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**University of Birmingham**

**School of Biosciences**

Investigation of Gibberellin Signalling and the Effect of *N*-acyl Homoserine Lactones  
on Germination in *Physcomitrella patens* and an Investigation of the Interaction  
between PGE<sub>2</sub> and Phospholipase B1 in a *Cryptococcus*/Macrophage Infection  
Model

A Research Thesis Submitted by:

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*For the degree of:*

**MRes in Molecular and Cellular Biology**

*Under the Supervision of:*

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*And*

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August 2014

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This project consists of two research projects undertaken over the course of one year.

Part one of this thesis looks at gibberellin signalling in the early land colonising plant *Physcomitrella patens* and the effect of bacterial quorum sensing molecules, *N*-acyl homoserine lactones, on germination. Gibberellins are a crucial plant growth regulator that controls germination as well as other important processes. To date, no gibberellins have been identified in the moss *Physcomitrella* but an important precursor in the gibberellin biosynthesis pathway, *ent*-kaurene, is present in large quantities. Using a strain that lacks the synthase for this protein we have studied the germination rate compared to the wild type. Further to this AHLs have been previously identified as having diverse effects on plants including root architecture. We have examined the effect of different chain length and substitution states of AHLs on *Physcomitrella* germination rates.

Part two of this thesis looks into host-pathogen interactions of the fungal pathogen *Cryptococcus neoformans*, an opportunistic pathogen that can be life threatening to individuals with HIV/AIDS. This project looks into the role of eicosanoid production by the fungal pathogen on characteristics associated with macrophage infection. PLB1 is involved in virulence traits of *Cryptococcus* including cell wall stability, intracellular survival and dissemination as well as prostaglandin production. We have studied how this effects pathogen-macrophage interactions including intracellular proliferation rates, phagocytic uptake and cell size with the addition of exogenous PGE<sub>2</sub>.

## **Abstract- Part One**

Investigation of Gibberellin Signalling and the Effect of *N*-acyl Homoserine Lactones on Germination in *Physcomitrella patens*

Supervisor: Dr Juliet Coates

The emergence of land colonisation and multicellularity represent two key events in plant evolution and study into these mechanisms could potentially lead to better manipulation of plants for food production and biofuels. This study investigates the role of hormones and bacterial signals on early land plant germination. Gibberellins (GA) are plant hormones important in seed plants for germination but their role in the moss species *Physcomitrella patens* is still controversial. GA is synthesised via *ent*-kaurene found in *Physcomitrella patens* in large quantities. Using a mutant strain lacking the *ent*-kaurene synthase we have looked at the effect on germination rates. Furthermore, *N*-acyl homoserine lactones (AHLs) are bacterial quorum sensing molecules shown to affect root architecture in *Arabidopsis*. Growing *Physcomitrella* in the presence of AHLs we studied the effect on germination. Moss that lacked the *ent*-kaurene synthase germinated slower than wild type spores but to the same potential. Additionally, long-chain AHLs (C8-C12) promoted spore germination regardless of substitution states. We have concluded that gibberellin signalling in moss affects germination and that *Physcomitrella* can react to *N*-acyl homoserine lactones produced by soil bacteria.

### **Key Words:**

*N*-acyl homoserine lactones  
*Physcomitrella patens*  
*ent*-kaurene  
Germination  
Quorum Sensing

## Abstract- Part Two

Investigation of the Interaction between PGE<sub>2</sub> and Phospholipase B1 in a *Cryptococcus*/Macrophage Infection Model

Supervisor: Professor Robin May

*Cryptococcus* is a life-threatening intracellular fungal pathogen in immunocompromised individuals. Phospholipase B1 has been identified as an important virulence factor in *Cryptococcus* and is critical for dissemination and intracellular survival. Phospholipases also free lipid mediators, such as arachidonic acid, a precursor used to synthesise eicosanoids, including prostaglandins, which *C. neoformans* makes endogenously. The *Cryptococcus plb1* knockout produces significantly less prostaglandins than the wild type and is characterised by a reduced intracellular proliferation rate and cell size increase over 18 hours. Using the murine macrophage-like model cell line, J774, with *C. neoformans* and a knockout strain, *plb1* and a reconstructed PLB1 strain, *plb1<sup>rec</sup>*, PGE<sub>2</sub> was added during infection and we looked into the effect on the intracellular proliferation rates as well as other characteristics including cell size, phagocytic uptake and viability. We found that by adding 0.6 ng/ml PGE<sub>2</sub> during infection we could rescue the IPR of the *plb1* mutant to that of the wild type and *plb1<sup>rec</sup>* strain and increased intracellular survival of *plb1* over 18 hours. PGE<sub>2</sub> is important for *Cryptococcus* proliferation inside macrophages, but does not rescue the cell size increase normally seen in the *plb1* mutant.

### Key Words:

PGE<sub>2</sub>  
phospholipase B1  
*Cryptococcus neoformans*  
prostaglandins  
eicosanoids

## **Acknowledgments**

I would like to thank Dr Juliet Coates and Professor Robin May for their supervision during these projects, along with their labs and especially Eleanor Vesty and Robert Evans for their help and guidance during this research.

I would also like to thank my housemates and course members Will Hughes, Emily Dixon, Izzy Davis, Amy Whitbread and David Hardy for their help and friendship during this project.

I would also like to thank my family for constantly supporting me emotionally and financially during my studies.

