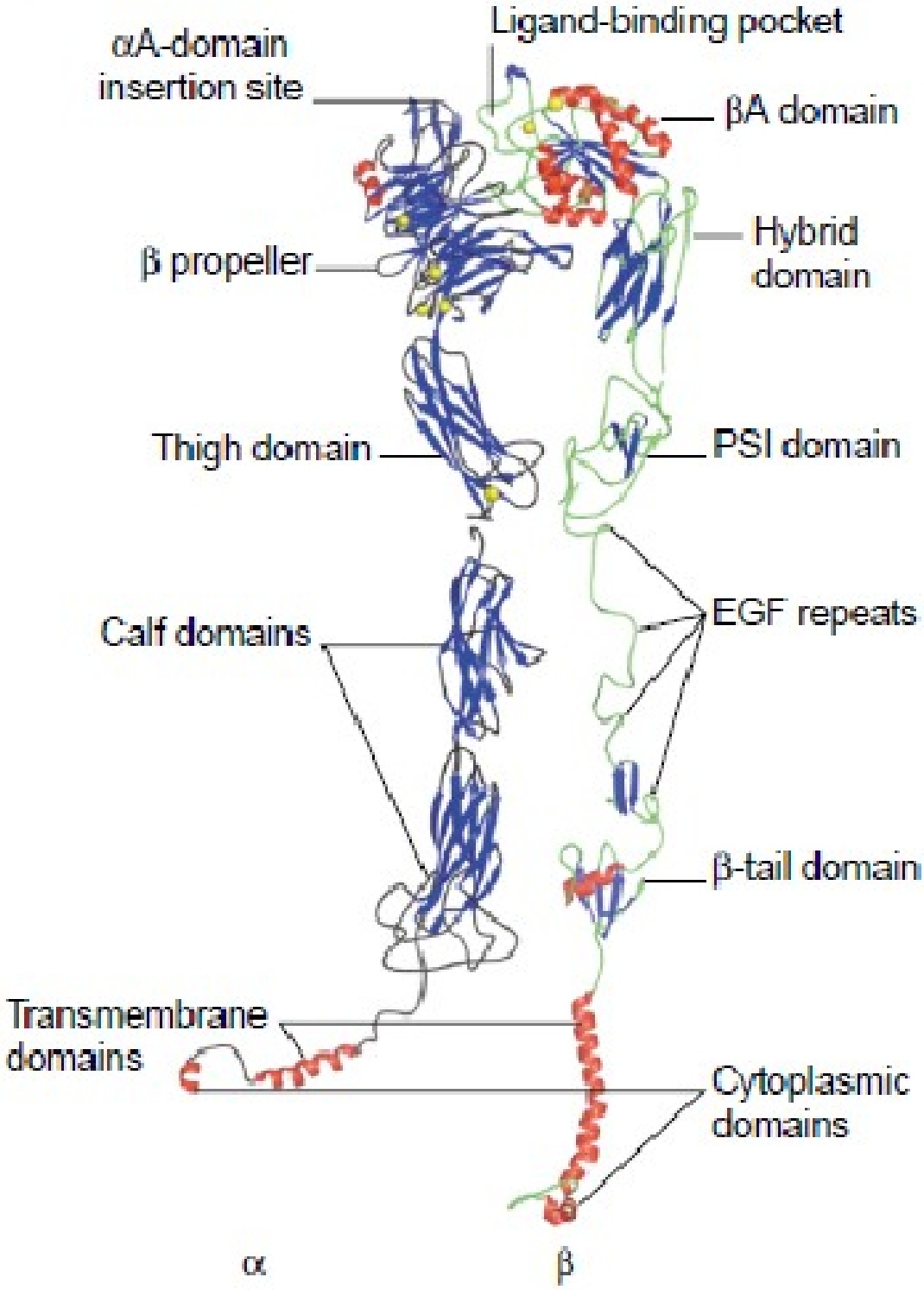

Figure 6: Straightened conformation of the Integrin αVβ3. A helix's are shown in red, β strands blue, loops grey (α) and green (β). Metal ions are shown as yellow spheres (Humphries et al, 2003).

other important information about the tissue and the nearby cells. Integrins are considered to be the primary means of communication between the ECM and a cell (Giancotti et al, 1999). Integrins are thought to provide bidirectional signalling through the membrane (Xiong et al, 2002; Giancotti et al, 1999; Humphries et al, 2003). Outside-in signal transduction is where binding of the integrin to ECM elements leads to the transduction of a signal into the cell, and inside-out signal transduction is where the binding of cytoplasmic factors to the integrins cytoplasmic domains regulates the extra cellular functions of the integrin (Xiong et al, 2001; Xiong et al, 2002; , Giancotti et al, 1999). *In vivo* different integrins are thought to work together with other receptors such as growth factor receptors and the insulin receptor, with different integrins being shown to co-localize with different receptors (Giancotti et al, 1999).

This co-localization has been show to increase the sensitivity of both the integrin and the receptor and it is postulated that the co-localization results in both being partially activated (Giancotti et al, 1999).

The primary organizational structure associated with integrins is the focal adhesion. This is a region of the membrane where integrins clump together with other receptors (Giancotti et al, 1999), these adhesion sites are large enough that they can be observed on the plasma membrane (Wozniak et al, 2004). A focal adhesion site consists of a number of different integrins which are linked via their cytoplasmic domains to a mixture of different kinases and structural proteins which connect them up to the actin cytoskeleton. Focal adhesion sites are dynamic and seem to be critical for proper integrin function (Wozniak et al, 2004).

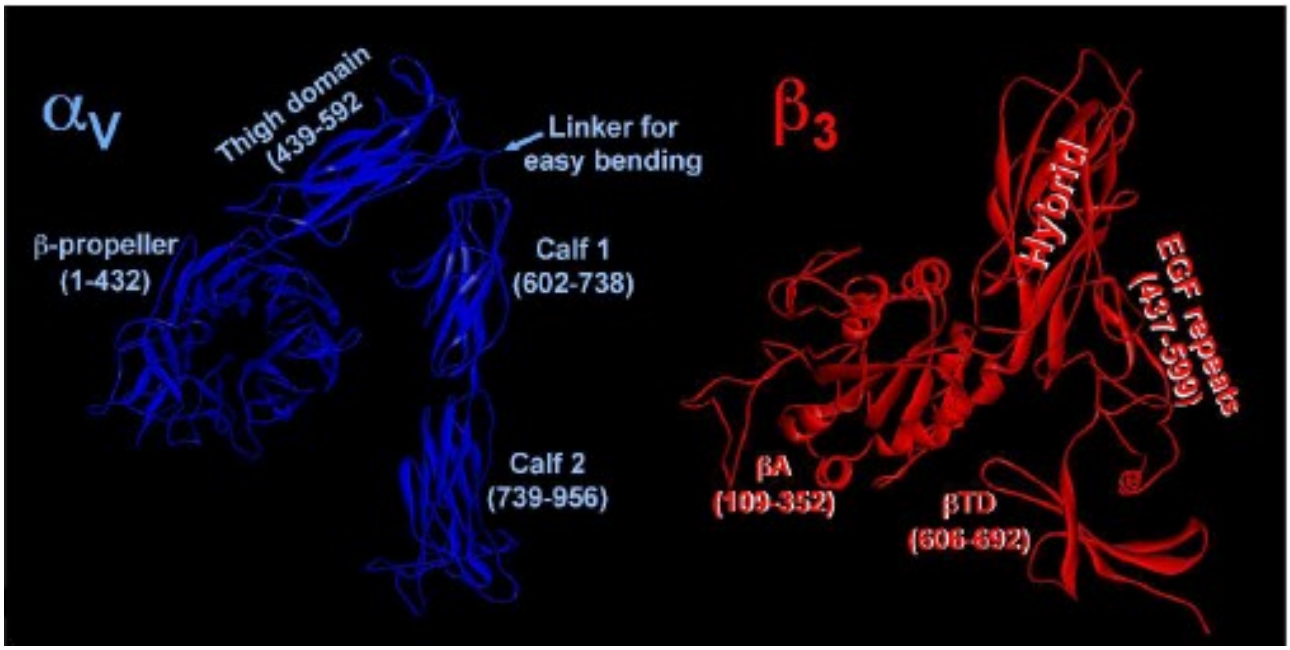

Figure 7: Crystal structures of the subunit conformations in an inactive integrin. The α_V subunit clearly shows the 7 bladed β propeller and the extreme angle at which the knee bends. The β_3 subunit is less precise but provides and indication of how the subunits link.

Integrin Structure

The best way to describe the structure of an integrin is to say that it is similar to the lower half of a “human amputated at the waist” (Figure 6) (Humphries et al, 2003). The α and β subunits (Figure 7) that form each integrin are linked at the “waist” of the integrin in a non covalent manner forming a globular region that is responsible for binding the ligand. The remainder of each subunit forms a leg which extends down to the plasma membrane where it forms a foot, which extends through the membrane into the cytoplasm. Various kinases and structural proteins bind to this cytoplasmic “foot”region (Xiong et al, 2001; Humphries et al, 2003).

The α subunit consists of a 7 bladed β propeller (NH domain) which is the main region of attachment between the α and β subunits (Xiong et al, 2001), in the αA subunits the A domain is present at the top of this region (Figure 6). The β propeller then connects the “thigh domain” the first of the three large β-sandwich domains which form the leg. Between the “ thigh domain” and the first of the two “calf domains” is a highly flexible region referred to as the “knee”. After the knee the two β-sandwich Calf domains form the rest of the leg and connect to the “foot” the α helix transmembrane domain and the cytoplasmic domain (C domain)(Humphries et al, 2003).

The β subunit consists of the βA domain which links to the α subunits β-propeller and is responsible for ligand binding in integrins lacking an αA domain. The β subunit leg consists of a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, 4 epidermal growth factor (EGF) like repeats and a cystatin-like fold. The transmembrane and cytoplasmic “foot” region consists of an α helix. The β subunit “knee” region consists of

the hybrid domain, the PSI domain and two EGF repeats (Humphries et al, 2003).

Integrins have 3 major conformations (Figure 8) which correspond to different levels of activation. The first is the bent conformation (Figure 8a) the inactive form of the integrin, the state in which the integrin is not bound to a ligand and is unable to do so. It is thought that the integrin is held in this conformation by the presence of a salt bridge linking the cytoplasmic domains of the α and β subunits. In this conformation the integrin is bent over at the “knee”. The binding of an appropriate cytoskeletal or signalling molecule is thought to break the salt bridge causing a change in conformation of the integrin, resulting in it shifting to its primed conformation (Figure 8b) at which stage it is able to bind to ligands. In this primed state the integrin appears to be “standing up” but with the “legs still somewhat bent” (Humphries et al, 2003). The final activated form of the integrin is when a ligand binds. This causes a change in the conformation in the “waist” region of the integrin which leads to a straightening of the “leg regions” of each subunit (Figure 8c) and the extension of part of the transmembrane domain into the cytoplasm. This change in conformation is thought to activate kinases and signaling molecules associated with the cytoplasmic regions of the integrin leading the activation of the appropriate signalling pathways (Blystone, 2004).

Ligand binding

Due to the two different forms of the α subunit, different integrins bind to ligands via one of two methods. In integrins with an αA domain the αA domain is responsible for binding the ligand. The ligand is bound to the integrin via coordination to a metal ion usually thought to be of Mg or Mn. In this system a carboxyl residue of the ligand completes the coordination sphere for a metal ion. This occurs at the Metal ion

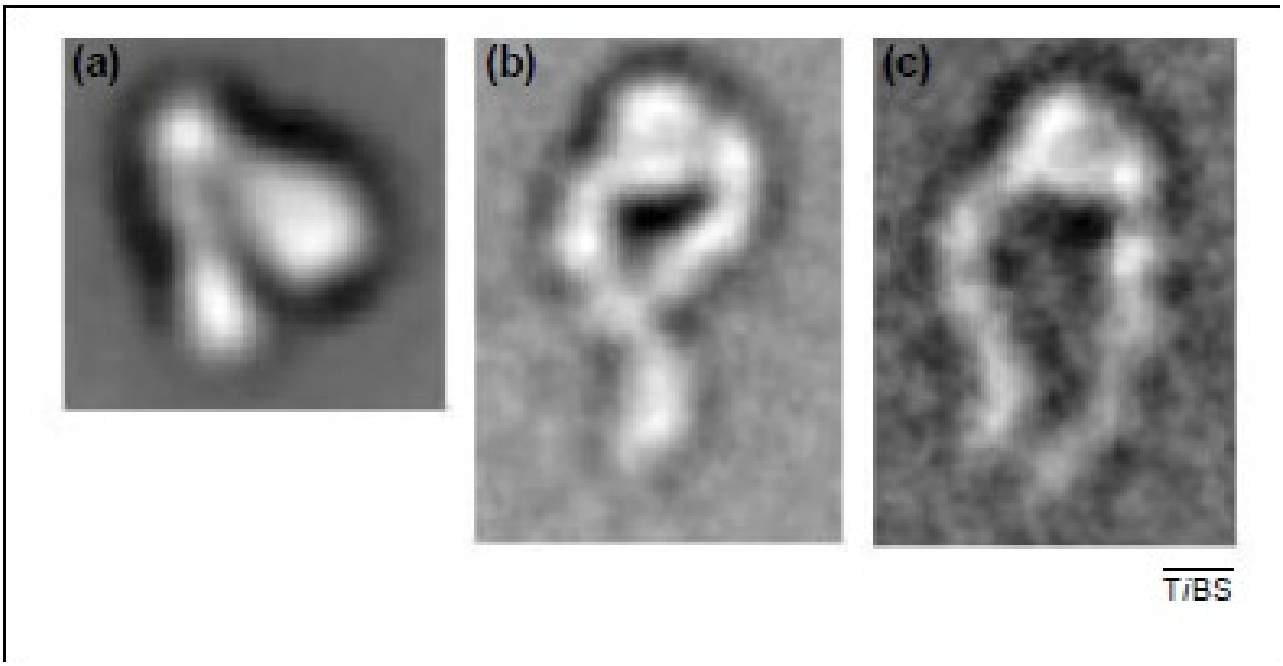

Figure 8: Average electron microscope images of different αVβ3 activation states of the extracellular domains. (a) Bent inactive conformation, (b) primed conformation, (c) ligand bound conformation.(Humphries et al, 2003)

dependent adhesion site (MIDAS), where via a conserved, 5 amino acid, motif with the ligand acid residue providing a 6^{th} coordination site to stabilise the ion This is supported by additional hydrogen bonding between the ligand and the integrin and secondary binding at ligand specific sites on each integrin.

For integrins lacking the αA domain the initial binding occurs via the β subunits βA domain. The βA domain is similar to the αA domain. In this type of integrin the ligands carboxyl residue inserts between the β-propeller and the βA domain where it coordinates with the βA MIDAS site in the same manner as the αA binding. However in the βA domain a second metal ion binding occurs, the ligand dependent metal ion binding (Xiong et al, 2002). The binding of the metal ion to the MIDAS site causes a change in conformation in the the βA domain which forms a second coordination sphere allowing the coordination of a second metal ion. Again this is thought to be followed up by additional binding at ligand and integrin specific sites (Humphries et al, 2003). This causes a change in conformation which is transmitted through the subunit “legs” causing the extension of the transmembrane domains into the cytoplasm leading to the activation of the integrin associated signalling molecules and kinases and thus the signal cascade (Blystone 2004).

Integrins also have important roles in the regulation of many critical cellular functions. Integrin activation and deactivation of various pathways can be linked to the control of the Cell Cycle, Cell Death, Cell Growth, and Cell Shape (Giancotti et al 1999). Integrins also play and important role in cell migration.

Many cell types move via an ameboidal method (Heath & Steinberg, 1999) with the cell first extending a lamellipodia which attaches to the external environment and samples it to determine if the environment is suitable for the growth of the organism (Giancotti et al 1999). If the environment is suitable the cell then moves itself along after the lamellipodia. If the environment is not suitable it can instead move in another direction. For the cell to push itself along after the lamellipodia it needs an anchor point or strong base attached to the external environment that it can exert force against, to propel the cell forward without compromising the structural integrity of the cell. The anchor must also connect to the actin cytoskeleton as that will be the primary source of the force. Integrins are one of these adhesion molecules (Giancotti et al, 1999), being able to bind to material in the external environment and forming focal adhesions which are connected to the cytoskeleton. This provides a solid platform for the cytoskeleton to "push" against. Therefore, in cell migration, integrins play several important roles. Primarily as a means of transmembrane signaling between the external environment (for example the ECM) helping to identify if the external environment around the lamellipodia is suitable. Secondarily, due to their ability to bind to the external environment, clump and form focal adhesions linked to the cytoskeleton, they can form the anchor points or base needed by the cell for the cytoskeleton to exert force against, to provide the motive force for the cell (Giancotti et al, 1999; Blystone, 2004). In hyphal organisms it is assumed that the Integrin like proteins which are associated with the plasma membrane (Chitcholtan & Garrill, 2005) act to link the cytoskeleton to the plasma membrane and the cell wall. The cell wall effectively replaces the external environment or ECM that integrins bind to in animal cells. This would allow the cytoskeleton to be anchored to a structurally sound external structure against which force could be generated, without risk of rupturing the cell. The cell wall is a suitable

structure due to its strength. The force generated could be used for a number of different purposes, ranging from moving the cytoplasm along behind the growing tip or supplying the protrusive force to keep the tip expanding at low levels of turgor pressure. What the exact role of integrin like proteins is in hyphal organisms has yet to be determined, although there is evidence that it is related to tip growth [ref:Kenny]. The existence of integrin like proteins is also supportive of the tube dwelling Amebae hypothesis for tip growth reviewed by Heath and Steinberg (1999).

Thus understanding integrins may be of considerable importance in understanding the growth of hyphal organism, should integrin like proteins prove to be common and widespread among hyphal organisms. Also greater understanding of the structure and sequence of hyphal integrin like proteins will be of great use in determining their role and importance in tip growth.

Methods:

Methods

Materials

Microscope and capture software

A Olympus IX70 Microscope, Olympus Mercury Burner, Coolsnap camera and software were used for both DIC (Differential Interference Contrast) and Epi-Fluorescent image capture and microscope work.