## Abbreviations- Part One

ABA: Abscisic Acid

AHL: *N*-acyl homoserine lactones

C10-HSL: *N*-Decanoyl-L-homoserine lactone

C12-HSL: *N*-Dodecanoyl-L-homoserine lactone

C4-HSL: *N*-Butyryl-L-homoserine lactone

C6-HSL: *N*-Hexanoyl-L-homoserine lactone

C8-HSL: *N*-octanoyl-L-homoserine lactone

CCD: Carotenoid Cleavage Dioxygenase

CPS/KS: *ent*-kaurene synthase

CPS: *ent*-copalyl diphosphate synthase

CTL: Control

*Ent*-K: *ent*-kaurene

GA: Gibberellic Acid

GA<sub>9</sub> ME: GA<sub>9</sub> Methyl Ester

KS: *ent*-kaurene synthase

NAE: *N*-acylethanolamides

OH: Hydroxy Group

OXO: Ketone Group

PGR: Plant Growth Regulator

QQ: Quorum Quenching

QS: Quorum Sensing

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

SEM: Standard Error of Mean

WA: Water Agarose

WT: Wild Type



## **Abbreviations- Part Two**

AA: Arachidonic Acid

Ab: Antibody

APP1: Antiphagocytic Protein 1

CSF: Cerebrospinal fluid

EET: Epoxyeicosatrienoic

EP: E-Prostanoid

FBS: Foetal Bovine Serum

GPCR: G-protein Coupled Receptor

IFN: Interferon

IL: Interleukin

IPR: Intracellular Proliferation Rate

LPS: Lipopolysaccharide

NSAID: Non-steroidal Anti-inflammatory Drugs

PAMP: Pathogen Associated Motif Pattern

PBS: Phosphate Buffered Saline

PG: Prostaglandins

PI: Propidium Iodide

PKC: Protein Kinase C

PMA: Phorbol myristate acetate

PRR: PAMP recognition receptors

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

Th1: T-helper 1

Th2: T-helper 2

TLR: Toll like receptor

TNF: Tumour Necrosis Factor

WT: Wild Type

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# **Part One: Investigation of Gibberellin Signalling and the Effect of *N*-acyl Homoserine Lactones on Germination in *Physcomitrella patens***

## **1.1 Overview**

Green plants are used directly and indirectly for almost everything; climate mitigation, atmospheric oxygen, fuel, food and fabrics. A growing population and climate change is leading to a reduction in arable land and food security is now one of the biggest issues facing the world today. The Earth's population is predicted to rise to 9 billion by 2050 and food production will have to double to cope with this increase (www.fao.org). To tackle this issue will require the collaboration of research from many areas both to increase yields and reduce loss from disease and pests. For instance, mosses are highly plastic and are able to survive in diverse habitats from deserts to the Antarctic (Prigge and Benzanilla 2010)(Patiño *et al.* 2008). Understanding how this occurs may mean we can apply this to crop plants so they can grow in areas they normally would not.

All plants reproduce via desiccation-resistant structures, either seeds like angiosperms or spores like the bryophytes and pteridophytes (Patiño *et al.* 2014). From these structures new plants germinate to bring about the next generation. The environmental and hormonal control for the germination in seeds has been well studied but many factors remain unknown. Biosynthesis pathways of hormones controlling germination, gibberellins and abscisic acid, have been identified but the downstream regulation of these systems requires further study (Nonogaki *et al.* 2010) and even less is known about germination in spores.

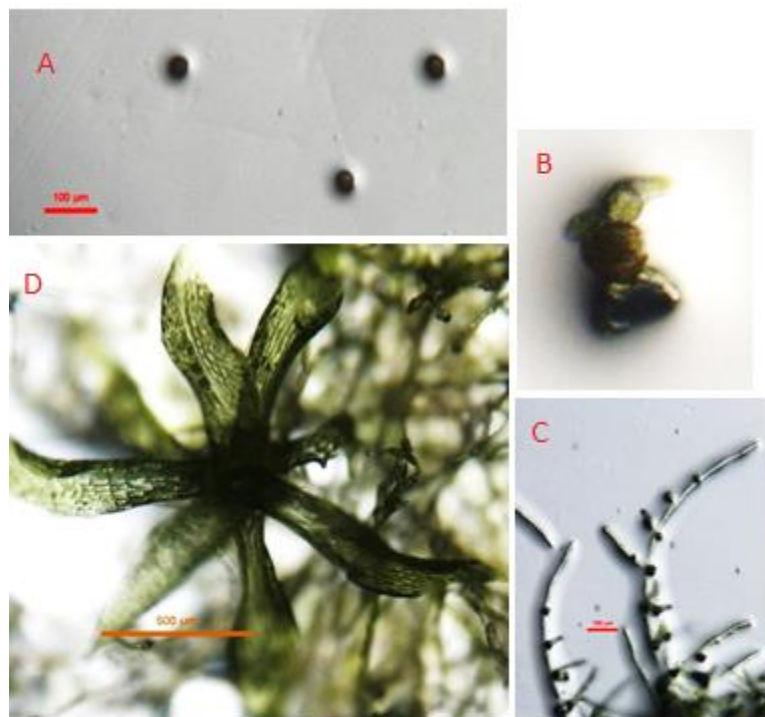
*Physcomitrella patens*, a member of the bryophyte family, illustrates the earliest land colonising plant that diverged before the evolution of vascular plants around 470 million years ago (Bonhomme *et al.* 2013) and evolutionarily bridges the gap between marine algae and higher plants (Zimmer *et al.* 2013). The jump to land colonisation was a massive turning point in plant evolution and led to increased complexity. By increasing our knowledge of the basic biological processes in the plants around us we are more likely to be able to use these for agricultural success in the future.

Since the sequence of *Physcomitrella patens* was published in 2008 (Zimmer *et al.* 2013)(Rensing *et al.* 2008) there has been much more interest in this organism and has allowed for further study. This study focuses on the germination control in the bryophyte, *Physcomitrella*, focusing on the role of gibberellins and precursors of the gibberellin biosynthesis pathway. Further to this, the project also focuses on the effect of bacterial signals on *Physcomitrella* germination. *N*-acyl homoserine lactones have been shown to have various effects on plants including changes in protein regulation (Rad *et al.* 2008) and root architecture (Ortiz-Castro *et al.* 2008).

## **1.2 *Physcomitrella*: A Model Organism**

The early divergence from vascular plants makes *Physcomitrella* a useful tool in plant evolutionary studies. Its simple morphology and life cycle make it ideal for laboratory experiments and many plant growth regulators (PGRs) are conserved in both mosses and angiosperms plants including auxin, ABA and cytokinins (Sabovljević *et al.* 2014)(Schaefer and Zryd. 2001)(Ross and Reid. 2010), which are important in

controlling plant development. Mosses have a simple architecture; their leaves are only a single cell thick, thus not requiring stomata and gaseous exchange occurs via diffusion (Cove *et al.* 2005). Mosses produce spores instead of seeds. Unlike angiosperms, mosses spend the majority of their life cycle in a haploid state rather than diploid. This makes *P. patens* ideal for genetic studies as there is only one copy of a gene to knockout so mutants are relatively simple to make (Cove *et al.* 2005). Targeted mutagenesis has been used so successfully in bacteria and yeast is also



**Figure 1. The life stages of *Physcomitrella patens* from spores to gametophyte production. A.** Un-germinated spores on BCD media. **B.** Newly germinated spore. **C.** Filamentous growth of caulonemal cells. **D.** Leafy gametophyte structure. Images produced by Sarah Needs. (2013).

possible in *Physcomitrella* (Schaefer. 2002); this is unique amongst land plants and targeted mutagenesis in higher plants remains problematic (Osakabe *et al.* 2010).

When spores germinate protonema filaments are produced, initially consisting of chloronemal cells as shown in Figure 1B. These cells are so named because they contain a high number of mature chloroplasts (Cove *et al.* 2005). Some of these cells differentiate into caulonemal cells which contain less chlorophyll and undergo diagonal division (Cove *et al.* 2005). Small buds branch from the caulonemal cells which eventually become the leafy gametophyte (Figure 1d). *Physcomitrella* are monoicous and have separate male and female organs within the same plant, producing antheridia and archegonia respectively (Cove *et al.* 2005). Instead of pollen mosses produce motile, flagellated spermatozoids. When water splashes onto the moss the spermatozoids can swim and fertilise the female organ. Self-fertilisation is the main way *Physcomitrella* produces diploid sporophytes, which goes onto then produce the thousands of haploid spores by meiosis which are held in the sporophyte (Cove *et al.* 2005)(Prigge and Benzanilla. 2010). Sporophyte formation is induced by low temperatures and short days (8:16 light: dark; Cove *et al.* 2005). Once mature sporophytes are formed they 'plump up' and turn a dark brown colour and eventually burst to release spores; for the following experiments intact sporophytes were removed from the plant and allowed to air dry before use in experiments.

Seeds and spores can be seen as analogous organs and share key similarities; they are both desiccation resistant structures and the structure from which the embryo emerges, however, they are developmentally different and arise at different points in the life cycle. Further, spores are haploid and produced via meiosis events in the sporophyte and seeds are diploid.

### 1.3 Germination Control

The mechanisms controlling seed germination are many and complex; some of these are environmental, such as water, light and temperature, ensuring seeds germinate when environmental conditions are favourable. There are also intrinsic factors, phytohormones. (Heddon and Phillips 2000)(Holdsworth *et al.* 2008). Phytohormones are essential for correct growth, development and communication in the developing and mature plant and many are highly conserved within the plant kingdom.

Timing of when seeds 'decide' to germinate is crucial for correct development and survival and therefore subsequent reproduction. The two main pathways that have been identified in controlling seed germination are those of gibberellic acid (GA) and abscisic acid (ABA). These hormones act antagonistically, GA promoting seed germination and ABA suppressing it (Hirano *et al.* 2008)(Sakata *et al.* 2010). Crosstalk between these hormones is important; an increase in GA is associated with a decrease of ABA (Xi. *et al.* 2010). Although ABA is best known for its importance in seed dormancy, a period when seeds do not germinate whether or not environmental conditions are favourable (Penfield *et al.* 2012), it is also present in moss (Anterola *et al.* 2008)(Sabovljević *et al.* 2014). However, it has not been verified whether spores have a formal dormancy period like seeds. When spores are shed, providing conditions allow, they will most likely germinate (Glime 2007). ABA was first found to be associated with leaf abscission, although it is now known this is only a minor role. ABA inhibits seed germination by blocking the uptake of water (Manz *et al.* 2005). Water uptake during germination occurs in three phases, a rapid initial uptake of water

is followed by a levelling off. After germination is complete, i.e. the radicle has pushed through seed coat, a third phase of rapid water uptake occurs. ABA inhibits this third phase of water uptake (Manz *et al.* 2005). The effect of ABA has been studied in mutant systems. An ABA lacking mutant maize produces seeds that germinate while still attached to the plant, a process called vivipary (Raven *et al.* 1999). This is undesirable on crop plants and it means the seed cannot be harvested or used the following year. ABA is also involved in stress tolerance, including freeze tolerance (Cove. 2006) and desiccation resistance. As early land plants mosses would have faced a myriad of stresses, one of the main being desiccation due to separation from its water source. ABA could be an early desiccation tolerance control in seedless plants and a conserved signal that has evolved different uses like the stabilisation of seeds.

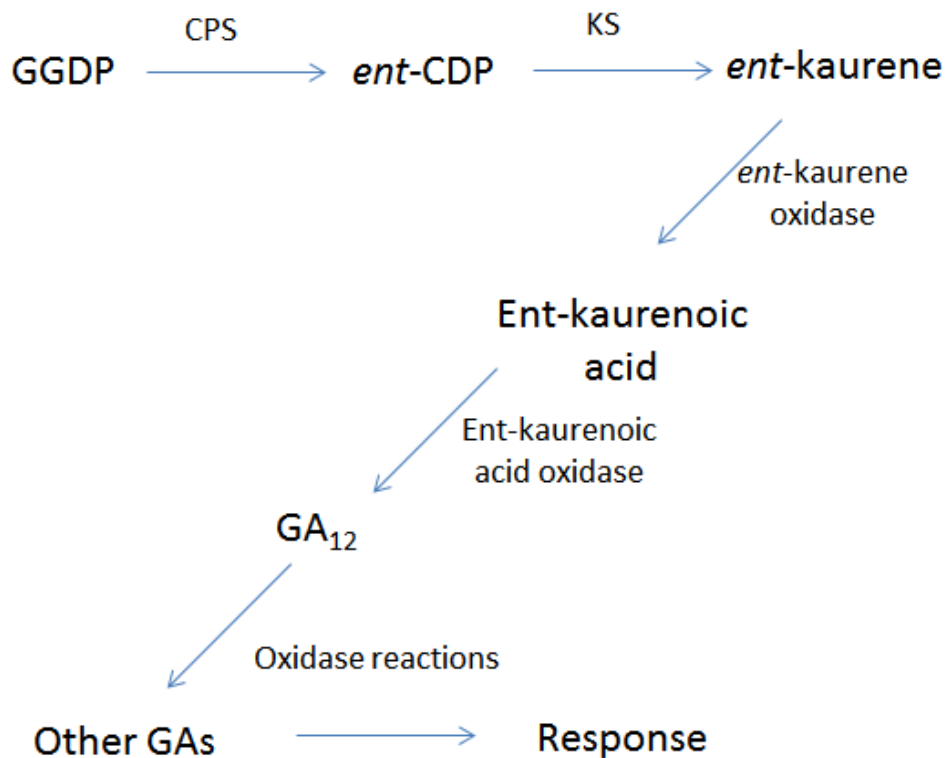
Gibberellins are tetracyclic diterpenes that control a number of different responses in plants including germination and cell elongation (Ross and Reid. 2010). The first GA to be identified was from the pathogenic fungus, *Gibberella fujikuroi*, responsible for 'foolish seedling disease' in rice that caused huge amounts of stem elongation (Eckardt 2002). To date around 126 gibberellins have been identified in plants, bacteria and fungi (Gomi and Matsuoka 2003). Most have no known function and few have shown biological activity in plants aside from GA<sub>3</sub>, GA<sub>4</sub> and GA<sub>1</sub> (Hua *et al.* 2008). The first step in gibberellin biosynthesis occurs via production of *ent*-kaurene by terpene synthases shown in Figure 2.