Stock Cultures:

Phytophthora cinnamomi

Stock cultures of *Phytophthora cinnamomi* (University of Canterbury stock culture c216) were maintained at 25°C in the dark on peptose-yeast-glucose (PYG) agar (3 g/l glucose, (Merck), 1.25 g/l bactopeptone (Gibco BRL, UK), 1.25 g/l yeast extract (Gibco BRL, UK), and 20g/l bacteriological agar (Oxoid, UK) made up in nano-pure water). For sub cultures an agar plug with hyphae growing on it, was taken from the growing edge of the culture.

Achlya bisexualis

Stock cultures of *Achlya bisexualis* Coker (a University of Canterbury stock culture, isolated from *Xenopus laevis* dung) were maintained at 20°C in the dark on peptose-yeast-glucose (PYG) agar (3 g/l glucose, (Merck), 1.25 g/l bactopeptone (Gibco BRL, UK), 1.25 g/l yeast extract (Gibco BRL, UK), and 20g/l bacteriological agar (Oxoid, UK) made up in nano-pure water). For sub cultures an agar plug with hyphae growing on it was taken from the growing edge of the culture.

Experimental Culture preparation:

Two cultures of *Phytophthora cinnamomi* were prepared for experimental work, one of these was for investigations of invasive growth and the other was for investigations of non-invasive growth. The *Phytophthora cinnamomi* cultures were grown at 25°C in the dark. Two cultures of *Achlya bisexualis* Coker (a University of Canterbury stock culture, isolated from *Xenopus laevis* dung) were also used for experimental work, again one was an invasive culture and the other a non-invasive culture. *Achlya bisexualis* cultures were grown at 20°C in the dark. Cultures used for experimental work were between 4-7 days old after which they covered 40-80% of the plate, and thus were assumed to still be healthily growing hyphae. Both organism's cultures were prepared in the same manner as detailed below.

Non-Invasive Cultures

The non-invasive plates were prepared by placing a circle of colourless cellophane (John Sands brand) on a PYG agar plate. Prior to use the cellophane was prepared by boiling 3 times for 10 minutes in nanopure water and autoclaved, to remove any manufacturing residues. Previous work from this lab has indicated this is a necessary prerequisite that ensures hyphal growth. The hyphal plug of the organism was then placed in the center of the cellophane circle.

Invasive Culture:

The invasive plates were prepared by placing a circle of sterile boiled cellophane on PYG agar. A hyphal plug was then placed in the center of the cellophane face down and the plate was evenly covered with 6ml of molten 4% Low Melting Point agarose (LMP agarose in dH_20). This had the effect of providing a layer of agarose above the

cellophane and the culture through which hyphae would grow (invasively i.e. through the media).

Imaging Methods

Criteria for Imaging of Hyphae

For experiments involving microscope work a simple criteria was decided one for determining what hyphae would be imaged. Hyphae that met the criteria would be imaged those that didn't were observed but neither epi-fluorescent nor DIC images were taken.

Hyphae to be imaged were required to meet these requirements:

- Good staining through out the hyphae
- Low to moderate background fluorescence in the field of view.
- No overlap with other hyphae near on the focal plane
- Cytoplasm in good condition with out significant distortions
- No significant distortion to the hyphae structure
- Hyphae wall intact, with no signs of bursting in the on branches or at tips
- Decent focus for the hyphae tip

Image Processing

All image processing was done in Adobe Photoshop, The GIMP (The GNU Image Manipulation Program) and Image Pro Plus 4. Processing consisted of rotation and

alignment of images, level adjustments, cropping, and conversion to greyscale.

Growth Rate Measurements Image Pro Plus

The sequential images produced with the coolsnap camera were aligned manually in The GIMP and cropped to produce a layered Photoshop file, showing the growth of the hyphae. The layers were then split into individual files and imported into Image Pro Plus. A 30µm scale line from an objective micrometer (0.01mm Olympus) was used to calibrate Image Pro Plus. Then each hypha was measured and the results exported to excel for analysis.

Actin Experiments

Actin Staining

For staining hyphae were prepared by cutting 1cm squares from the growing edge of the organism and these were placed in welled slides and 700µl of PYG broth (3 g/l glucose, (Merck), 1.25 g/l bactopeptone (Gibco BRL, UK), 1.25 g/l yeast extract (Gibco BRL, UK) made up in nano-pure water) was added. The samples were then allowed to recover for 2 hours at room temperature in the dark. After this time the PYG broth was replaced with 700µl fixing solution (4% formaldehyde (Sigma) with 0.5% methylglyoxal (Sigma) in pipes buffer) and the hyphae were left to fix for 40 minutes in the dark. The samples were then rinsed 2 times for 5`minutes in buffer and then stained by incubating in the dark, at room temperature, for 30mins with 400µl of dilute Phalloidin tagged with the fluorescent dye Alexa 488. A control was incubated in the same conditions for 30mins with Pipes buffer (50mM pipes pH 6.8). The samples were washed twice for 15 minutes with 10ml of Pipes buffer. Then were prepared with the addition of anti-fading solution

(0.1% w/v of *p*-phenylenediamine dihydrochloride) prior to observation under the Microscope.

Actin Staining: Latrunculin B

For samples treated with LatB (Latrunculin B, Sigma) the Actin staining method described above was used with an additional step. After the recovery step the broth was replaced with 700µl of PYG broth containing LatB at the specified concentration and incubated in the dark at room temperature for 30 minutes. For the control the broth was replaced with fresh PYG broth.

Growth Rate Experiments

Growth Rate measurements

Plates were prepared according to the technique listed in “Culture preparation”. For LatB plates the invasive method was used with the specified amount of LatB being mixed to the molten LMP agrose, before it was poured onto the plate. Measurements were made by marking the center and the edge of the inoculums. From this 6 evenly spaced lines were then drawn on the bottom of the plate to it's edge. The distance the hyphae had moved along each line was noted, and the six values were then averaged to give an average growth rate.

Growth Rate: Microscope method

For growth rate experiments 1 cm square samples were cut at the edge of the mycelial margin from both invasive and non-invasive plates. The samples were placed in welled

slides and 700µl of PYG broth was added to the sample. They were then allowed to recover at room temperature in the dark for 2 hours. The samples were then observed under the microscope. On the microscope a 0.6 x 0.6mm square of agar was placed onto the sample to anchor the sample. Healthy hyphae, identified by regular or smooth cytoplasm [reference] and the distinct presence and movement of cellular organelles, were identified and observed for 5 minutes to determine if they were currently growing. An initial image was taken, via the attached coolsnap camera, and a second after a further 5 minutes. If a comparison of the images showed that the hyphae was growing and was stable in the field of view having not significantly “drifted” then the hyphae was selected for continued observation. If the hyphae did not meet the afore mentioned requirements another was selected and the process repeated, after 3 attempts the sample would be abandoned and the process begun on another. If the hyphae had met all requirements then a time zero image was taken followed by additional images every 2 minutes. At T8-T10 (eight to ten minutes after the initial image) if the hyphae was still growing then it would be used for the second stage of the experiment. The PYG broth was removed and replaced with fresh PYG broth containing a specified amount of Latrunculin B and the next image was taken this image was referred to as L0. The hyphae was then observed for a minimum of a further 30 minutes with images taken every 2 minutes. Once hyphal extension ceased, the interval for images was increased to every 5 minutes.

Integrin-like Proteins

Integrin-like protein staining.

For experiments involving staining of integrin-like proteins, a 1 cm square sample was cut from the growing edge of the culture, this was done for both invasive and non-invasive cultures.

The samples were placed in welled slides and attached to the side of the well via the application of molten LMP agarose (0.02g LMP agarose in 1ml dH_20). Once the samples were attached to the side of the well 700µl of PYG broth (3 g/l glucose, (Merck), 1.25 g/l bactopeptone (Gibco BRL, UK), 1.25 g/l yeast extract (Gibco BRL, UK) made up in nano-pure water) were added to the well, and the sample was allowed to recover for 2 hours at room temperature (23°C) in the dark. After the recovery period the broth was replaced with 700µl of fixing solution (4% formaldehyde with 0.5% methylglyoxal in saline buffer containing 60mM Pipes pH 6.8, EGTA 2mM, $MgCl_2$ 2mM, KCl 0.27mM and NaCl 137mM) and allowed to incubate for 40 minutes in the dark. The sample was then washed 5 times for 5 minutes with 700µl of washing solution (saline buffer with 0.1% v/v Tween 20 added) on an orbital shaker and incubated for 15 minutes with 400µl Driselase digestion solution (saline solution with 10mg/ml Driselase (Sigma)) at room temperature. After digestion the samples were then washed twice for 5 minutes and incubated for 20 minutes with 400µl permeabilising solution (saline solution with 0.1% v/v Triton X-100) at room temperature. The samples were washed twice more for 5 minutes and blocked for 30 minutes with blocking solution (saline solution with 2.5% w/v skim milk powder), then washed 4 times for 5 minutes and incubated overnight with 400µl of diluted (1:100-1:1000) primary antibody, mouse anti-Integrin β4 [Manufacturer name/details] at 4°C (fridge). The control was incubated overnight with 400µl of the antibody dilution solution. The samples were then washed 3x for 5 minutes and

incubated with 400µl of dilute (1:500-1:1000) secondary antibody (Molecular Probes Goat Anti-mouse, tagged with Alexea 488) for 2 hours in the dark at room temperature. After this they were washed 7 times for 5 minutes with 700µl washing solution followed by 3 times with 15ml of washing solution, both washes were carried out in the dark. The samples were then moved to clean welled slides and anti-fading solution (0.1% w/v of *p*-phenylenediamine dihydrochloride) was added to the samples to protect against photo-bleaching.

The same method was used for both *Achlya bisexualis* and *Phytophthora cinnamomi* samples.

Western blot: Integrin like proteins

Thirty plates of *Phytophthora cinnamomi* were grown on PYG covered with cellophane and frozen with liquid nitrogen, then ground to a powder in extraction buffer (50mM Tris-HCl pH 6.2, 10mM NaF, 2mM PMSF, 10mM β-mercapethanol, 7mM EDTA, 20% v/v glycerol, 250mM sucrose). The slurry was resuspended in additional extraction buffer and centrifuged at 10,000g for 30 minutes at 4°C; the supernatant was further centrifuged at 100,000g for 2 hours at 4°C. The resulting pellet was resuspended in additional buffer, and samples of the resulting suspension, along with the previous soluble crude fractions, were precipitated on ice via the addition of trichloroacetic acid (TCA).

The resulting precipitates were mixed with 5x Laemmli buffer and heated in a boiling water bath for 5 minutes. The proteins were then separated via SDS PAGE on a 5% stacking gel and 12% polyacrylamide separating gel. The proteins were transferred overnight to a nitrocellulose membrane in buffer (15.6mM Tri-Base and 120mM glycine)

at 40V at 4°C. The membrane was examined for protein transfer via staining with 0.1% w/v Ponceau Red for 20 minutes. With evidence of protein transfer the membrane was rinsed with the kit washing solution [kit details] then washed 2 times for 15 minutes with washing solution and once with nanopure water for 10 minutes. The membrane was blocked for 60 minutes at room temperature with the [kit] blocking solution, washed 3 times for 10 minutes with washing solution, once with nanopure water and incubated overnight with the diluted primary antibody mouse monoclonal IgG1 anti-Integrin β4 at 4°C. The membrane was visualized by Western Breeze™ (Western Immunodetection System, Invitrogen).

Results

Results

Actin Staining

DIC images of growing *Phytophthora cinnamomi* hyphae

The hyphae (Figure 9) typically showed regular/smooth cytoplasm and the presence of organelles or vesicles. For growth experiments hyphae were chosen based on regular/smooth cytoplasm, and the observable movement of organelles/vesicles. These structures appear as clear raised points (base of A, top B, centre C), or dark dots in the cytoplasm (A, B, D, E, F). The hyphae have a pointed apex though this is less distinct than in other organisms such as Achlya bisexualis . The *Phytophthora cinnamomi* hyphae also grow in 3 dimensions, moving up and down through the focal pane as well as side to side when growing. Movement in the vertical axis can be seen in B, C and E (Figure 9) where at the base of each image the hyphae as started to move out of the focal plane. F shows a change in direction of growth or movement on the horizontal axis. *Phytophthora cinnamomi* hyphae regularly show a tendency to branch. This would initiate when a swelling formed on the side of the hyphae, branches occur in any direction. If the branch occurs close to the apex of the hyphae growth at the original apex would decrease as the new branch started. Branches further back did not significantly affect the growth rate of the original apex.

Around thirty living hyphae were observed and the images in Figure 9 are representative sample.

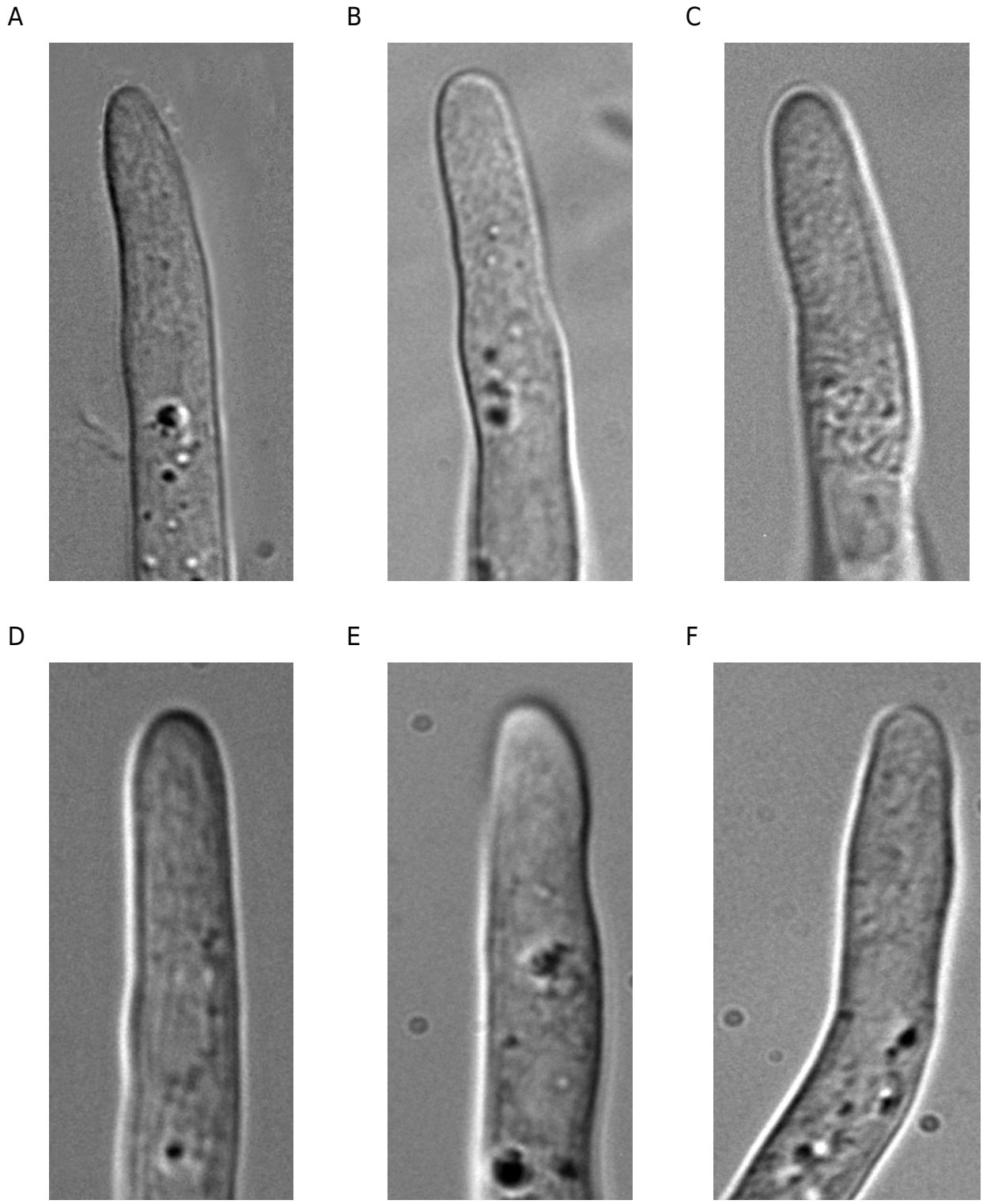

Figure 9: Hyphae A-F are DIC images of healthy growing hyphae. They show a pointed tip and regular cytoplasm. Loss of focus for Images B, C and E due to movement on the vertical plane.

Actin Stained Controls: Invasive Conditions

Figure 10 shows hyphae that were grown in invasive conditions and stained with Alexa Phalloidin, as described in the actin staining method (Page 42).

For samples grown in invasive conditions and stained with Alexa Phalloidin over forty hyphae were examined. Those shown in Figure 10 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

Hyphae A-E (Figure 10) show strong punctate staining back from the hyphal apex A, B, D, and E show a distinct area of low staining at the apex forming an actin depleted zone. Image C lacks a clear actin depleted zone with punctate staining further back from the apex than other hyphae. Image F is the invasive control showing a lack of punctate fluorescence, and fairly constant autofluorescence through out the hypha though a decrease is present near the apex as would be expected with the decrease in cellular material to autofluorescence.

The apparent variation in the location of the punctate staining in the stained hyphae is primarily due to the limited focal plane of the microscope. Hyphae A, D and E share a similar focal plane with punctate staining occurring at the edges of the hypha, and a lower level of diffuse staining just below the apex. Hyphae B, and C at a different focal plane showing more central punctate staining and a strong region of diffuse staining below the apex.

All hyphae show regular/smooth cytoplasm in the DIC with the presence of organelles/vesicles which is indicative of healthy hyphae and good chemical fixation.