GA is negatively regulated by DELLA proteins, which are so named because of the conserved DELLA sequence at the N-terminus of the protein (Yasumura *et al.* 2007). GA binds to GA<sub>1</sub> which complex promotes the interaction with DELLA proteins.

This targets the DELLA proteins for degradation via polyubiquitination, therefore removing the down-regulation of GAs and allowing GA signalling (Ross and Reid. 2010). DELLA proteins are also important in the cross talk between GA and ABA in *Arabidopsis* although this process remains to be elucidated (Hirano *et al.* 2008). *Physcomitrella* contains two putative DELLA proteins in its genome; PpDELLAa and PpDELLAb, the increase in DELLA proteins is probably down to gene duplication (Yasumura *et al.* 2007). In spite of this discovery the classic GA-GID1-DELLA interaction does not occur in *Physcomitrella* (Ross and Reid. 2010). The DELLA proteins found in this moss are more divergent than the proteins found 'higher up' in the phylogenetic tree and the DELLA site at the N-terminus that interacts with GID-1 is not as conserved as lycophytes and higher plants, possibly suggesting another function of these proteins (Chen *et al.* 2013)(Yasumura *et al.* 2007).

In spite of this, initial findings have shown that PpDELLA knockouts show promotion of germination (E. Vesty. Unpublished raw data. 2013), which is what would be expected if gibberellin control was conserved. Hirano *et al.* (2008) speculates that it is *ent*-kaurene that could be the key in the evolution of gibberellin signalling in larger vascular plants.





**Figure 2. Gibberellin biosynthesis from geranylgeranyl diphosphate (GGDP) to general GAs in *Arabidopsis*.** CPS- *ent*-copalyl diphosphate synthase. KS- *ent*-kaurene synthase.

#### 1.4 Gibberellins in moss

In contrast to angiosperms, *Physcomitrella* only has one enzyme that synthesises *ent*-kaurene from GGPP, copalyl synthase/kaurene synthase (PpCPS/KS) is bifunctional and expresses both CPS and KS activity (Chen *et al.* 2011). At some point these enzymes split forming separate CPS and KS enzymes in angiosperms (Chen *et al.* 2011). *Ent*-Kaurene (*ent*-K), a precursor in gibberellin biosynthesis pathway has been identified in moss (Hayashi *et al.* 2010). Gibberellins have also been isolated in ferns, another ancient land plant that produces spores suggesting the GA pathway could be conserved in all land plants.

Other studies have previously looked into the effect of *ent*-kaurene on *Physcomitrella* germination. A study by Anterola *et al.* (2009) described that germination was reduced if no *ent*-kaurene being synthesised. However, a later paper by Hayashi *et al* (2010) reported no difference between the wild type and a *Ppcps/ks* knockout mutant *Physcomitrella* that produced no *ent*-kaurene. There is still controversy around this subject which we aim to shed light on. One problem with the study of germination is nomenclature. As previously described we will refer to germination as the spore distension, this occurs when the cell wall bursts and the early chloronema is just visible. Anything after this event is termed growth, not germination. Further, germination rate is the speed at which the population germinates to near 100% and germination potential is the ability of a population to germinate to near 100%.

In the study by Anterola *et al.* (2009) using *Physcomitrella* and AMO 1618, a chemical that blocks the enzyme copalyl diphosphate synthase/kaurene synthase (CPS/KS) that produces *ent*-kaurene, and therefore any downstream gibberellins. They found AMO 1618 inhibited germination of the moss spores. By adding GA<sub>3</sub>, a biologically active GA in *Arabidopsis* and *ent*-kaurene they hoped to rescue this phenotype, although GA<sub>3</sub> had no effect, whereas adding *ent*-kaurene resulted in a partial rescue of germination. However, the data does not show the speed at which spores germinated or what the final germination potential reached was. In our study germination rates at different time points from 0% germination to final germination potential of the samples will be collected. Furthermore, there were no solvent controls for these experiments. The solvent that the hormones and AMO-1618 were dissolved in may have had an effect on spore germination as we have found methanol and DMSO have an inhibitory effect on spore germination.

Hayashi *et al.* (2010) further investigated the role of *ent*-kaurene using a knockout strain that lacked the *cps/ks* gene in *Physcomitrella* and could therefore not synthesise *ent*-kaurene. Although this study focused mainly on the protonemal development of the mutant germination of the wild type and mutant were also measured but only on day 10. They found that there was no difference between the wild type and the *cps* knockout. However, at this point germination was just greater than 50%; if there was an effect on early germination this measurement would not see it. Since a consensus on this subject has yet to be reached, this will be one of the focus points of this study.

### **1.5 Introduction to AHLs**

*N*-acyl-homoserine lactones (AHLs) are quorum sensing (QS) molecules produced by Gram negative bacteria (Klein *et al.* 2009) and are by far the best studied QS molecules. QS is a method by which bacteria communicate with each other that leads to changes in gene regulation, and consequently their behaviour, dependent on population density (Tait *et al.* 2009). Quorum sensing regulates a variety of crucial functions in bacteria including biofilm formation (Dickschat. 2010), virulence factors, conjugation, hydrolytic enzyme secretion and antibiotic production (Rad *et al.* 2008)(Gonzalez and Venturi 2013). AHLs allow cells to communicate and therefore act as a community rather than individuals (Heeb *et al.* 2012).

AHLs have received interest as a possible target for antimicrobials. Many Gram negative pathogenic bacteria only cause disease once they have reached sufficient numbers and form a biofilm, the formation of the biofilm makes the bacteria harder to kill using antibiotics. If biofilm formation can be disrupted this may inhibit the infection process. AHLs from pathogenic bacteria have already been detected in human

infections (Davis *et al.* 2010). This delay in upregulation in virulence factors is a way of remaining undetected by the hosts' immune system until the bacteria has enough numbers to cause disease. It is now known that eukaryotic cells can perceive and react to these signals (Rad *et al.* 2008).