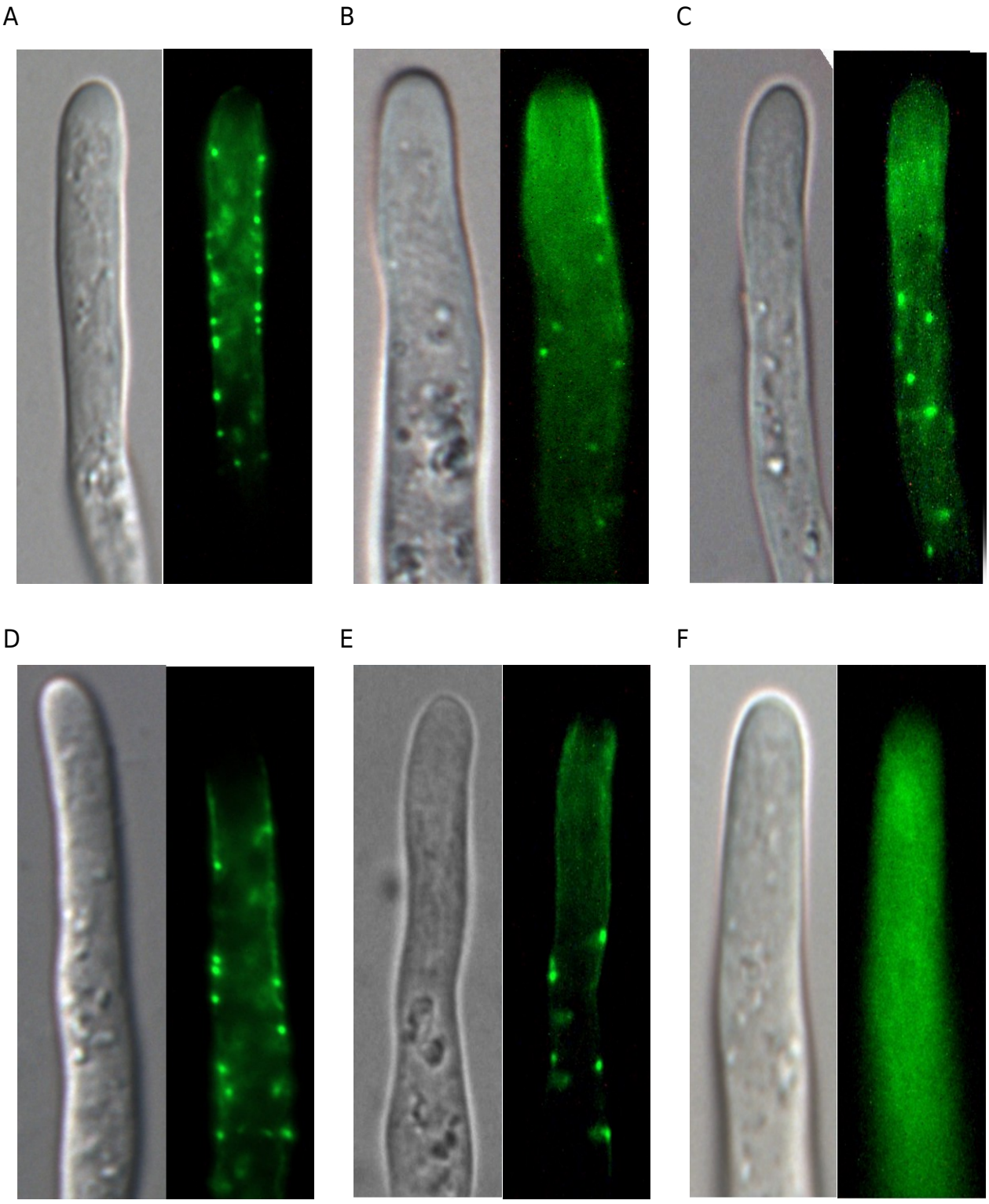

Figure 10: Hyphae A-E were grown in invasive conditions fixed and stained with Alexa Phalloidin. Image F is a control hypha grown in invasive conditions and fixed it was not stained before viewing with the microscope.

Actin Stained Controls: Non-invasive Conditions

Figure 11 shows hyphae grown in non-invasive conditions and stained with Alexa Phalloidin, as described in the actin staining methods (Page 42).

For samples grown in non-invasive conditions and stained with Alexa Phalloidin over forty hyphae were examined. Those shown in Figure 11 are a representative sample of those observed and meet the criteria to be imaged (Page 41). Hyphae A-E (Figure 11) show strong punctate staining in the sub-apical region and an area of low staining for an actin depleted zone at the apex, all hypha also show strong staining in bands along the walls of the hypha. Hypha A shows a strong region of diffuse staining in the sub-apical region and punctate staining throughout the rest of the hypha. Hyphae B, C and E show punctate staining throughout the hypha. Hypha D shows large areas of overlapping punctate staining along the edge of the hypha with very little central punctate staining. Hypha F shows the control which is an unstained hypha, grown in non-invasive conditions. The hypha lacks punctate staining and has a constant level of autofluorescence through out. It lacks an area of low fluorescence at the apex as is present at the apex of the stained hyphae.

The DIC images show a decrease in the regularity/smoothness of the cytoplasm and a lack of distinguishable organelles/vesicles, compared to both the invasive hyphae (Figure 10), and growing hyphae (Figure 17), indicating less than ideal fixing. The DIC for Image F is also slightly out of focus due to the low level of signal for the control compared to the stained hyphae, the controls were focused first under epi-fluorescent conditions after which the DIC was taken, this combined with the hypha growing through the plane of focus result in difficulties when attempting to focus for both fluorescent and DIC conditions.

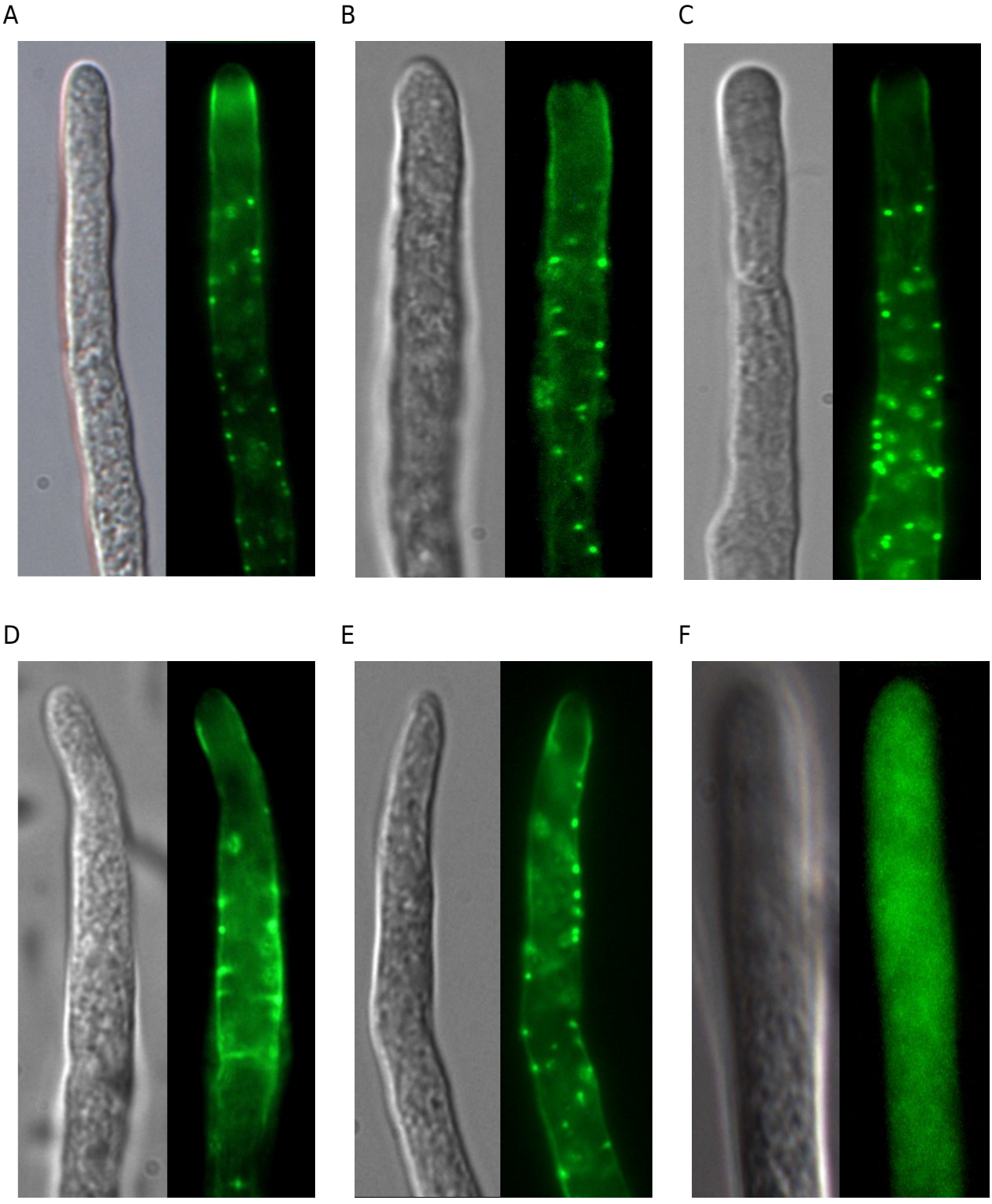

Figure 11: Images A-E show hyphae grown in non-invasive conditions stained with Alexa Phalloidin, after fixing. Image F shows an unstained control.

Actin Stained Controls: 0.1% DMSO Control in Invasive Conditions

DMSO controls were grown as described in the “Latrunculin B Actin Staining” methods (Page 43), however instead of incubation in PYG Broth containing Latrunculin B, they were incubated for the same amount of time in PYG broth containing DMSO at the specified concentration.

For hyphae that were exposed to 0.1% DMSO hyphae, shown in A-D (Figure 12), show strong punctate staining. Hyphae A, C, and D also show and zone of low staining at the apex indicative of an actin depleted zone. The level of punctate staining is similar to that of the control. Hypha B shows punctate staining throughout the hypha with no prominent actin depleted zone. Hypha E is a control grown in invasive conditions and stained with Alexa Phalloidin.

The DIC images for A and D show a regular/smooth cytoplasm though organelles/vesicles can not be clearly identified, while hyphae B and C show a more irregular/rough cytoplasm compared to the control. Hypha B also lacks the more pointed tip present in the other hyphae and controls (Figures 9, 10 and 11).

For samples treated with 0.1% DMSO and stained with Alexa Phalloidin over a dozen hyphae were examined. Those shown in Figure 12 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

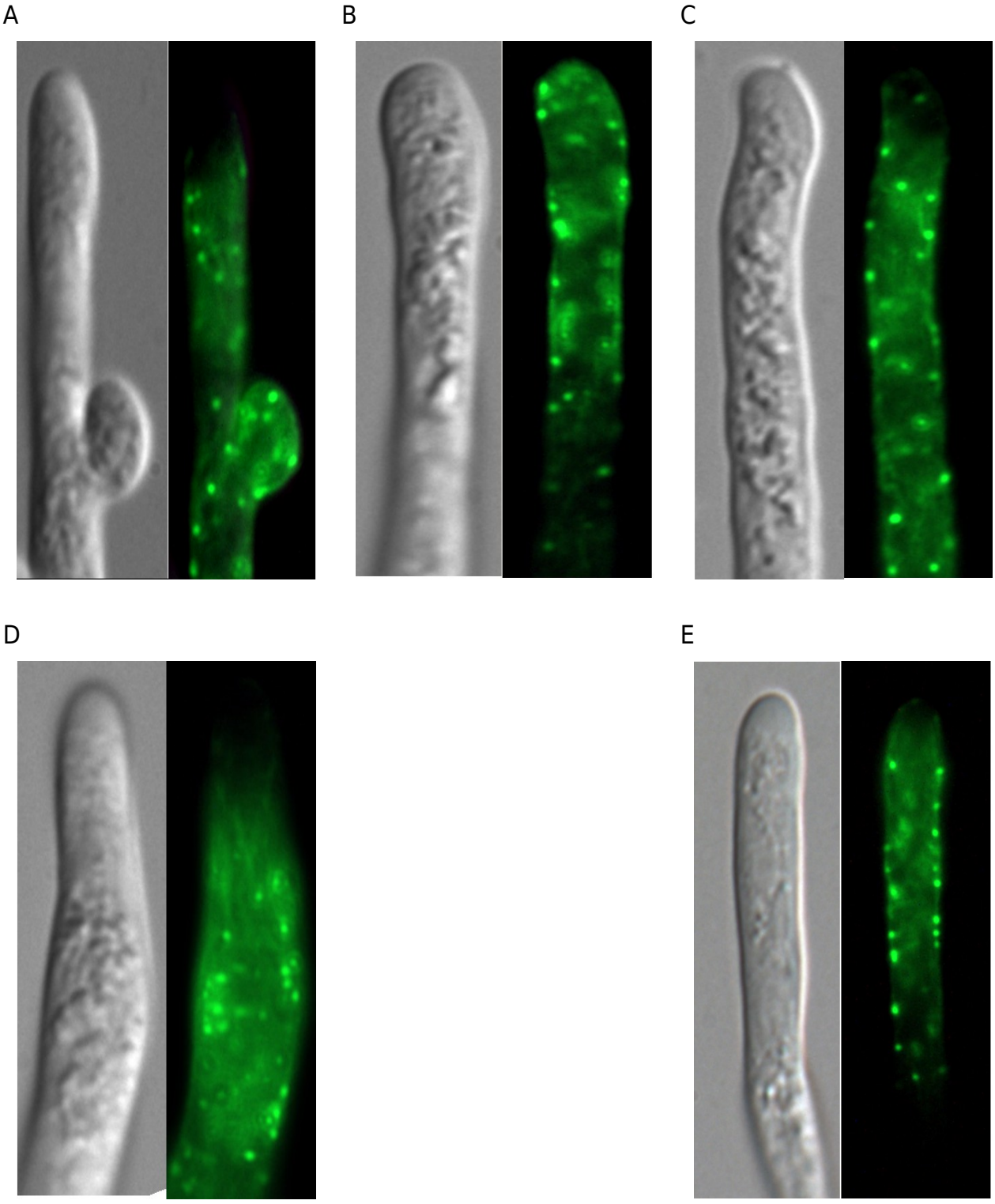

Figure 12: Images A-D are hyphae grown in invasive conditions then incubated with 0.1%DMSO in PYG Broth and stained with Alexa Phalloidin. Image E is an actin stained hypha grown in invasive conditions.

Actin Stained Controls: 1% DMSO Controls in Invasive Conditions

DMSO Controls were grown as described in the “Latrunculin B Actin Staining” methods (page 43), however instead of incubation in PYG Broth containing Latrunculin B, they were incubated for the same amount of time in PYG broth containing DMSO at the specified concentration.

Hyphae A-D (Figure 13) show strong punctate staining in the sub-apical region. Hypha A, C and D also show a decrease in staining the apex of the hyphae suggestive of an actin depleted zone. Hypha B while showing the strong punctate staining lacks an actin depleted zone at the tip, where a rim of staining is present along the cell periphery. Hyphae B and C both show a noticeable area of diffuse staining in the sub apical region. Compared to the control hypha the 1% DMSO treated hyphae may have a lower level of punctate staining.

The DIC images A, C and D have a regular/smooth cytoplasm, and visible organelles/vesicles associated with good fixing and healthy hyphae. They also have the more pointed tip associated with growing hyphae, while hypha B has a more rounded structure and less regular/smooth cytoplasm.

For samples treated with 1% DMSO and stained with Alexa Phalloidin over a dozen hyphae were examined. Those shown in Figure 13 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

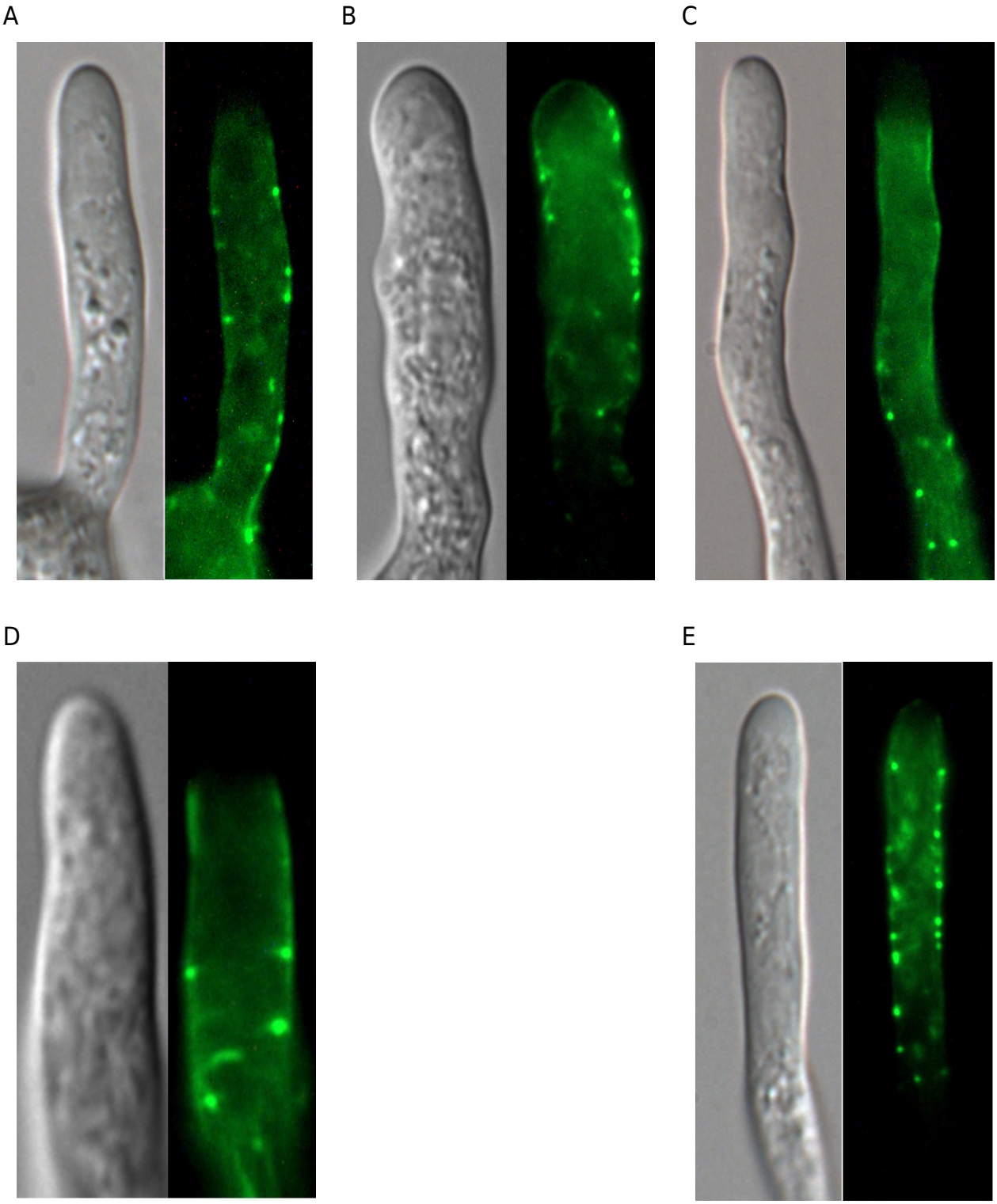

Figure 13: Images A-D are 1% DMSO controls that were incubated for 30mins in PYG Broth with 1% DMSO, fixed and stained with Alexa Phalloidin. Image E is a standard control hypha grown in invasive conditions and stained with Alexa Phalloidin.

Actin Staining: Latrunculin B 0.5ug/ml

Samples were prepared as described in “Actin Staining: Latrunculin B” with 0.5ug/ml of Latrunculin B in PYG broth, from hyphae grown in invasive conditions (Page 43).

Hypha A, and B (Figure 14) lack punctate staining and an actin depleted zone at the apex, having a low level of diffuse staining throughout the hypha. Hyphae D and E lack significant punctate staining but do show a single large aggregate of stain amongst the diffuse stain which fills most of the hyphae, similar features are also observed in other hyphae not shown in Figure 14. They also lack any strongly defined region of low stain at the tip, indicating the loss of any actin depleted zone. Hypha C shows strong punctate staining which was present in a smaller number of stained hyphae at this level of Latrunculin B, however near the tip the punctate staining becomes more diffuse and the tip lacks a region of low actin staining.

The DIC images for hyphae A, B, D, and E show a low level of swelling compared to the control with the apex and sub-apical region of the hyphae expanding compared to the region of hypha further back, Hypha C does not show this swelling. Also the cytoplasm of all Latrunculin B treated hyphae excepting C shows, more irregularities/roughness or a possible accumulation of organelles and vesicles in the hyphae, compared to the control.

For samples treated with 0.5ug/ml LatB and stained with Alexa Phalloidin around 30 hyphae were examined. Those shown in Figure 14 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

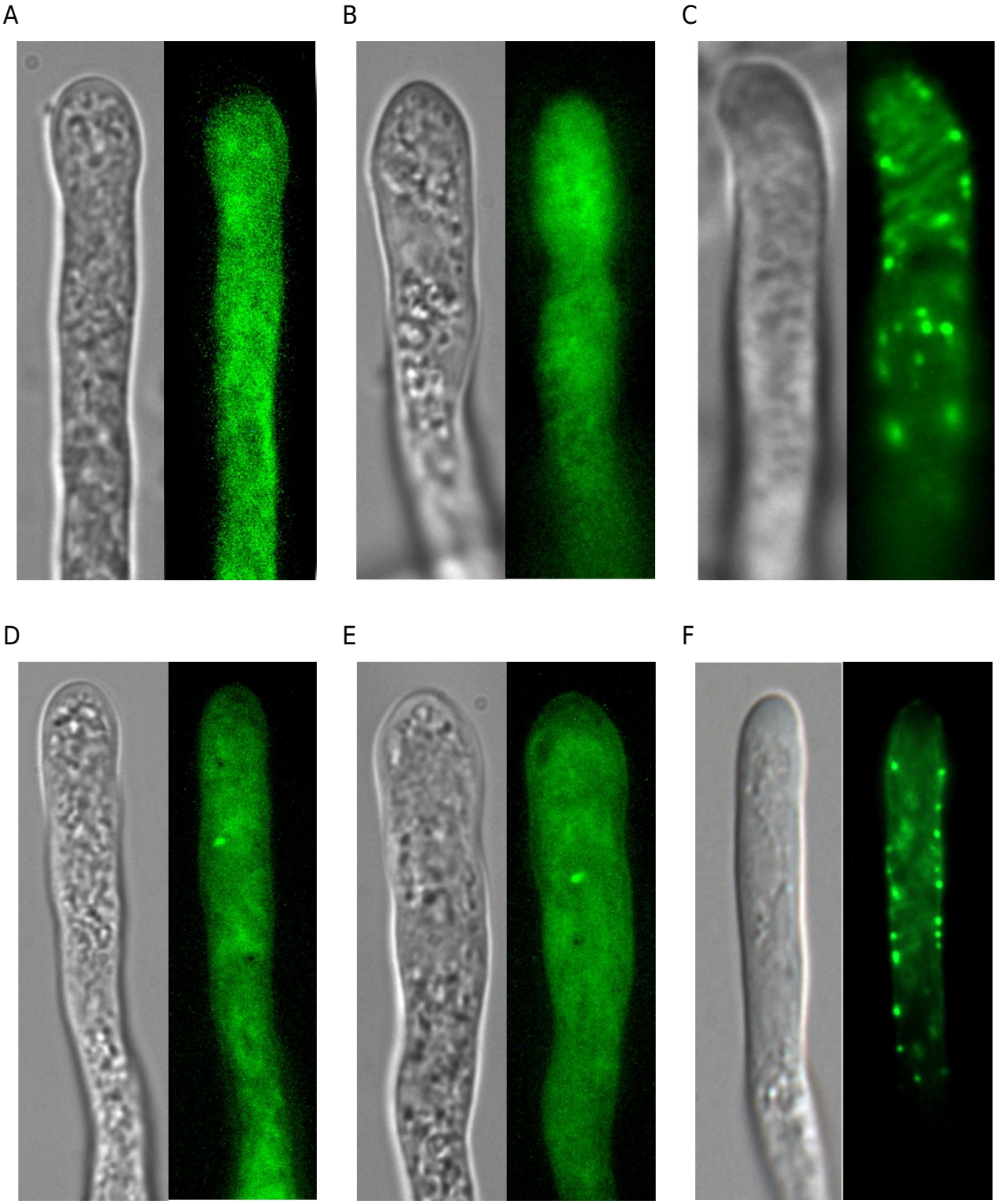

Figure 14: Hyphae A-E were stained with Alexa Phalloidin after exposure to 0.5ug/ml LatB. Image F is an Alexa Phalloidin stained Control.

Actin Staining: Latrunculin B at 1ug/ml

Samples were prepared, as described in “Actin Staining: Latrunculin B” using 1ug/ml of Latrunculin B in PYG broth, from hyphae grown in invasive conditions (Page 43).

Hyphae A, C, D and E (Figure 15) show punctate actin staining in the sub-apical region. Hyphae A and E also show a region of low staining, an actin depleted zone at the apex of the hypha, both also show the greatest levels of punctate staining. Hyphae C and D show punctate staining at the apex, with C showing punctate staining through out the rest of the hyphae while D shows much lower levels of punctate staining through out the rest of the hyphae. Hypha D does show a very large aggregate of staining similar to those seen in images C and D (Figure 14) of hyphae treated with 0.5ug/ml Latrunculin B. Hypha B (Figure 15) shows high levels of diffuse staining similar to those seen at other concentrations of Latrunculin B.

The DIC images for all the treated hyphae show an increase in irregularities in the cytoplasm or an accumulation of the organelles and vesicles compared to the control. Images D and E also show a low level of the apical swelling that is present at the other concentrations of Latrunculin B.

For samples treated with 1ug/ml LatB and stained with Alexa Phalloidin around 20 hyphae were examined. Those shown in Figure 15 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

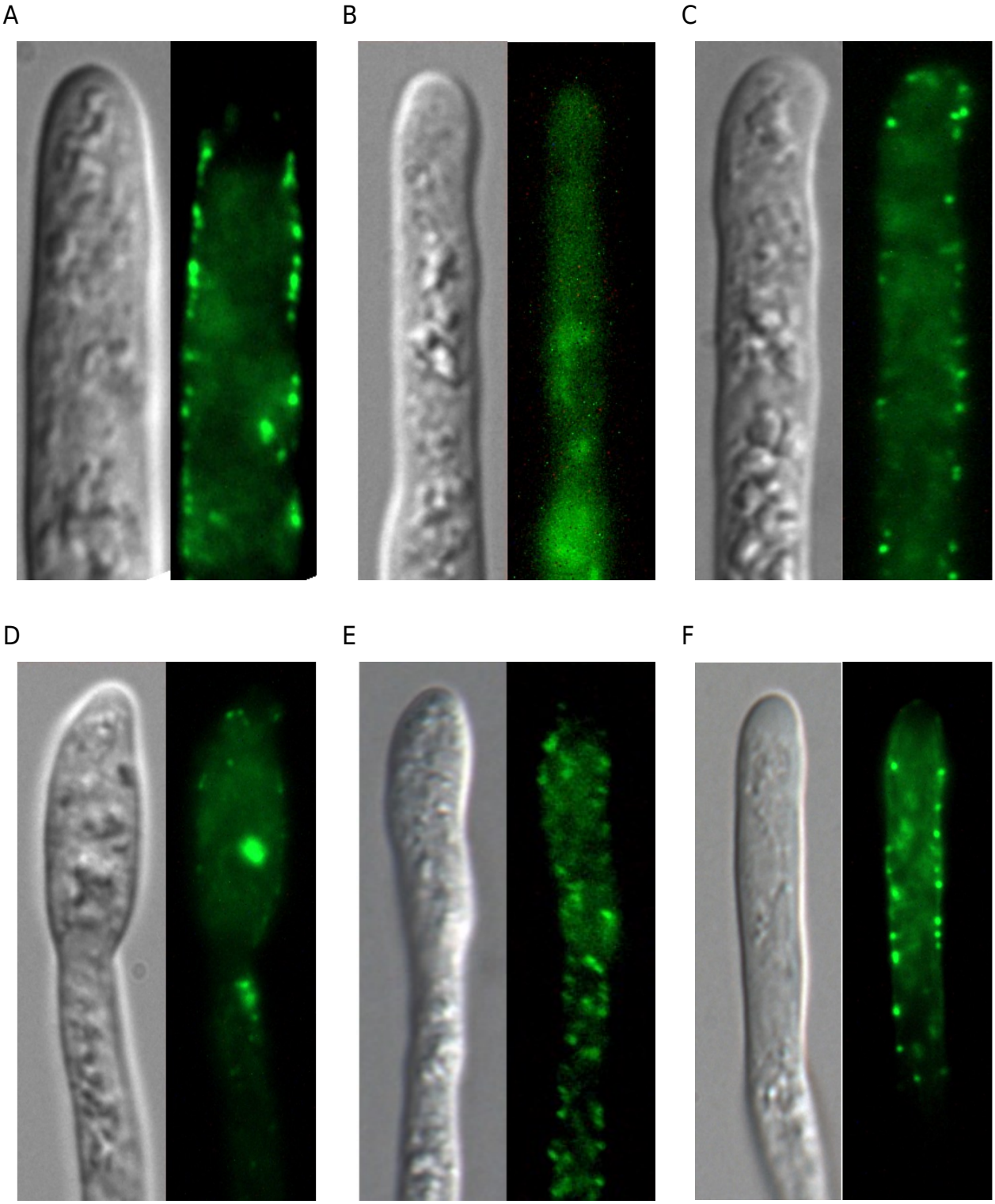

Figure 15: Hyphae A-E, Alexa Phalloidin stained hyphae after treatment with 1ug/ml LatB. Image F Alexa Phalloidin stained control.

Actin Staining: Latrunculin B at 5ug/ml

Samples were prepared, as described in “Actin Staining: Latrunculin B” using 5ug/ml of Latrunculin B in PYG broth, from hyphae grown in invasive conditions (Page 43).

At 5 ug/ml Latrunculin B all treated hyphae A, B, C, D, and E (Figure 16) show a loss of punctate staining and an actin depleted zone at the apex. Staining is diffuse through much of the hyphae. Hyphae A, and B still show some “patchiness” in the staining but it is visibly different from punctate staining present in the control images.

The DIC images show that significant apical swelling has occurred compared to the controls, and lower concentrations of Latrunculin B. Also hyphae A, C and D show that the swelling is not even leading to distortions of the hypha shape. The images also show an increase in irregularities in the cytoplasm or the possible accumulation of organelles and vesicles apical and sub-apical regions of the hyphae compared to the control.

For samples treated with 5ug/ml LatB and stained with Alexa Phalloidin around 30 hyphae were examined. Those shown in Figure 16 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

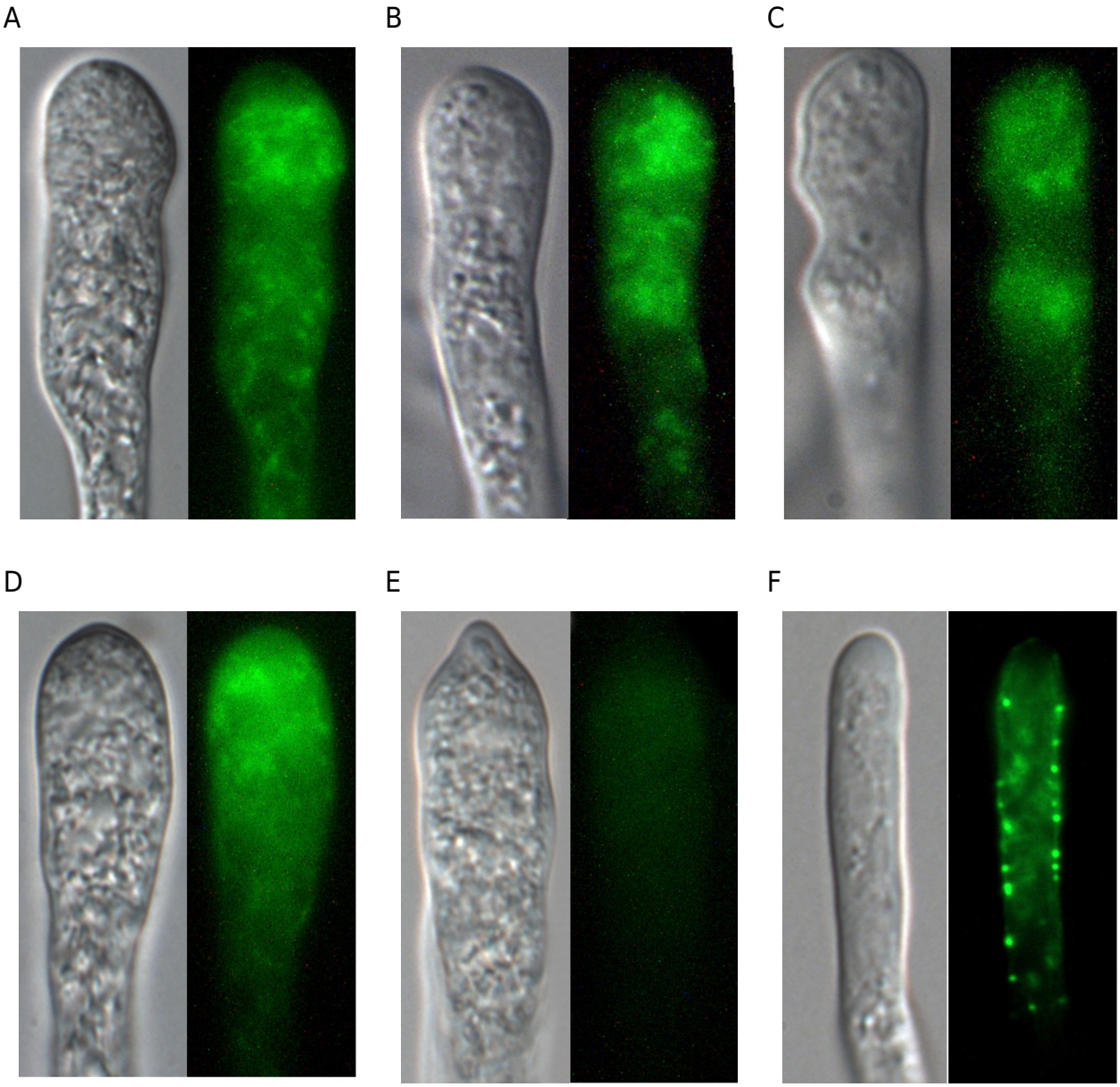

Figure 16: Hyphae A-E are Alexa Phalloidin stained hyphae grown in invasive conditions then incubated with 5ug/ml LatB in PYG broth. Image F is a control stained with Alexa Phalloidin.

Actin Staining: Latrunculin B at 10ug/ml

Samples were prepared, as described in “Actin Staining: Latrunculin B” using 10ug/ml of Latrunculin B in PYG broth, from hyphae grown in invasive conditions (Page 43).

Samples treated with 10ug/ml Latrunculin B, as shown by hyphae A-E (Figure 9), show a complete lack of punctate staining in any region of the hypha. Instead a low level of diffuse staining, staining is present throughout the swollen region compared to the control image F (Figure 9) and the actin controls in Figures 10 and 11. The diffuse stain is also less “patchy” than that observed in hyphae treated with 5ug/ml Latrunculin B, as seen in Figure 16 (images A, B, and C). The hyphae A-E (Figure 9) also lack any distinctive region of low staining or actin depleted cap near the apex of the hyphae as can be seen in the control example and in Figures 10 and 11.

Both the DIC images and the epi-fluorescent images of hyphae A-E (Figure 9) show distinct irregular apical and sub-apical swelling compared to the controls. Hyphae A-D show irregularities in the cytoplasm or an accumulation of organelles or vesicles in the apical and sub-apical regions compared to the controls.

For samples treated with 5ug/ml LatB and stained with Alexa Phalloidin around 30 hyphae were examined. Those shown in Figure 9 are a representative sample of those observed and meet the criteria to be imaged (Page 41).