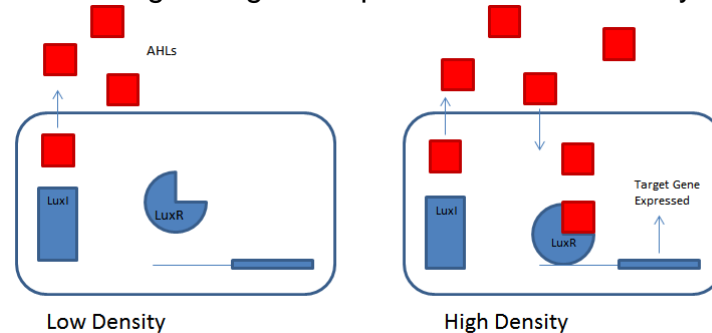
AHLs have also been shown to affect various plant processes with most research focussed on the effects on roots. Some stimulates responses involved in stresses like adventitious root growth (Bai *et al.* 2012), while others effect lateral root growth (Ortiz-Castro *et al.* 2008).

## **1.6 AHLs: Structure and Signalling**

AHLs share a conserved homoserine lactone ring and a hydrophobic fatty acid chain that can vary in length between C4-C18 (Bai *et al.* 2012). The acyl side chain can be un-substituted or have an oxo or hydroxyl group at the C3 position. Due to the variations of structure for AHLs this leads to a large number of possible combinations so the question of specificity arises. To consider this, first the regulation of AHLs needs to be studied.

AHLs are produced constitutively at low levels and then increase as population increases and accumulate extracellularly until it reaches a threshold limit which leads to a change in gene expression as depicted in Figure 3 (Gonzalez and Venturi. 2013). Importantly the changes in gene expression must be greater than the catabolism of the molecule produced (Winzer *et al.* 2002) (Williams *et al.* 2007 a).

Quorum sensing was first discovered in the bioluminescence bacteria on fish (Anetzberger *et al.* 2009). Thus the model system used to describe the QS is the LuxI/R which regulates the changes in gene expression. LuxI is the synthase that produces



**Figure 3.** AHL signalling in bacteria at low and high density cell populations. Produced by S. Needs based on information from Choudhary and Schmidt-Danner. (2010).

the AHL and the LuxR protein being the transcriptional regulator that binds both AHL and DNA (Gonzalez and Venturi. 2013). Short chain AHLs can diffuse into cells; there is evidence that long chain AHLs require active transport to gain entry (Dong *et al.* 2001). AHLs bind to the modular LuxR protein causing a conformational change resulting in dimerization (Dong *et al.* 2001) of the protein and binding to promoter DNA at the lux box. Subsequently, RNA polymerase is recruited resulting in the transcription of the target gene. In other systems LuxR can also act as a repressor by blocking access of RNA polymerase to the DNA and dissociating from DNA when an AHL binds. As well as genes for virulence and biofilm formation, among others, LuxI is commonly up-regulated to produce more AHLs (Gonzalalez and Venturi. 2013). LuxR proteins respond best to the AHL that is produced by its cognate LuxI protein. Bacteria can have more than one LuxR/I homologue resulting in the production of a mixture of AHLs. The diverse homology of LuxR proteins has meant it has been hard to identify conserved residues in the binding site and find molecules that could bind AHLs in

bioinformatic studies alone (Kock *et al.* 2005). However, Gnanendra *et al.* (2012) has identified conserved residues in the binding pocket of LuxR proteins. Using protein modelling information and available crystal structures of four LuxR proteins from different organisms identified a tyrosine and aspartic acid residue in the active site of the LuxR involved in binding the lactone ring of its cognate AHL.

Not all species contain all components of this LuxI/R signalling model. Some bacteria only express the LuxR type proteins and lack the synthase that produces the AHLs suggesting they can perceive signals from other bacterial species; these are termed LuxR solos (Subramoni and Venturi. 2009).

### **1.7 LuxR-Family Solos**

Research into AHL synthesis and regulation has previously focused on bacterial monocultures (Ryan and Dow. 2008), whereas *in situ* bacteria are part of complex communities made up of many different species that are in competition with each other. Around 20% of Gram negative bacteria that do not produce AHLs encode a LuxR-family protein and others encode more LuxR proteins than LuxI proteins (Subramoni and Venturi 2009), allowing them to sense exogenous AHLs expressed by other bacteria. For example, *Salmonella sp.* encodes the LuxR homologue SdiA which binds to exogenous AHLs produced by neighbouring bacteria, after binding genes controlling resistance to complement killing are expressed (Ryan and Dow. 2008).

However, there are a number of bacteria that contain LuxR-family proteins that do not bind AHLs. In the plant pathogen *Xanthomonas sp.* which affects a variety of different plants, two LuxR-family proteins have been studied, OryR and XccR. These

proteins are involved in virulence and are activated by an unknown plant molecule, possibly an AHL mimic. When OryR is activated this drives the production of cell-wall degrading enzymes that facilitates entry into the plant and finally pathogenesis (Subramoni and Venturi. 2009). It seems rational that bacteria that associate with plants would react to molecules produced by them. These LuxR-family proteins are not as specific as those with cognate LuxI proteins and are characterised by the absence of conserved residues (Subramoni and Venturi. 2009) leading to more flexible binding to molecules that have similar structures to AHLs. The flexibility and its dissemination in a large number of species lends itself to interspecies and interkingdom signalling.

### **1.8 Interkingdom Signalling – AHL Signalling and Plants**

One of the most obvious effects of bacterial interactions with plants involves green macroalgae including *Ulva sp.* as they require bacterial signals for correct development, when grown axenically they form a mass rather than the filamentous growth seen in the sea (Twigg *et al.* 2014). In marine systems the signals are freely diffusible and have other effects. The marine algae *Ulva* also produces motile zoospores that settle on bacterial biofilms. They are attracted to these biofilms by the AHLs produced by these bacteria and that they act as chemo-attractants. Wheeler *et al.* (2006) found that zoospore swimming decreases in the presence of AHLs, with the most effective at attracting being 3-oxo-C12-HSL and longer chain AHLs were overall better than shorter chains (Wheeler *et al.* 2006). In other experiments AHL signalling was disrupted and attraction to the bacterial biofilms was lost (Tait *et al.* 2005).