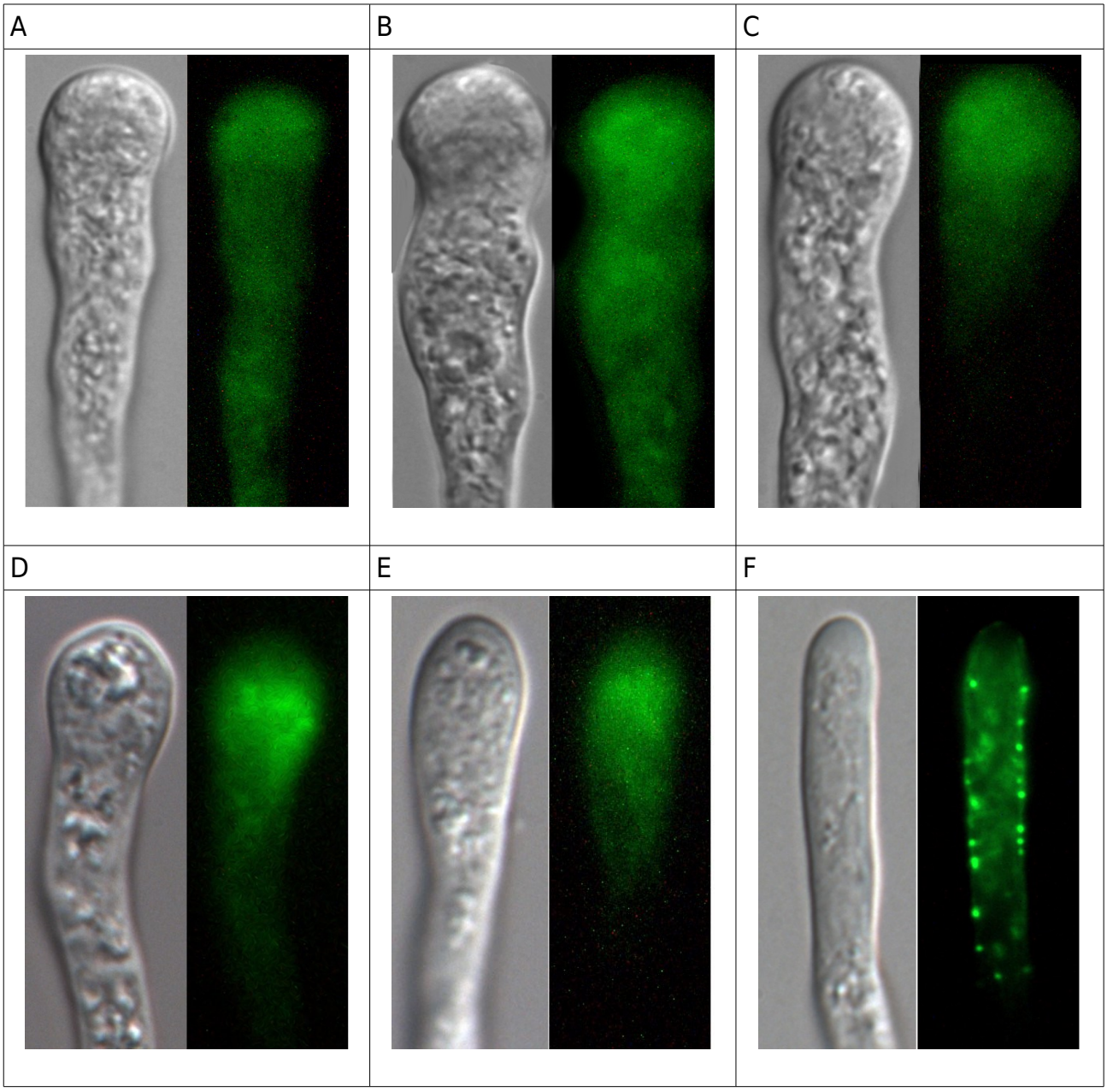

Figure 17: Hyphae A-E are hyphae grown in invasive conditions, and exposed to 10ug/ml Latrunculin B before staining with Alexa Phalloidin. Image F is a control grown invasive conditions, fixed, and then stained with Alexa Phalloidin.

Growth Rates

Plate Growth rates:

Plates were prepared as described in “Growth Rate Measurements” (Page 43). Treated plates had the specified amount (5µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml) of Latrunculin B added to the molten LMP agarose. The samples were then allowed to grow for 120hours during which time measurements were taken as described in the afore mentioned method.

Growth rates with the various treatments are summarized in Table 1.With the addition of 5µg/ml, 2µg/ml, 1µg/ml and 0.5µg/ml of Latrunculin B in the invasive agarose layer no growth of *Phytophthora cinnamomi* was observed in the treated plates during the 120hour period or indeed over the next 4 weeks. Additional invasive and non-invasive plates assembled at the same time utilizing the same media and stock culture as the inoculum, and grown in the same conditions, without the addition of the Latrunculin B grew. Stock cultures of *Phytophthora cinnamomi* grown as described in “Stock cultures: *Phytophthora cinnamomi*” (Page 39) and grown in the specified conditions had an average growth rate of 2.1µm/min.

Invasive cultures of *Phytophthora cinnamomi* grown as described in “Invasive cultures” (Page 40) and grown at the specified conditions had an average growth rate of 2.59µm/min.

Non-invasive cultures of *Phytophthora cinnamomi* grown as described in “Non-Invasive cultures” (Page 40) and grown at the specified conditions had an average growth rate of 2.64µm/min. These values were measured to give an indication expected growth rates for samples observed while examining the effects of Latrunculin B on growth rate.

	Growth Rates	
Plate Type	Average µm/min	N (sample size)
5µg/ml LatB Invasive	0	1
2µg/ml LatB Invasive	0	1
1µg/ml LatB Invasive	0	2
0.5µg/ml LatB Invasive	0	2
Stock	2.1	4
Invasive	2.59	5
Non-Invasive	2.64	5

Table 1: Average growth rates for Phytophthora cinnamomi under various conditions.

Growth Rate Results: Invasive Conditions 5µg/ml Latrunculin B

Hypahe were prepared following the method detailed in the “Growth Rates Microscope” method (Page 43) using the specified level of Latrunculin B (5µg/ml). Once the data was collected it was processed in “The GIMP” (GNU Image Manipulation program) and Photoshop then analyzed in Image Pro Plus 4.5 and the data exported to Excel where it was graphed.

For invasively grown hyphae at 5µg/ml the initial growth rates varied between 2.8µm/min and 0.79µm/min. Growth rates for all hyphae (Figure 18) were variable before the addition of Latrunculin B. With the addition of Latrunculin B hyphae 1, 2 and 4 showed a similar response with growth ceasing between 4-6minutes after it's addition. Hyphae 1 continued to show some changes in growth rate after the initial cessation. Hyphae 3 responded differently with an increase in growth rate after the addition of Latrunculin B followed by a more gradual decrease in the rate of growth. With growth ceasing at approximately 30minutes after the introduction of Latrunculin B.
For all hyphae once directional growth ceased it was replaced with swelling of the apical and sub-apical regions of the hypha.

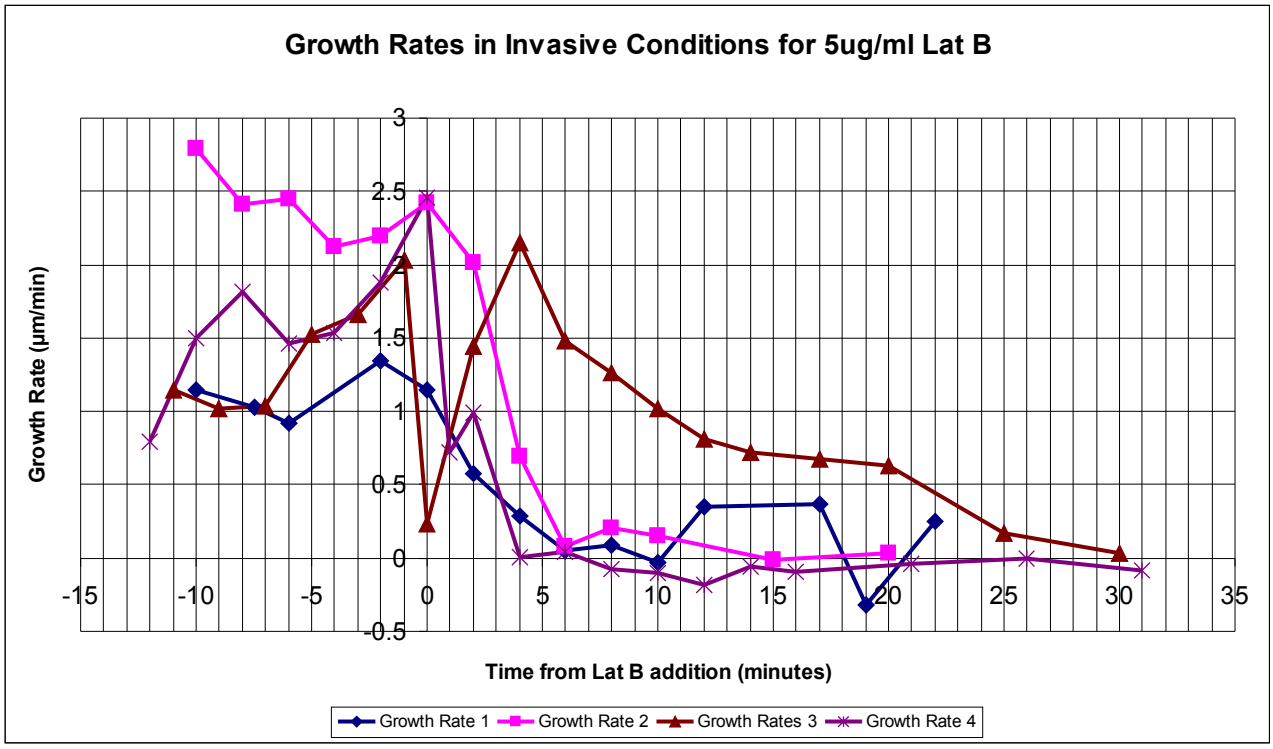

Figure 18: Growth Rates in µm/min against time for invasive hypahe with 5µg/ml LatB

Non Invasive conditions 5µg

Hyphae were prepared following the method detailed in the “Growth Rates Microscope” method (Page 43) using the specified level of Latrunculin B (5µg/ml). Once the data was collected it was processed in “The GIMP” (GNU Image Manipulation program) and Photoshop then analyzed in Image Pro Plus 4.5 and the data exported to Excel where it was graphed.

For hyphae grown in non-invasive conditions before treatment with 5µg/ml LatB (Figure 19) the initial growth rates were between 1.3µm/min (sample 1) and 2µm/min (sample 4).

Growth rates for all hyphae, up until the addition of Latrunculin B were variable. With the addition of 5µg/ml Latrunculin B all hyphae responded in a similar manner. Growth rates immediately began to decline, in a nearly identically manner with directional growth ceasing for all hyphae between 3 and 4minutes after the addition of the Latrunculin B. For all hyphae once directional growth ceased it was replaced with swelling of the apical and sub-apical regions of the hypha.

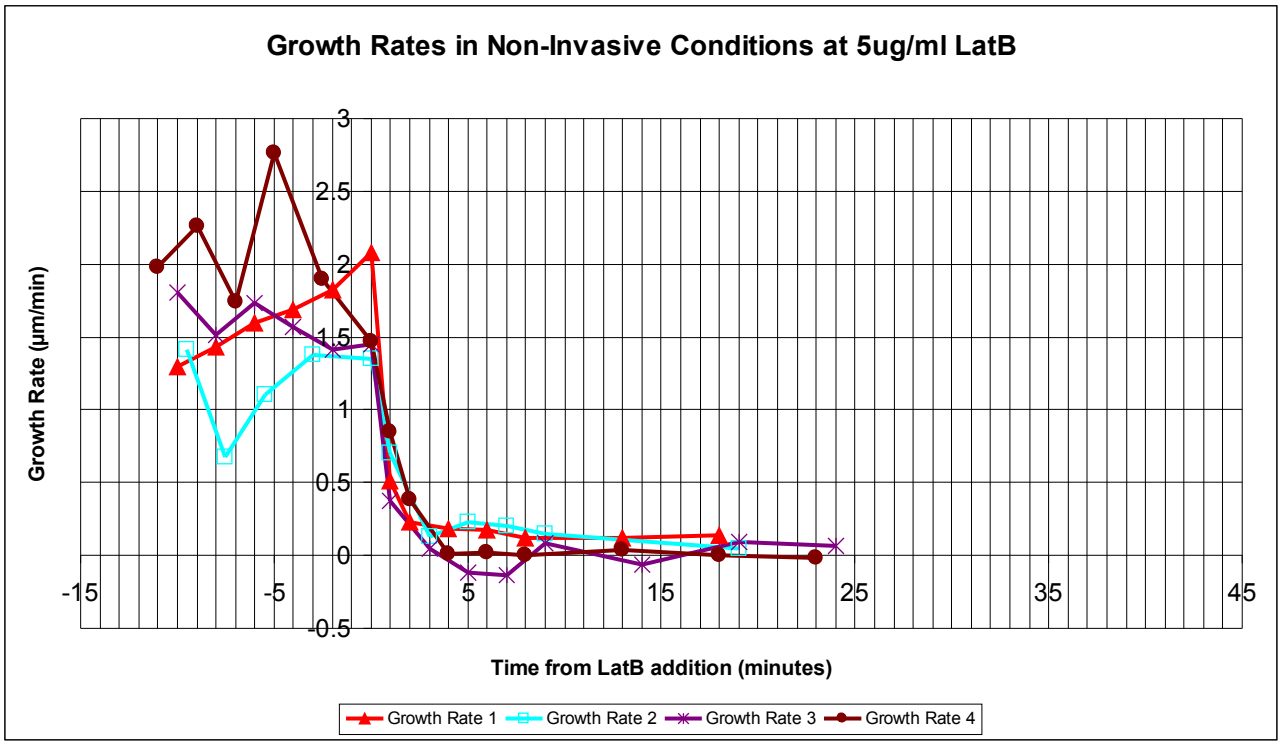

Figure 19: Growth Rates in µm/min against time for non-invasive hyphae with 5µg/ml LatB

Invasive conditions 0.5µg

Hyphae were prepared following the method detailed in the “Growth Rates Microscope” method (Page 43) using the specified level of Latrunculin B (0.5µg/ml). Once the data was collected it was processed in “The GIMP” (GNU Image Manipulation program) and Photoshop then analyzed in Image Pro Plus 4.5 and the data exported to Excel where it was graphed.

For hyphae grown in invasive conditions (Figure 20) before treatment with 0.5µg/ml Latrunculin B, the initial growth rates varied between 0.6µm/min and 2.6µm/min. Growth rates for all hyphae, up until the addition of Latrunculin B were variable. After the addition of 0.5µg/ml Latrunculin B all hyphae responded in a similar manner with a gradual decrease in growth rates until cessation of directional growth occurred between 7 and 10minutes after Latrunculin B addition. For hyphae 3 and 4 the decrease in growth rate was variable with short term increases and decreases in the growth rate. For all hyphae once directional growth ceased it was replaced with swelling of the apical and sub-apical regions of the hypha.

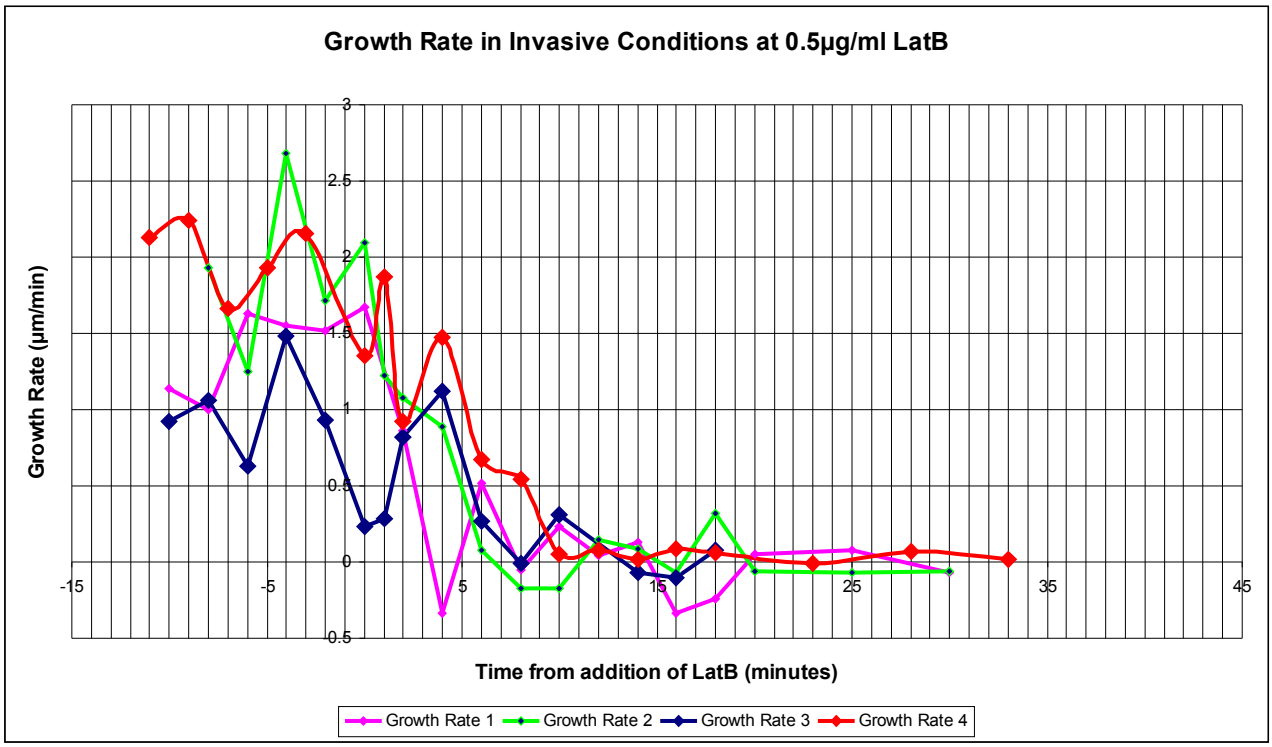

Figure 20: Growth Rates in µm/min against time for invasive hyphae with 0.5µg/ml LatB

Non-Invasive Conditions 0.5µg

Samples were prepared following the method detailed in the “Growth Rates Microscope” method (Page 43) using the specified level of Latrunculin B (0.5µg/ml). Once the data was collected it was processed in “The GIMP” (GNU Image Manipulation program) and Photoshop then analyzed in Image Pro Plus 4.5 and the data exported to Excel where it was graphed.

For hyphae grown in invasive conditions (Figure 21), growth rates before the addition of Latrunculin B varied between 0.8µm/min and 2.8µm/min. Growth rates for all hyphae, up until the addition of Latrunculin B were variable. With the addition of 0.5µg/ml Latrunculin B growth rates followed one of two patterns. Growth rates for hyphae 2 and 3 immediately began to decrease with cessation of growth occurring at approximately 12minutes for hyphae 2 and 3minutes for hyphae 3 after the addition of Latrunculin B. For hyphae 1 and 4 the growth rate initially increased abruptly reaching approximately 2.7µm/min before rapidly declining, with both hyphae ceasing directional growing 4minutes after the addition of Latrunculin B.
For all hyphae once directional growth ceased it was replaced with swelling of the apical and sub-apical regions of the hypha.

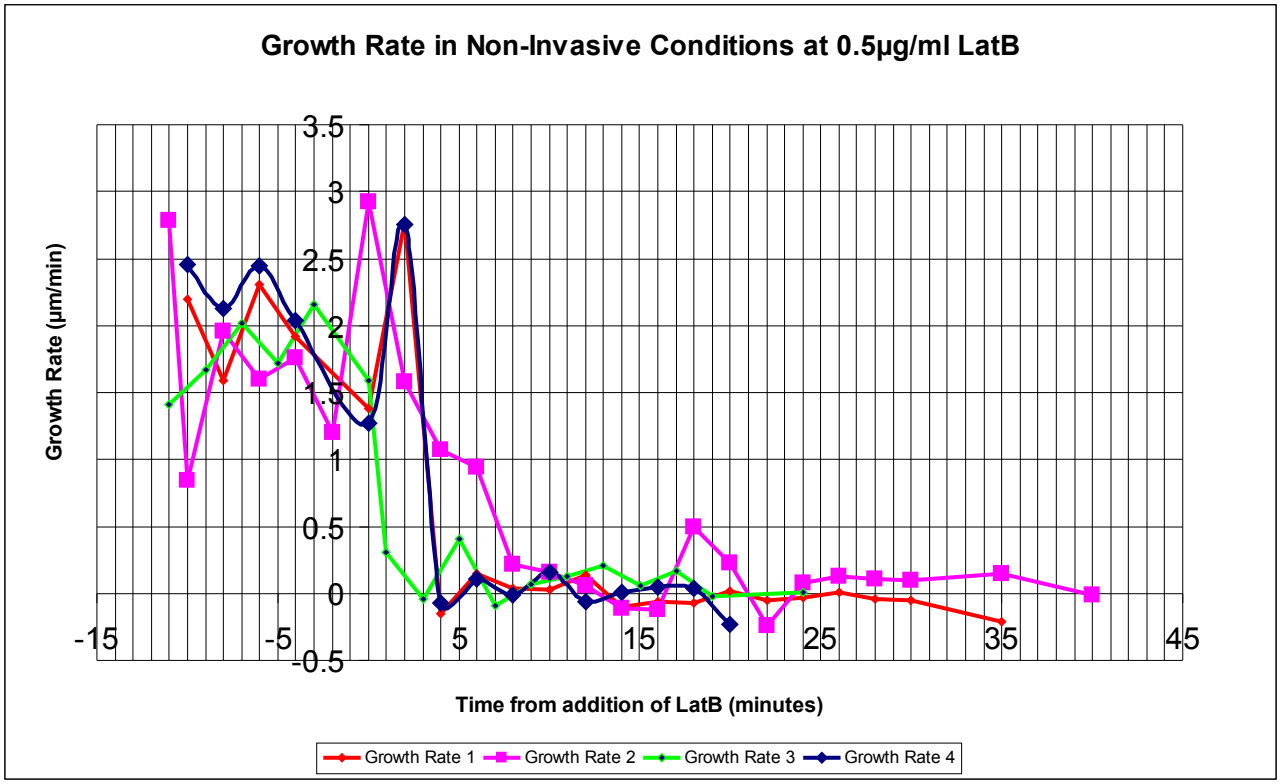

Figure 21: Growth Rates in µm/min vs time for non-invasive hyphen with 0.5µg/ml LatB

Growth rates Invasive and Non-Invasive.

After merging the data sets into two sets Invasive and Non-invasive conditions it can be seen that the non-invasive conditions (Figure 22) have significantly less variation after the addition of Latrunculin B with 5 out of the 8 samples showing remarkably similar profiles. All conditions show considerable variation occurring in growth rates before the addition of Latrunculin B. Also for 7 out of the 8 samples directional growth ceased between 3-4minutes after addition of LatB with out regard to the concentration used. The exception occurring with a hyphae where directional growth ceases approximately 10minutes after the introduction of Latrunculin B.

For the invasive conditions (Figure 23) the profiles are a lot more varied with directional growth ceasing for 6 out of the 8 samples between 6 and 10minutes after the introduction of Latrunculin B. The remaining hyphae cease direction growth between approximately 4 and 30minutes after introduction of Latrunculin B regardless of concentration of Latrunculin B used.

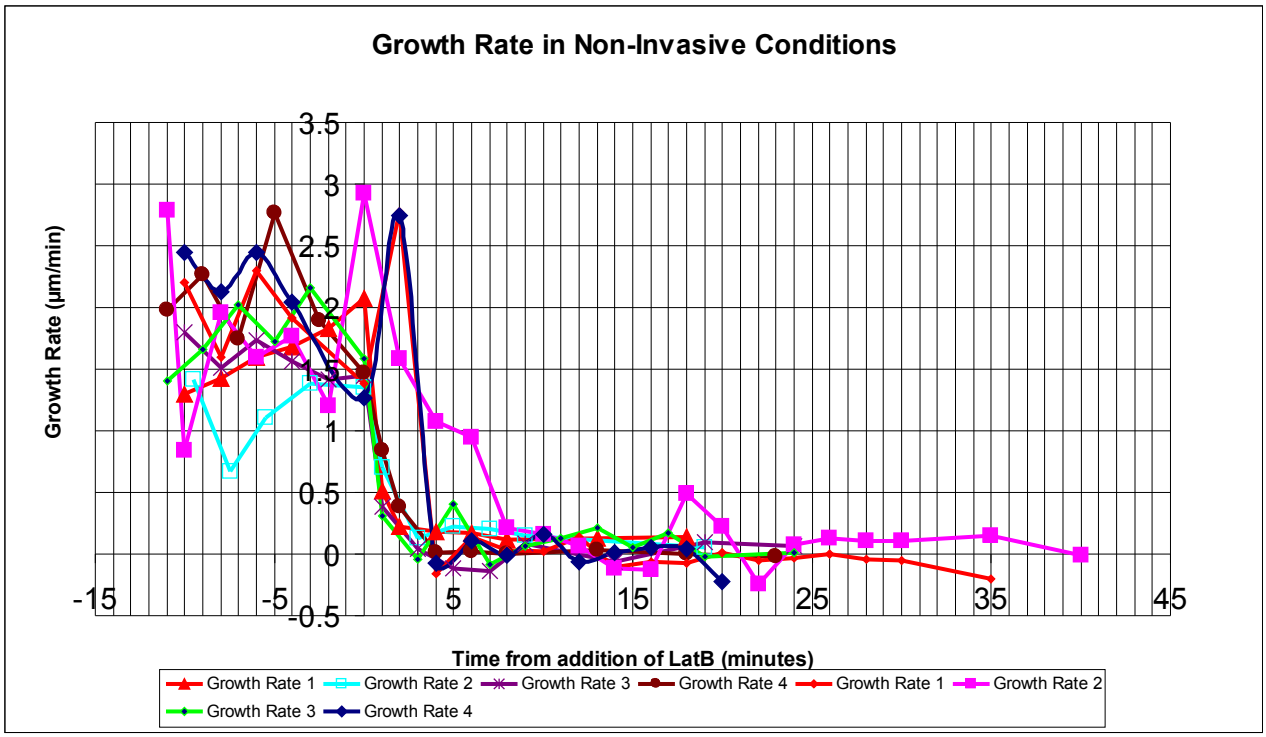

Figure 22: Merged Graph of Non-Invasive Growth rate samples, includes both 5µg/ml and 0.5µg/ml LatB samples.

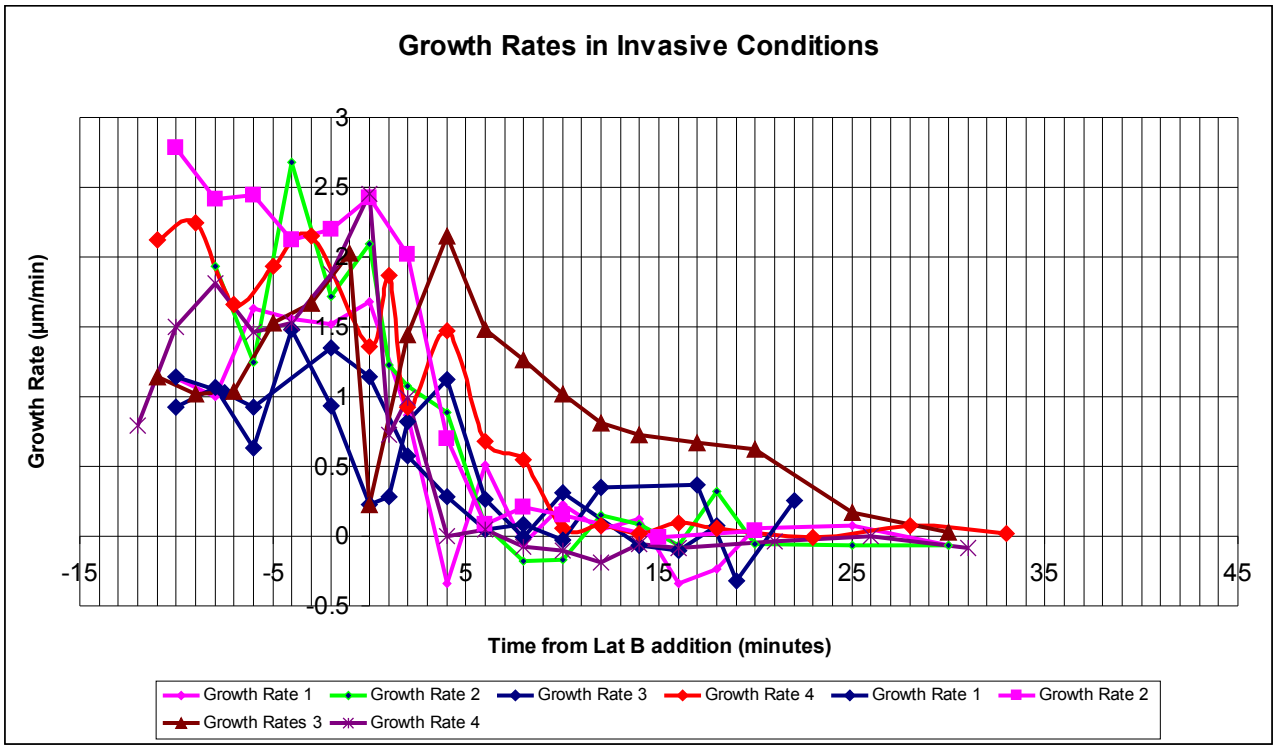

Figure 23: Merged Graph of Invasive Growth rate samples, includes both 5µg/ml and 0.5µg/ml LatB samples.

Growth Rates: Growth

Figures 24and 8 are examples of the affect Latrunculin B has on hyphae in invasive and non-invasive conditions. For both images the star marks the point at which Latrunculin B is introduced to the system. After the introduction of Latrunculin B a rapid change in the shape of the hyphae tip from slightly pointed to rounded, and the cessation of directional growth can be seen. Followed by the apical and sub-apical swelling that replaces directional growth, and the resulting change to the apical cytoplasm as vacuoles, vesicles and organelles accumulate.

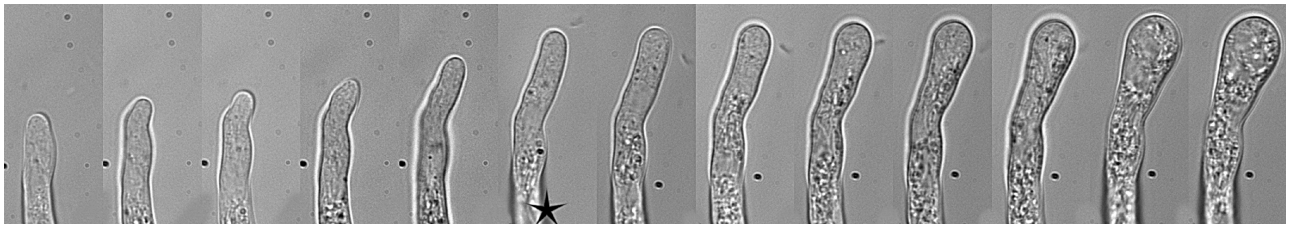

*Figure 24 Sequential images of Hyphae growing in non-invasive conditions before and after the addition of 5µg/ml LatB Star or * indicate the addition of LatB (Times in minutes: 0, 2, 4, 6, 8, 11*, 12, 14, 16, 18, 20, 25, 30)*

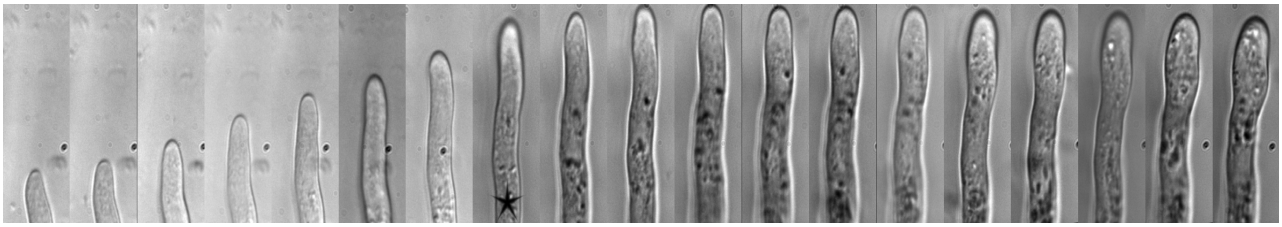

*Figure 25: Sequential images of Hyphae growing in invasive conditions before and after the addition of 0.5µg/ml LatB Star or * indicate the addition of LatB (Times in minutes: 0, 2, 4, 6, 8, 10, 12, 14*, 16, 18, 20, 22, 24, 26, 28, 30, 35, 40)*

Integrin like Protein Experiments

Anti-Integrin β4 stained *Phytophthora cinnamomi*

Phytophthora cinnamomi hyphae in Figure 26 were prepared as described in the method "Integrin-like protein staining" (Page 45) with mouse anti-integrin β4 antibody and stained with goat anti-mouse antibodies tagged with Alexa 488. The hyphae in Figure 3 are a representative sample of the approximately 160 hyphae examined.

The DIC Images for hyphae A, B, D, E, and F (Figure 26) show hyphae with regular cytoplasm in the sub-apical region and the slightly pointed shape tip characteristic of healthy *Phytophthora cinnamomi* hyphae. The hyphae in DIC image C shows somewhat irregular cytoplasm but no gross distortions and has the typical slightly pointed shaped tip of a healthy hypha. Hyphae A and F shows a similar pattern of fluorescence to the controls (Figure 27), with diffuse florescence with out regions of patch staining though out the hyphae and a reduction of florescence in regions where the DIC image shows organelles or vesicles. Hypha E shows a similar diffuse pattern of staining however a couple of small fluorescent patches are also present, located towards the edge of the cytoplasm but not at it. Hyphae B, C and D (Figure 26) all show a variable "patchy" pattern of staining similar but somewhat stronger than that observed in the control image C (Figure 27). The patches are variable in size, number and strength and are generally associated with the centre of the cytoplasm rather than the edge as seen in the integrin plaques in the stained *Achlya bisexualis* hyphae (Figure 28). Hypha B also shows a halo of florescence at the hyphal tip similar to the control hyphae B (Figure 27). This halo is thought to be an artifact generated by anti-fading solution receding from the

hyphae and leaving a halo of solution around it which “bends” or focuses the background florescence at the tip of the hyphae generating the observed halo. In some hyphae (data not shown) florescence seems to be associated with specific organelles or potentially a contaminating organism, such florescence can be significantly brighter to the point where it drowns out the florescence of the hyphae. Addition of kanamycin to the solutions to prevent contamination simply decreased the quality of staining and had no effect on the presence of the contaminate.

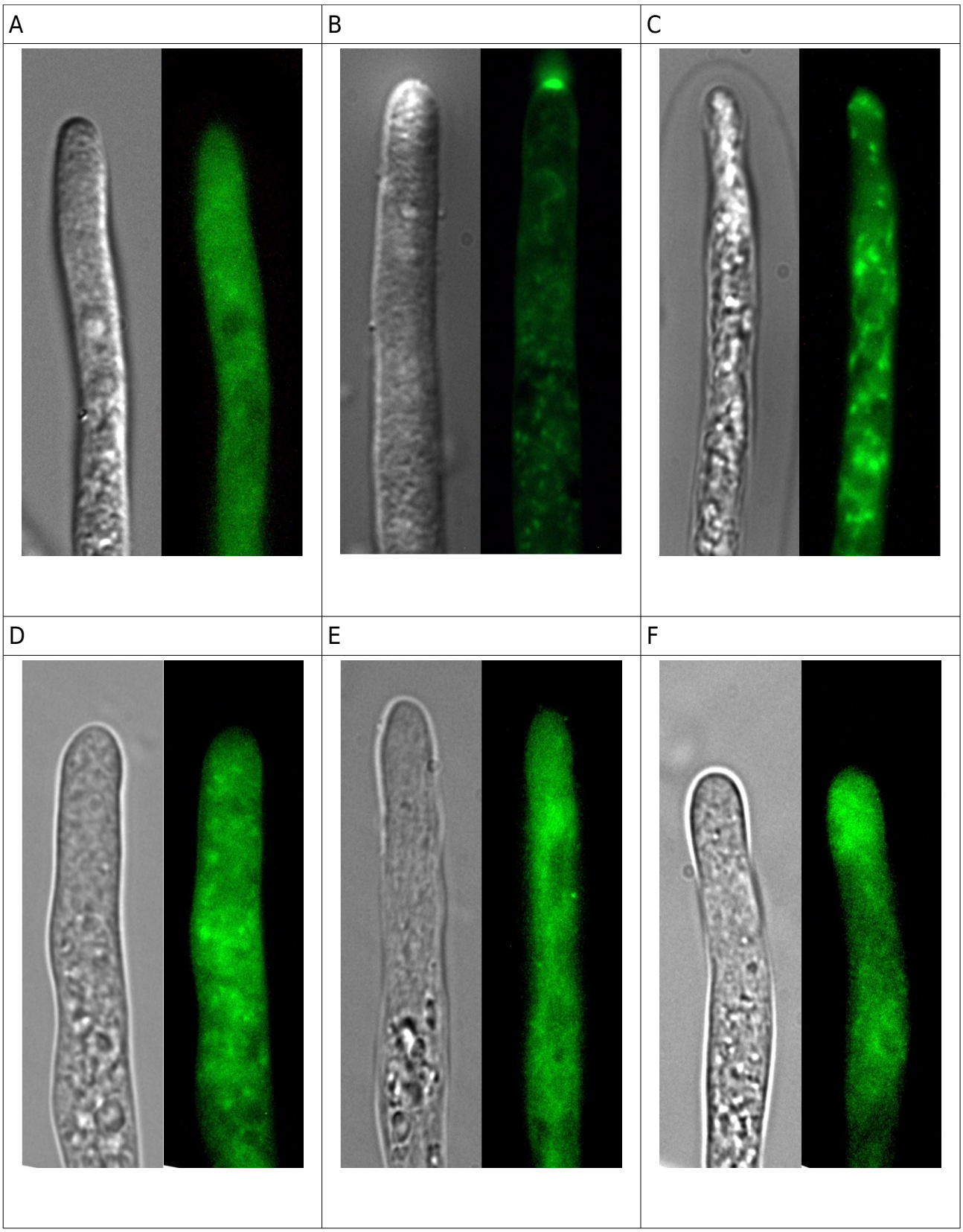

Figure 26: Phytophthora cinnamomi hyphae treated with mouse anti-Integrin β4 antibodies and stained with goat anti-mouse antibodies. Images A-F show the range of observed patterns for stained hyphae. Hyphae A-C are non-invasive, D-F invasive

Controls: Stained *Phytophthora cinnamomi*

Phytophthora cinnamomi hyphae as shown in Figure 27 were prepared following the Integrin-like protein staining method previously described (Page 45) then incubated with secondary Antibody in place of the primary antibody. Images A-F in Figure 1 are a representative sample of the approximately 80 control hyphae observed.