AHL-plant interactions have been mostly studied in angiosperms and many of the effects have been seen in root architecture, although evidence from barley has shown

long chain AHLs are transported all the way to the shoots (Sieper *et al.* 2013). Many bacteria found in the rhizosphere produce AHLs it seems sensible that many of the effects AHLs would have are present in root architecture. Since *Physcomitrella* does not have a classic root system like *Arabidopsis*, greater effects may be seen. AHLs have different effects on different plant species depending on chain length and dose.

Ortiz-Castro *et al.* (2008) studied the effects of different AHLs on root architecture of the model angiosperm *Arabidopsis thaliana*, using AHLs with chain lengths ranging from 4-14C long at concentrations from 12-192  $\mu\text{M}$ . Short chain AHLs had little biological effect, but medium to long chain length AHLs exhibited the most biological activity. C10-HSL was found to be the most active on *Arabidopsis* resulting in decreased primary root growth and increased root hair formation. This effect was dose-dependent with more root hairs formed at concentrations greater than 24  $\mu\text{M}$ . Other studies have additionally looked at the effect AHLs have on roots using *Arabidopsis*. In a study by Rad *et al.* (2008) they studied the transcriptome of *Arabidopsis* after treating with C6-HSL and found up-regulation of growth associated proteins in just 4 hours alongside root elongation.

Bai *et al.* (2012) studied the effect of AHL application on adventitious root growth; usually a stress response in low water conditions, which is an auxin-dependent process. In this study they tested a number of different AHLs with un-substituted and oxy groups with various chain lengths at concentrations ranging from 0 – 500 nM and looked at adventitious root formation of mung beans (*Vigna radiate*). They found that 3-oxo-C10-HSL at 100 nM had the greatest effect at stimulating adventitious root growth but at greater concentrations the AHL effect became inhibitory (Bai *et al.* 2012). This looks like 3-oxo-HSLs with long chains had a greater biological effect and that



plants were more sensitive due to effects seen at nM range rather than at the  $\mu\text{M}$  range used in the Ortiz-Castro *et al.* (2008) experiment. They also found  $\text{H}_2\text{O}_2$ , NO and cGMP were up-regulated during this process, the same second messengers that are also up-regulated during auxin signalling (Ortiz-Castro *et al.* 2008). Using NO and cGMP scavengers to remove these molecules they found that this inhibited the effect of 3-oxo-C10-HSL. Thus they concluded that the AHL signal is transduced using these messengers and that the change in root architecture was an auxin-dependent process.

As well as root architecture AHLs have been implicated in symbiotic mycorrhizal relationships (Markmann *et al.* 2008). In one such study to look into the control of AHLs on symbiotic relationships Zheng *et al.* (2006) looked at the Rhizobia species, *Mesorhizobium tianshanense*, which encodes LuxR/I homologs. Using a mutant strain that lacked the ability to produce AHLs they compared the amount of nodulation of a legume host plant of the wild type and mutant 2 months after inoculation with the bacterium. While the wild type reached maximal nodulation the mutant strain exhibited no nodulation of the plant at all. It was also found that root hair adherence; the first step in nodulation was greatly reduced in the mutant compared to the wild type (Zheng *et al.* 2006).

AHLs are structurally related to several molecules produced by plants, alkamides and *N*-acylethanolamides (NAE's), and more distantly strigolactones. Like AHLs, alkamides and NAE's have been shown to affect the root architecture in *Arabidopsis* (Ortiz-Castro *et al.* 2008). Strigolactones also share some functional similarities to AHLs. Strigolactones have been shown to act like QS molecules in moss, regulating protenemal branching and colony extension. Strigolactone biosynthesis starts with the action of a carotenoid cleavage dioxygenase (CCD) (Nambara and

Marion-Poll, 2005). Proust *et al.* (2011) have shown that when *Physcomitrella* lacks this enzyme the colonies formed are not as distinct as WT and continue to spread out. They also found that the WT strain secretes strigolactones into the extracellular media so they use strigolactones as a way of sensing where other colonies are. Strigolactones and AHLs have both been implicated in mycorrhizal interactions in plants (Proust *et al.* 2011). Due to the similarities in the structures of alkalamides and AHLs it has been hypothesised that AHLs can regulate responses in plants too.

Plants and bacteria have evolved together; it seems practical that plants and bacteria would have a system to perceive signals produced by one another and many plant associated bacteria have been shown to produce AHLs. Some plants use this to their advantage by using quorum quenching or anti-QS molecules that disrupt signalling in bacteria and if the bacteria is pathogenic results in the halting of virulence factor production (Koh *et al.* 2005).

## **1.9 Quorum Quenching**

Many bacteria produce quorum quenching molecules to inhibit the activity of neighbouring cells. Some species of plants like the marine alga *Delisea pulchra* also produce quorum quenching molecules like halogenated furanones which are structurally similar to short chain AHLs. These bind directly to the LuxR protein of the bacteria and signal for it to be degraded, thus down regulating the signal. This could be in response to the negative effects of bacterial biofilms on the surface of the algae (Manefield *et al.* 2002).

This finding has received much interest in both medical and agricultural areas. If plants can be engineered to produce such quorum quenching molecules it will stop many pathogenic bacteria from infecting plants. However, it would also have a detrimental effect on the beneficial bacteria such as the nitrogen fixing bacteria (Choudhary and Schmidt-Danner. 2010).

### **1.10 Project Aims**

One of the aims of this study was to study gibberellin signalling in *Physcomitrella*. These were undertaken by looking at the effect of *ent*-kaurene on *Physcomitrella* germination rates and morphology by using a *Ppcps/ks* knockout strain and compare these to the wild type. Confocal microscopy was also used to analyse the regulation of the CPS/KS gene in the mutant. A strain containing Yellow Fluorescence protein (YFP) linked to the CPS/KS promoter was used to study any changes in expression under different conditions. Further to this, RNA was extracted from *Physcomitrella* and the green algae, *Ulva sp.* for future transcriptome analysis. Further to this the role of dormancy in spores was briefly investigated.

As well as the effect of gibberellin signalling on germination, the effect of AHLs on germination rates was studied, a class of quorum sensing molecules produced by Gram-negative bacteria. As moss is not a vascular plant and grows nearer the ground than other plants AHLs from soil bacteria may play a bigger role in its physiology. Germination rates of wild type spores were compared to different amounts and types of AHL molecules.