The fluorescent images show the range of different of fluorescence patterns noted in the controls (Figure 27). Hyphae A and D show a weak fluorescence through out the hyphae. Images E and F show a higher level of a similar pattern of fluorescence. Image E does show a certain degree of unevenness in the florescence, with an area of stronger florescence near the apical end of the hyphae. Hyphae B and C show less common patterns of fluorescence observed in the control samples. Hyphae B shows a halo of strong fluorescence at the apex of the cell compared to the rest of the hypha. Hyphae C shows “patchy” fluorescence, with small regions of higher florescence present among the diffuse background florescence. The patches show no specificity and are distributed though out the hyphae, in the high staining of the sub-apical region they are less apparent due to the higher level of fluorescence. Also of note for all the hyphae are the regions of low staining which in hyphae A, B, E and F are associated with the presence of organelles of vesicles in that region as shown in the DIC images.

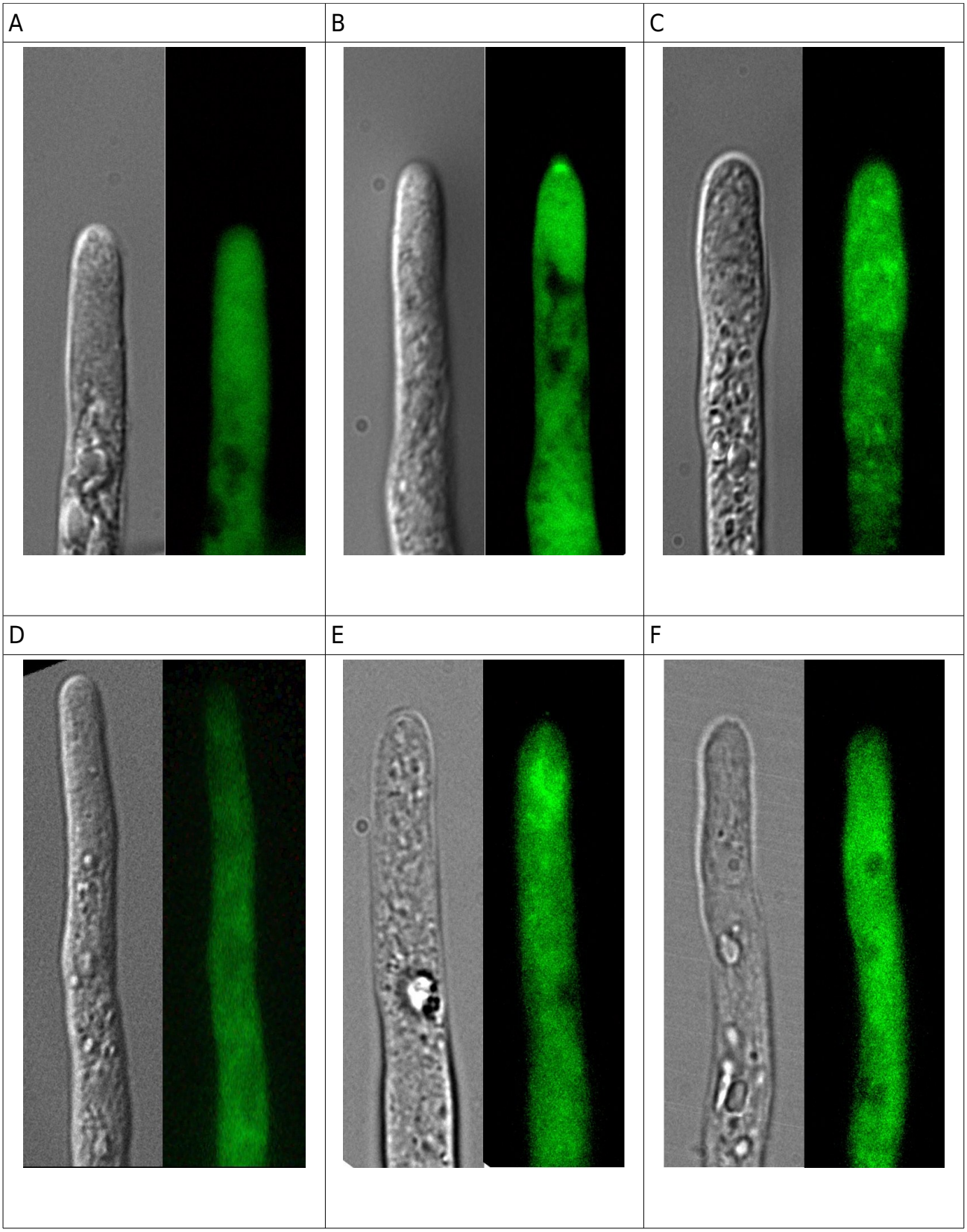

Figure 27: Phytophthora cinnamomi hyphae fixed and incubated with Goat Anti-Mouse secondary antibody,

Controls: Anti-Integrin β4 Stained *Achlya bisexualis*

Achlya bisexualis Hyphae in Figure 28 were treated as described in the Integrin-like protein staining method (Page 45), they are a representative sample of the approximately 15 hyphae observed.

Images A-D (Figure 28) show hyphae treated with mouse anti-integrin β4 antibody and stained with goat anti-mouse antibodies tagged with Alexa 488 florescent dye. Hyphae A shows 2 small stained plaque, indicated by the white markers, one in the sub-apical and one in the basal regions of the hyphae, both are associated with the edge of the hyphae. A high level of diffuse staining is present throughout the sub-apical region of the hyphae, then the diffuse staining decreases in the basal region. The DIC image shows some irregularities in the cytoplasm which has receded from the cell wall at the apex. Image B shows the same hyphae as A at a higher magnification. At the higher magnification hyphae B shows additional plaques compared to A which are also associated with the edge of the cytoskeleton or hyphae, also larger patches of staining can be observed along the periphery of the cytoplasm, and appears to be separate from the diffuse background florescence.

Hypahe C has a low level of diffuse fluorescence throughtout the hypha with small plaques again associated with the edge of the cytoplasm. The DIC image for C shows irregular cytoplasm. Hyphae D also shows the presence of plaques at the edge of the hyphae cytoplasm, and irregularities in the shape and cytoplasm of the hyphae.

Image E shows the basal region of a hyphae with low levels of diffuse fluorescence in the cytoplasm and high levels of clusters of plaques along the edge of the cytoplasm.

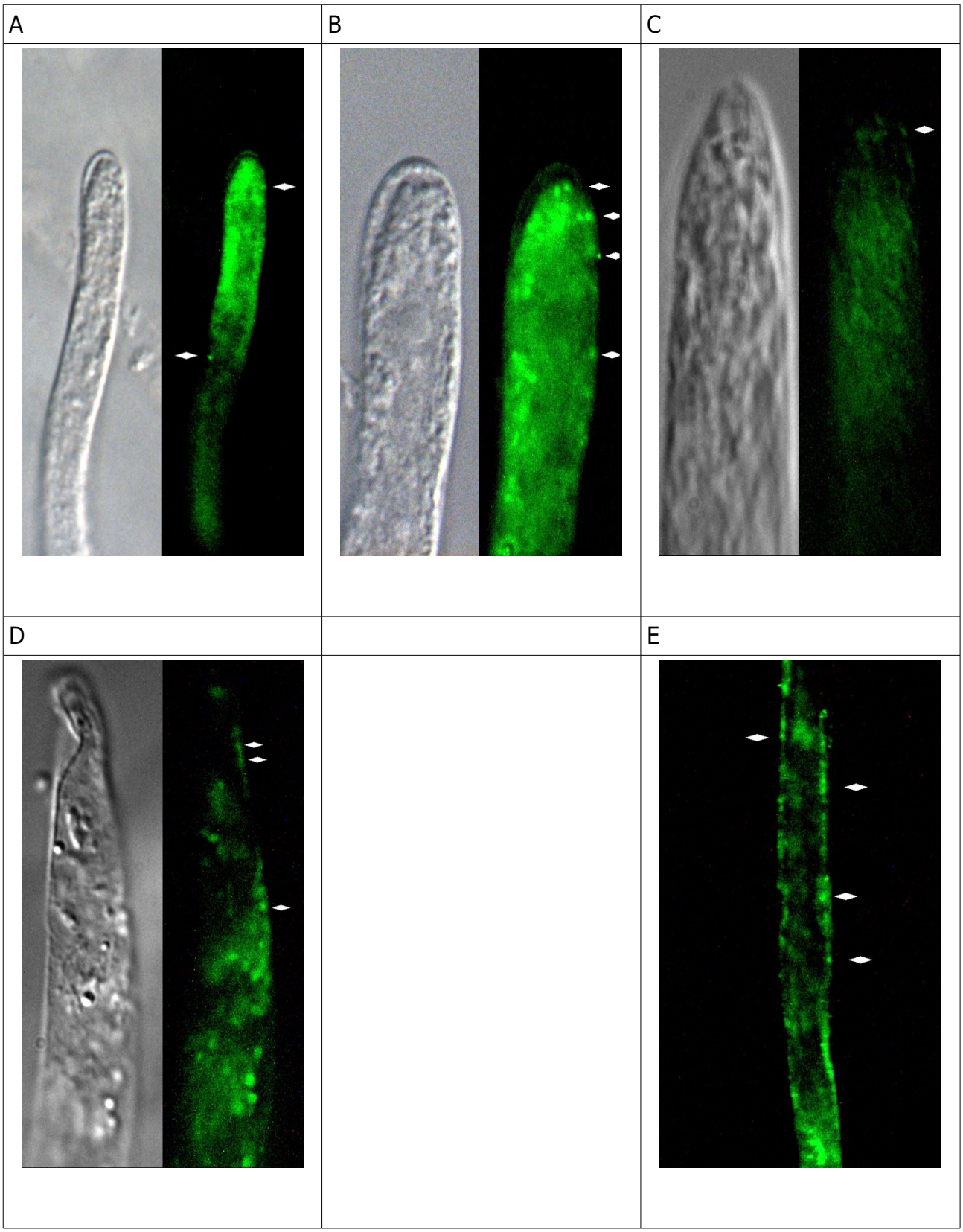

Figure 28: Achlya bisexualis hyphae stained with mouse anti Integrin β4 antibody and goat anti mouse antibody. Images A-D show the tips of stained hyphae. Image E shows the basal region of a stained hyphae White pointers indicate stained integrin plaques

Anti-Integrin β4 Western blots

Western blots were prepared as described in the “Western blot: Integrin like proteins” method (Page 46). Six western blots were prepared from three separate *Phytophthora cinnamomi* protein extractions, Figures 29 and 30 are representative of those blots.

The lanes on the gels are marker (A), soluble fraction (B), soluble fraction x2 (C), pellet (D), pellet x2 (E), pellet x3(F). The SDS-PAGE and Western Blot are from the same gel set.

For Figure 29, the SDS-PAGE, the marker in lane A is clearly visible, and protein bands can be seen for all lanes though for B and D they are faint. For lanes C, E, and F the protein bands are clearer but streaking has occurred. Other SDS-PAGE’s showed both clear bands and lacked streaking.

For Figure 30, the Western blot, the marker is clearly visible (lane A), and a non-specific protein smear can be seen for lanes E and F, with nothing being visible in the other lanes. There is a fair level of noise through out the blot. Other Western blots showed no reoccurring features apart from the marker, and a varying degree of noise.

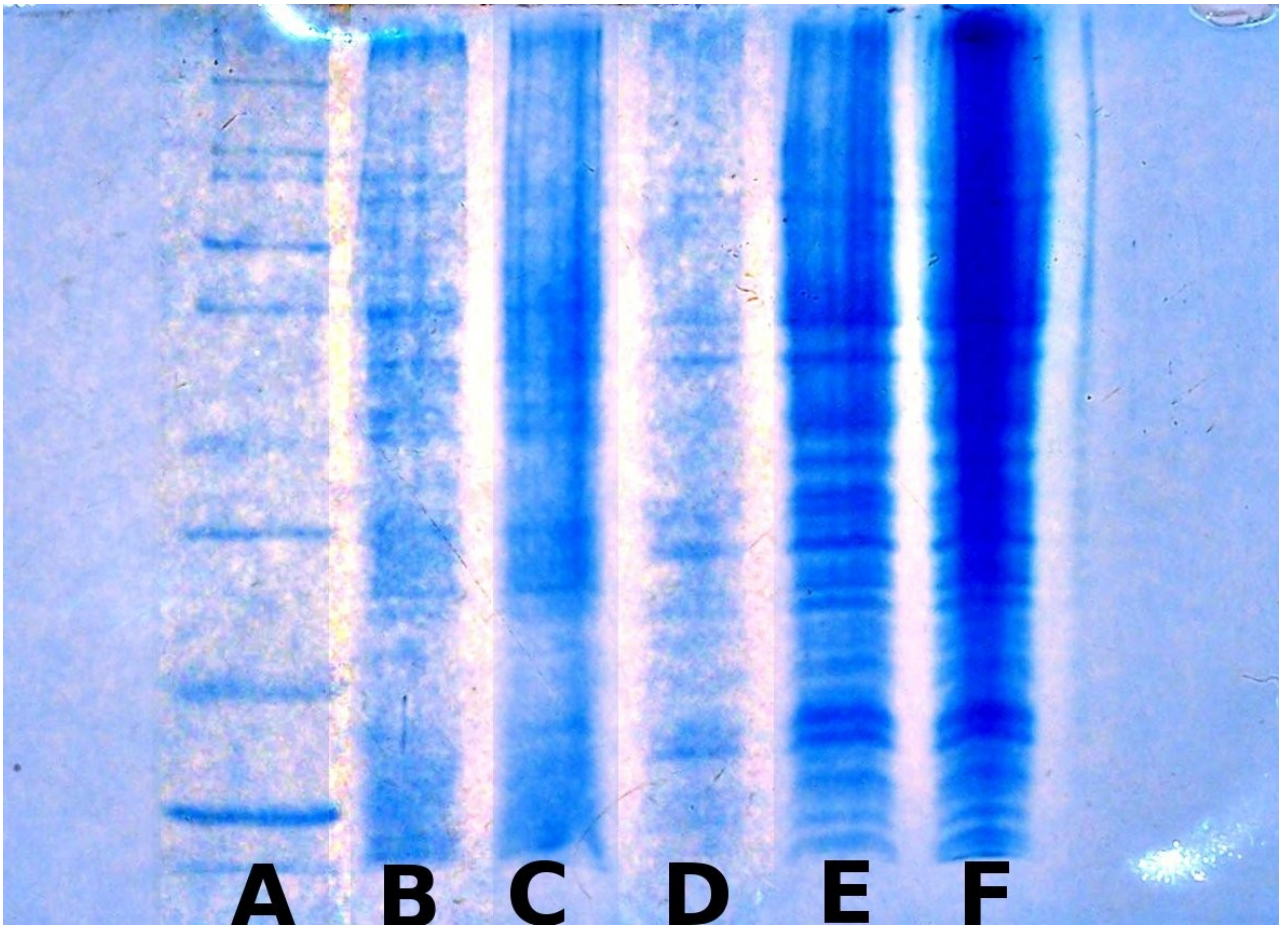

Figure 29: SDS Page of Phytophthora cinnamomi protein extracts. Lane A is the Marker, lanes B-F protein extracts

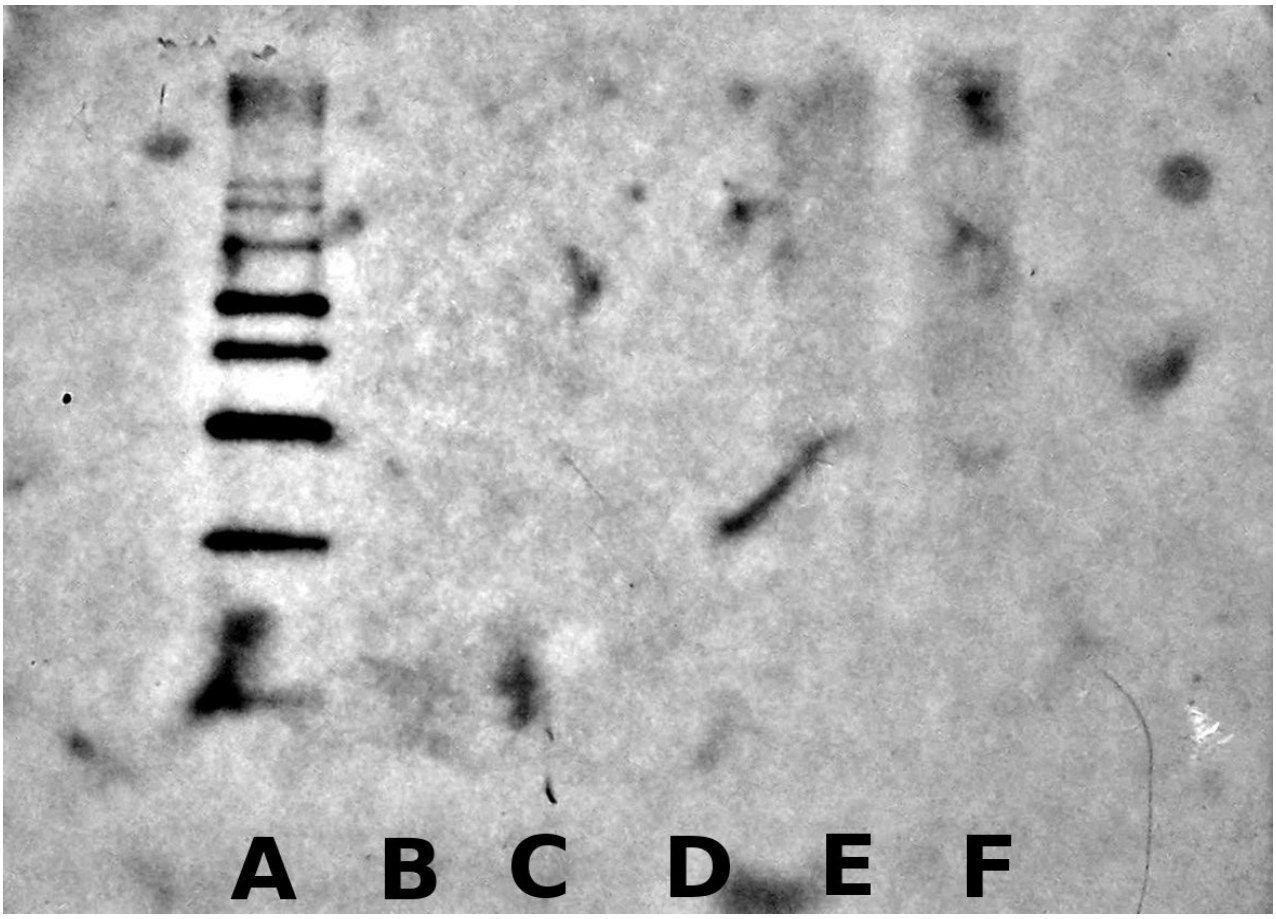

Figure 30: Western of Phytophthora cinnamomi protein extracts incubated with mouse anti-integrin β4 antibody. Lane A is the Marker, Lanes B-F are protein extracts at various concentrations.

Discussion

The effects of Latrunculin B on the actin cytoskeleton.

Interest in the role of actin in tip growth in hyphal organisms has lead to studies examining it in a range of species including but not limited to oomycetes *Saprolegnia ferax* (Gupta & Heath 1997; Heath and Harold 1992; Heath et al 2000; Jackson and Heath 1990), *Achlya bisexualis* (Heath and Harold 1992; Yu et al 2004; Walker et al 2006), and *Phytophthora cinnamomi* (Walker et al 2006; Jackson and Hardman 1998); eufungi *Geotrichum candidum* (Gupta & Heath 1997), *Neurospora crassa* (Heath et al 2000), *Aspergillus nidulans* (Torralba et al 1998); algae *Fucus distichus* (Kropf et al 1989) and yeast *Saccharomyces cerevisiae* (Ayscough et al 1997). These studies and many others have helped provide a solid basis for understanding the roles of F-actin in tip growth, and furthered our understanding of the viability, of the various proposed models of tip growth (Heath and Steinberg 1999; Money 1997).

Actin has been shown to be of critical importance in developing polarisation a critical feature of tip growth. Kropf in 1989 showed that actin disruption by cytochalasin A prevent the formation of the cell axis in the algae *Fucus distichus* (Kropf et al, 1989). In the oomycetes that drug has been used to suggest that F-actin is involved in cleavage of zoospores, as well as the contractile vacuole in *Saprolegnia ferax* and *Achlya bisexualis* (Heath and Harold 1992) as well as *Phytophthora cinnamomi* (Jackson and Hardham, 1998). F-actin has been shown to be required for the localisation of some proteins involved in budding (an analogous process to tip growth) in *Saccharomyces cerevisiae* (Ayscough et al, 1997). F-actin is essential in polarisation and hyphal tip

growth both oomycetes and eufungi (Heath et al 1999; Bachewich and Heath 1998; Torralba 1998) with disruption of F-actin leading to a loss of directional tip growth in hyphae, resulting in apical swelling in eufungi and sub-apical swelling in oomycetes.

In our research disruption of the F-actin by Latrunculin B in *Phytophthora cinnamomi* led to a cessation of directional growth and was followed by sub-apical swelling which correlates with the results from the previously mentioned studies. Disruption of F-actin can clearly been seen when comparing hyphae stained with Alexa Phalloidin in Figures 14, 15, 16, and 17 which had been treated with Latrunculin B at various concentrations, with those from control hyphae in Figures 10 and 11 which were not treated with Latrunculin B (LatB). The treated hyphae clearly show disruption of the distribution of F-actin with loss of the regions of punctate staining and fibrils which can be seen in the control hyphae. The degree of disruption is related to the concentration of LatB used with hyphae treated with higher concentrations showing a greater loss of specific staining and greater swelling. Comparison of the LatB treated hyphae (Figures 14-17) and the Controls (Figures 10 and 11) with hyphae that were treated with only Dimethyl sulfoxide (DMSO) show that the changes in staining patterns and hypha morphology are primarily due to the effect of LatB rather than its carrier, DMSO.

The treated hyphae in Figures 14-17 clearly show sub-apical swelling similar to the sub-apical seen in *S. ferax* (Bachewich and Heath 1998; Gupta and Heath 1997), and *G. candidum* (Heath et al 2003) and related to the apical swelling in *A. nidulans* (Torralba et al, 1998), and *N. crassa* (Heath et al 2000). The process of swelling can be seen in more detail in Figures 24 and 25 which show individual hypha growing over a 30-40minute time period during which LatB is added.

In *S. ferax* (Bachewich and Heath 1998), *A. nidulans* (Torralba et al, 1998), and *G. candidum* (Heath et al 2003) branching and limited growth has been observed during incubation with low concentrations of LatB or cytochalasin A, suggesting alternative means of growth or branching that are not dependent on actin. Neither growth nor branching was observed in the period, 50 minutes, during which *Phytophthora cinnamomi* treated with LatB was observed for. Also plates prepared with an invasive layer of LatB did not grow in 120 hour period (Table 1). This suggests that *Phytophthora cinnamomi* lacks alternative means of branch formation and growth which are not actin dependent. Alternately a longer period may be required for branches and alternate growth mechanisms to take effect. Some organelle or vesicle movement and continued swelling were observed throughout the 50 minutes the hyphae were observed for, which indicates the hyphae were still alive. We did not however attempt to allow recover of growth by replacing the LatB containing broth with fresh untreated broth as has been done in the studies previously listed.

With the loss of directional growth after treatment with the F-actin disruptor and the resulting swelling being observed in a range of hyphal organisms, studies have made some effort to determine what is happening in the hyphae. It is thought that the disruption of F-actin initially prevents the localised or targeted distribution of vesicles containing additional cell wall and plasma membrane materials and enzymes. This initial loss of localised distribution causes the loss in directional growth as the tip runs out of materials to extend the cell wall. After this further disruption to the distribution of F-actin leads to the deactivation of the actin dependent mechanism which prevents the exocytosis of cell wall vesicles in regions other than the hyphal tip. This results in uncontrolled exocytosis of cell wall vesicles in the sub-apical region. The contents of the

wall vesicles includes wall softening enzymes which in conjunction with the additional materials needed for wall growth, weakens the cell wall, expansion or swelling then is driven by turgor which is no longer regulated or resisted by the F-actin (Gupta and Heath 1997). With swelling occurring and F-actin no longer present to regulate or filter movement (Geitmann & Emons 2000), vacuoles, vesicles and some organelles begin to accumulate in the centre of the sub-apical region. This forces organelles such as Golgi bodies, which are usually found centrally in the cytoplasm of the sub-apical region to the periphery of the hypha, as can be seen in the study by Bachewich and Heath (1998). We would suggest that the resulting concentration gradient of vesicles produced by the Golgi bodies would lead to continued rapid exocytosis of wall materials. The continued exocytosis of wall materials allows for continued swelling and leads to localised regions of wall thickening (Bachewich and Heath, 1998).

While our experiments provide no additional insight to such processes, our data and observations could well be explained by such a model. Results from our growth rate experiments Figures 24 and 25 show a build up of vacuoles, vesicles and organelles at the centre of the sub-apical region after the addition of LatB and we observed continued swelling throughout our observational period which continued up to ~40minutes after the addition of LatB. We also note that at least in the observation period the sub-apical swelling was mainly restricted to the region of regular sub-apical cytoplasm. This may correspond with the location of Golgi bodies and does correspond with the region of diffuse stain F-actin fibrils observed in the control hyphae (Figures 10 and 11). The sub-apical region of regular cytoplasm can be observed in living hyphae (Figure 9), the stained control hyphae (Figures 10 and 11), the DMSO controls (Figures 12 and 13) and the time series images of growing hyphae (Figures 24 and 25). This sub-apical region of

cytoplasm is thought to migrate along behind the growing tip leaving behind it a thin layer of cytoplasm and numerous vacuoles, and is considered to be supportive of amebal growth (Heath and Steinberg, 1999).

While swelling in F-actin disrupted hyphae is thought to be driven by turgor pressure and the exocytosis of wall vesicles, there is still debate over the exact role of turgor in normal hyphal tip growth (Money 1997). An Amebae like growth model has been suggested as an alternative (Heath and Steinberg 1999). In the amebae model instead of turgor pressure providing the protrusive force needed to extend the cell wall the extension of F-actin filaments provides the force, this may be supplemented by additional force generated by actin binding proteins acting as motors and pushing the microfilaments forward (Money 1997). Heath and Steinberg (1999) provide a detailed review of the Amebae model while Money (1997) looks at the role of turgor and actin as an alternative source of force.

Related to the amebae model of growth Gupta and Heath (1997) suggested that actin may regulate the effect of turgor on tip extendibility in hyphae with normal turgor and in hyphae with low turgor acts as the source of protrusive force. They proposed this could be demonstrated by disrupting F-actin in a hypha with normal turgor, if F-actin regulated the extendibility of the hyphae this should lead to a transient increase in the growth rate, due to the unregulated turgor pressure driving growth, until it was constrained by a lack of cell wall materials due to disruption of the F-actin. If the F-actin was disrupted in a hyphae with low turgor growth would cease with out a transient increase in the growth rate, as the F-actin would no longer be able to provide the protrusive force for tip extension. This was then experimentally tested in *Saprolegnia ferax* and LatB with the

results confirming their hypothesis (Gupta and Heath 1997). A transient increase in growth rate was observed for hyphae with normal turgor followed by swelling. While for low turgor hyphae the growth rate immediately declined and no swelling was observed due to the lack of turgor pressure.

We also examined the effects of LatB on the growth rate of *Phytophthora cinnamomi* in both invasive and non-invasive conditions. Due to technical limitations we were limited to imaging the hyphae at one or two minute intervals, this results in a much coarser measure of growth rate changes than the 10second intervals managed by Gupta and Heath (1997). This coarser measurement of growth rate combined with a high degree of variability in the growth rate of *Phytophthora cinnamomi* in the conditions used, means we are unable to be as conclusive as in their study. Of the 16 hyphae observed (Figures 18-23) 5 hyphae showed a detectable increase in growth rate in the two minute period between the addition of LatB and the first image. Two hyphae grown in invasive conditions, and two in non-invasive conditions that were treated with 0.5ug/ml LatB and one invasive hyphae treated with 5ug/ml LatB. A number of hyphae showed an increase in growth rate at the addition of LatB which may have occurred in the 10 or so seconds required to take the image of the hyphae immediately after the addition of LatB. The observed increases are usually within the bounds set by the variability in growth rate observed for the ~10minutes before addition of LatB or close to the trend in growth before addition of LatB. Unfortunately this makes it difficult to determine from the collected data if this was a LatB induced increase in growth rate or simply due to the standard variability present *Phytophthora cinnamomi's* growth rate. While 5 hyphae do appear to show a transient LatB induced increase in growth rate the large 1 to 2 minute period over which the growth rate was measured makes it hard to say how significant or common this is. Further samples and smaller time period between measurements

would be needed to determine what exactly is occurring in *Phytophthora cinnamomi* tip growth immediately after the addition of LatB.

Recent studies in this lab utilising and improved fixative (Yu et al 2004) have identified an actin depleted zone in the F-actin cap present in oomycetes. This has been identified in two oomycetes, initially in *Achlya bisexualis* (Yu et al 2004) and more recently in *Phytophthora cinnamomi* (Walker et al 2006). An actin depleted zone had only been observed prior to that in had primarily been observed in pollen tubes, and once in oomycetes in a study where there was some concern that not all the F-actin had been stained due a lack of F-actin plaques being stained (Jackson and Heath 1990). In the study by Walker et al (2006) it was reported that the actin depleted zone was observed in 74% of invasive hyphae and 20% of non-invasive hyphae. It was suggested that this actin depleted zone may increase the protrusive force in the absence of an increase in turgor. As well as allow non-invasive growth at very low levels of turgor (Walker et al 2006).

In this study we observed the actin depleted zone in both invasive and non-invasive hyphae stained with Alexa Phalloidin (Figures 10 and 11). The actin depleted zone was not affected by DMSO (Figures 12 and 13) but was disrupted by all concentrations of LatB that were used (Figures 14-17). Based on our observations we would also suggest that for healthy non-invasive hyphae, with a tapered point and no noticeable cytoplasmic anomalies compared to living hyphae, the actin depleted zone was more common than the 20% of hyphae previously suggested (Walker et al 2006).This observed difference in the presence of the actin depleted zone in non-invasive hyphae, is potentially a result of our larger sample size for non-invasive hyphae (n=30)

compared to (n=15) used in Walker et al (2006). It may also partly be the result of the use of slightly different criteria for determining what healthy growing hyphae are and the difference in recovery period for our experiments, we allowed 2 hours and 30 minutes for the hyphae to recover in broth, after being cut, compared to the 1 hour recovery period used by Walker et al (2006).

Integrin like proteins in *Phytophthora cinnamomi*

In animal cells integrins are one of the most common and important classes of transmembrane receptors. They are also one of the primary structures for connecting the cytoplasmic skeleton to the external environment or extra cellular matrix. In the plasma membrane they form focal adhesions complexing with a variety of proteins that act to amplify transmembrane signals and provide anchorage for the cytoskeleton. The focal adhesions they form are thought to be critical components of amebal movement, by anchoring the cytoplasm to the external environment they allow force to be generated against the cytoskeleton for movement with out the risk lysing the cell.

As previously mentioned an amebal like model has been suggested for tip growth in plants and hyphal organisms (Heath and Steinberg 1999). If such a model is correct then a protein or a protein complex would be required to fill the transmembrane connective role of integrins in animals. Such a protein would need to provide a link between the cytoskeleton and the external environment, which in the case of plant and hyphal cells is the cell wall, to anchor the cytoskeleton. Anchor the cytoskeleton would allow the force needed for tip growth to be generated against the cytoskeleton without risking cell integrity.

Various studies utilising a number of integrin specific antibodies have shown that proteins epitopically similar to animal integrins are present in a range of organisms which utilise tip growth, including but not limited to the eufungi, *Neurospora crassa* (Degousee et al 2000); plants *Arabidopsis* (Swatzell et al 1999), *Lilium davidii, and Nicotiana tabacum* (Sun et al 2000); and oomycetes *Saprolegnia ferax* (Kaminskyj and Heath 1995), and *Achlya bisexualis* (Chitcholtan & Garrill 2005). A range of antibodies specific to various integrin subunits have been used targeting the subunits β_1 (Kaminskyj & Heath 1995; Degousee et al 2000; Sun et al 2000) α_V and β_3 (Sun et al, 2000), and β_4 (Chitcholtan & Garrill 2005). The studies have generally shown that these integrin like proteins (Ilps) are present at the apex of the cell and in some species also present in the sub-apical region (Degousee et al 2000).

In this study we examined Phytophthora cinnamomi for the presence of a β4 integrin like

protein utilising immunoflorescent microscopy and western blots. For Phytophthora cinnamomi we observed a range of florescent patterns in both the treated hyphae (Figure 26) and the control hyphae (Figure 27). Comparison of the control hyphae and the stained hyphae reveal no unique patterns of florescence in the stained hyphae. The most obvious pattern, the “patchy” staining visible in Figure 26 B, C, and D, is also present in the control hyphae Figure 27. The pattern of florescence also varies noticeable in appearance and location in the hypha compared to the staining pattern for β4 stained Achlya bisexualis hyphae Figure 28 C), the β4 staining pattern for Achlya bisexualis shown by Chitochalan and Garrill (2005), and those seen in the literature for other integrin like proteins (Degousee et al 2000; Kaminskyj & Heath 1995).
Western blots for a β4 integrin like protein were also prepared. However while the SDS-

PAGE gels showed, reasonable to good separation of proteins (example, Figure 29), and proteins were shown to have transferred to the membrane no response was observed on the developed blots (Figure 30).

Based on our data we suggest that β4 integrin like proteins are not present in the hyphae of *Phytophthora cinnamomi* although we advise caution as absence of evidence does not mean evidence of absence. Also this does not rule out the presence of integrin like proteins in *Phytophthora cinnamomi*. As is the case in animals a range of integrin like proteins have been observed in plants and hyphal organisms as we have mentioned previously. Also the exact range of integrin like proteins present in an organism has been shown to vary in plants. Sun et al (2000) found that α_V and β_3 integrin like proteins were strongly expressed in the tips of pollen tubes in *Lilium davidii* while α_V and β_1 were strongly expressed in the pollen tubes of *Nicotiana tabacum*. Thus we think that it is reasonable to suggest that differing integrin like proteins are likely to be present in oomycetes and that it is likely which ones are present and expressed is likely to vary especially between more distantly related oomycetes such as *Phytophthora cinnamomi* and *Achlya bisexualis* (Cooke et al 2000) that target different niches in the ecosystem.

Conclusion

In conclusion our study of the role of F-actin in tip growth and the effects of Latrunculin B on it in *Phytophthora cinnamomi* show that it is similar to that observed in other hyphal organisms. With F-actin being critical to the maintenance of directional tip growth in the organism and that the disruption of the F-actin elements, present in the hypha, causes rapid cessation of growth and sub-apical swelling. We also note that in the time we observed the treated hyphae they did not activate an alternative means of growth or branching that isn't dependent on F-actin as had been seen in other hyphal organisms. We have also shown that no β_4 integrin like protein is present in *Phytophthora cinnamomi* though the presence of some other integrin like protein is in no way ruled out by these experiments.

Overall our study has help increase our understanding of tip growth in Phytophthora cinnamomi.

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