We hypothesise that without *ent*-kaurene production germination rates will be slower than the wild type and that adding exogenous *ent*-kaurene and other GAs will rescue the *cps/ks* knockout phenotype.

## **2. MATERIAL AND METHODS**

### **2.1 Strains**

Wild type: *Physcomitrella patens* ecotype *Gransden2004*. Mutant strains were obtained from the Sørensen lab including YFP-*cps/ks* knockout and *Ppcps/ks mutant*.

### **2.2 Spore Collection and Storage**

Tissues was homogenised using a polytron tissue tearer in sterile laminar flow hoods and grown on autoclaved sterile peat plugs in Magenta pots under 24 hour light at 22 °C until gametophore structures developed. Plugs were transferred to 16:8 hour light:dark conditions at 15 °C until gametangia formed. Plugs were sprayed with sterile water to allow movement of the flagellated spermatozooids to the female organs and sporophytes are produced after. Mature sporophytes were large and dark brown and are removed using forceps under a dissecting microscope under sterile conditions. Sporophytes are allowed to air dry in eppendorfs, any remaining green tissue is removed after the sporophytes are dry. Sporophytes are kept in eppendorfs at room temperature in the dark for long-term storage.

### **2.3 Media**

Different BCD media was used for different experiments. Spore germination assays used IIII BCD media made up of 10 ml of each B (1 mM MgSO<sub>4</sub>), C (1.84 mM KH<sub>2</sub>PO<sub>4</sub>), and D (10 mM KNO<sub>3</sub>) solutions, 1 ml Trace Elements, 10 ml 5 mM ammonium tartrate, 5 ml CaCl<sub>2</sub> at 1M, 7.2 g agar per 1 l. For protonemal analysis tissue is grown on II BCD, for increased gametophore production tissue is grown on I BCD – 10 ml of each B, C and D media, 1 ml Trace elements, 10 ml 5 mM ammonium

tartrate, 1 ml CaCl<sub>2</sub> at 1M and 7.2 g plant agar per 1 L. For water agarose experiments 7.2 g agarose was added to 1 L distilled water.

## **2.4 Spore Germination Assays**

### Plate preparation

Spores were plated onto III BCD agar plates. Plates were poured under sterile conditions in a laminar flow hood. Cellophane sheets were placed in between circular filter paper. Distilled water was added to avoid cellophane wrinkling. These were autoclaved and placed at 4 °C to allow cooling. Autoclaved cellophanes were placed onto the agar plates using sterile forceps.

### Addition of hormones and AHLs

AHLs were obtained from Cámara lab. Different concentrations of AHLs, 10 nM, 50 nM, 0.1 µM, 0.5 µM, 1 µM, 5 µM including water and methanol controls, were added to III BCD media. This was achieved by pouring 75 ml per plate needed into a sterile conical flask. The AHLs or hormones were added (500 µl) to this to give the desired final concentration. The media was mixed by swirling and the pouring into the plates. AHL solutions were dissolved in methanol and stored at -80 °C. Spores were plated as usual (See Preparing and Plating Spores). Plates were cold treated for 3 days unless stated otherwise in the text. The same protocol was used for GA<sub>9</sub> experiments. Plates were made no more than 2 days before use and stored at 4 °C in dark conditions and cellophane on the day. All experiments were solvent matched. The amount of methanol present at the highest dose was calculated and extra methanol was added to the lower

concentration and the methanol control to assure every condition within an experiment contained the same amount of methanol.

### Spore Washing

A ratio of three sporophytes was used for every 10 BCD agar plates. Sporophytes were sterilised in a 25% bleach solution (25% bleach, 75% distilled sterile water) for 10 minutes. These were then washed for 10 minutes in sterile water under aseptic conditions, repeat 3 times.

### Spore Plating

The sporophytes were crushed in 200 µl of sterile water to release the spores and made up to amount needed for plates. 500 µl of the spore solution was plated onto the prepared BCD plates. Spores were cold treated on the plates for three days before being transferred to growth conditions in 22 °C under 24 hour light. Plates were first checked for germination on day 3. Germination was defined as the protonema first being visible. *Ppcps/ks* and wild type spores were matched by age to reduce any spore ageing or storage effects and at least two sporophytes were pooled in each experiment for each genotype. Each condition was based on a minimum of two plates, of which a minimum of 200 spores were counted on each plate at each time point.

## 2.5 Statistical Analysis

Student's T-Test was applied for each different condition on each day germination was recorded which used two samples with unequal variance with a two-tailed distribution. Results where the P value was < 0.05 were said to be significant.

## 2.6 Microscopy

Colony morphology analysis was conducted looking at spores plated on III BCD as previously described using NIKON SMZ1000 and created by NIS-Elements BR software. Figure 1E shows the average diameter of 150 randomly selected colonies for each condition on three different plates.

Confocal images were created using Leica confocal software. Homogenised tissue was grown on II BCD. 100 µl of water was put onto slides and individual colonies were taken and mounted onto the slide. A cover slip was placed gently over the top, taking care not to press down and distort the colony. Fluorescence, excitation at 488 nm excitation and 525 nm collection was used to detect YFP and natural fluorescence of chloroplasts. Images were taken using x40 objective.

## 2.7 RNA extraction of protonemal tissue and *Ulva* blades

RNA extraction of moss protonemal tissue was completed using BIOLINE RNA extraction kit as instructions indicate. Briefly, approximately 100 mg of wet protonemal tissue was frozen in eppendorfs and ground using a pestle and mortar. Lysis buffer was added and sample was filtered by centrifugation. 70% ethanol is added and the RNA is bound to an RNA plant column by centrifugation. The column was desalted and a DNase was added to digest DNA in the sample. After this the column is washed and finally the RNA is eluted in RNase free water. Samples were frozen at -20 °C.



## 2.8 RNA extraction of *Ulva* blades using TRIZOL method

Tissue was stored at 4 °C in Instant Ocean. 2 cm of *Ulva* blade was washed in distilled water and patted dry with tissue. The blade was crushed in liquid N<sub>2</sub> and 1 ml of Trizol (Invitrogen) was added to the homogenised blade and further crushed in the presence of liquid N<sub>2</sub>. Frozen Trizol and material was put into 1.5 ml eppendorfs and allowed to thaw. 250 µl was aliquoted into 4 new RNase free eppendorfs. 50 µl of 12M potassium acetate was added to give a final concentration of 0.2M and incubated at room temperature for 5 minutes. 200 µl of chloroform was added and shaken for 15 seconds and incubated at room temperature for 10 minutes. Samples were centrifuged at 4 °C at 12 000g for 15 minutes. The upper phase containing the RNA was pipetted off the intermediate and organic phases that contained the DNA and proteins, and put into new RNase free eppendorfs and 500 µl of isopropanol was added. Samples were frozen at -20 °C for 1 hour. Centrifuge at 4 °C at 12 000g for 10 minutes. Pellet was washed in 75% ethanol and centrifuged at 4 °C at 7 500g for 5 minutes. Ethanol wash was repeated. Pellet was dried at 50 °C in a dry oven for 10 minutes until the pellets turned clear. 20 µl of nuclease free water and incubated at 55-60 °C in an oven for a further 10 minutes and dissolved by pipetting the solution up and down. 2 µl samples were analysed by gel analysis and on Nano-drop looking at the 260/280 and 260/230 ratios to indicate purity of the sample. 1 µl of sample was loaded onto the nano-drop pedestal after a blank control of 1 µl distilled water as our sample was in RNase free water.

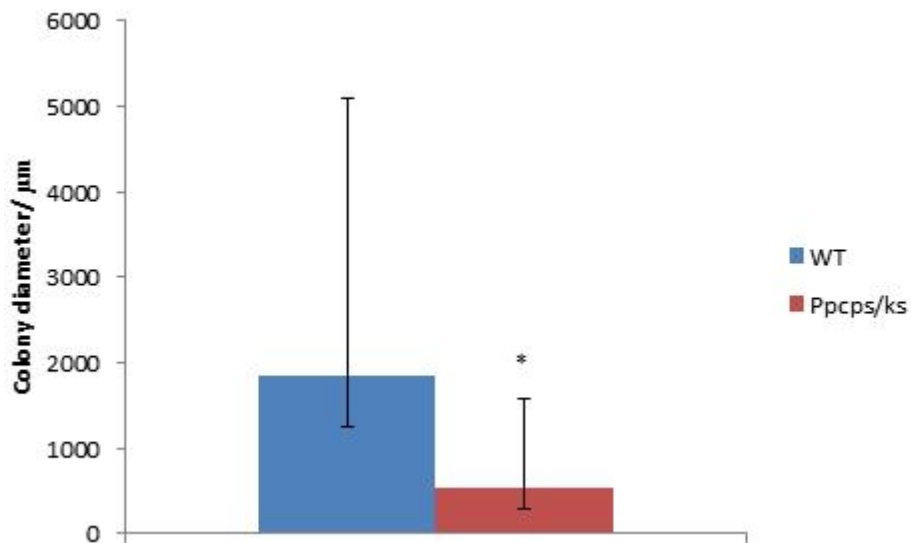
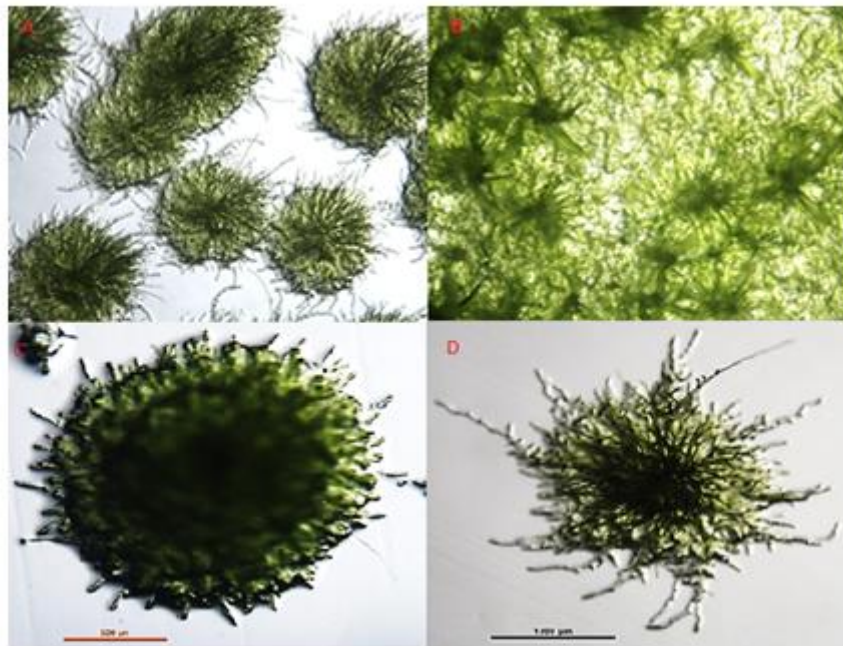
## 2.9 Gel Eletrophoresis

Gel electrophoresis was undertaken using 1% gel to visualise RNA samples. 4  $\mu$ l of RNA sample was loaded with 6X gel loading buffer.

### 3. Results

#### 3.1 *Ppcps/ks* mutant shows reduced caulonema development and smaller colony size

*Ent*-kaurene is a significant precursor in the gibberellin biosynthesis pathway in higher plants (Yamaguchi. (2008). Large amounts of *ent*-kaurene and *ent*-kaurenoic acid was produced in moss (Hayashi *et al.* 2010; Anterola *et al.* 2009). As reported in Hayashi *et al.* (2010) *Ppcps/ks* exhibits a different phenotype to that of wild type colonies. Colonies of wild type and *Ppcps/ks* spores were grown on BCD media for 10 days and morphology was analysed. Figure 4 A through D shows the *cps/ks* colony compared to the WT colony. The *Ppcps/ks* colonies shown on the left panels do not produce as many caulonemal filaments as the WT shown in the right hand panels. This results in a much rounder, compact colony with less branching. The diameters of colonies were compared by measuring the longest part of each colony; it was found that the WT had significantly larger colonies compared to the *Ppcps/ks*. Chloronemata is the filament type produced during initial phase in moss development after which caulonema develops. Caulonema have longer cells and fewer chloroplasts and it is from these cells which send that leafy buds develop into gametophores. Production of caulonema is lacking in the *cps/ks* knockout mutant. From this we confirm Hayashi *et al.* (2010) conclusion that *ent*-kaurene or a metabolite of *ent*-kaurene is involved in chloranemal to caulonema transition. Additionally, we are using a different, uncharacterised *cps/ks* allele mutation that acts similarly to the mutant used by Hayashi *et al.* (2010).



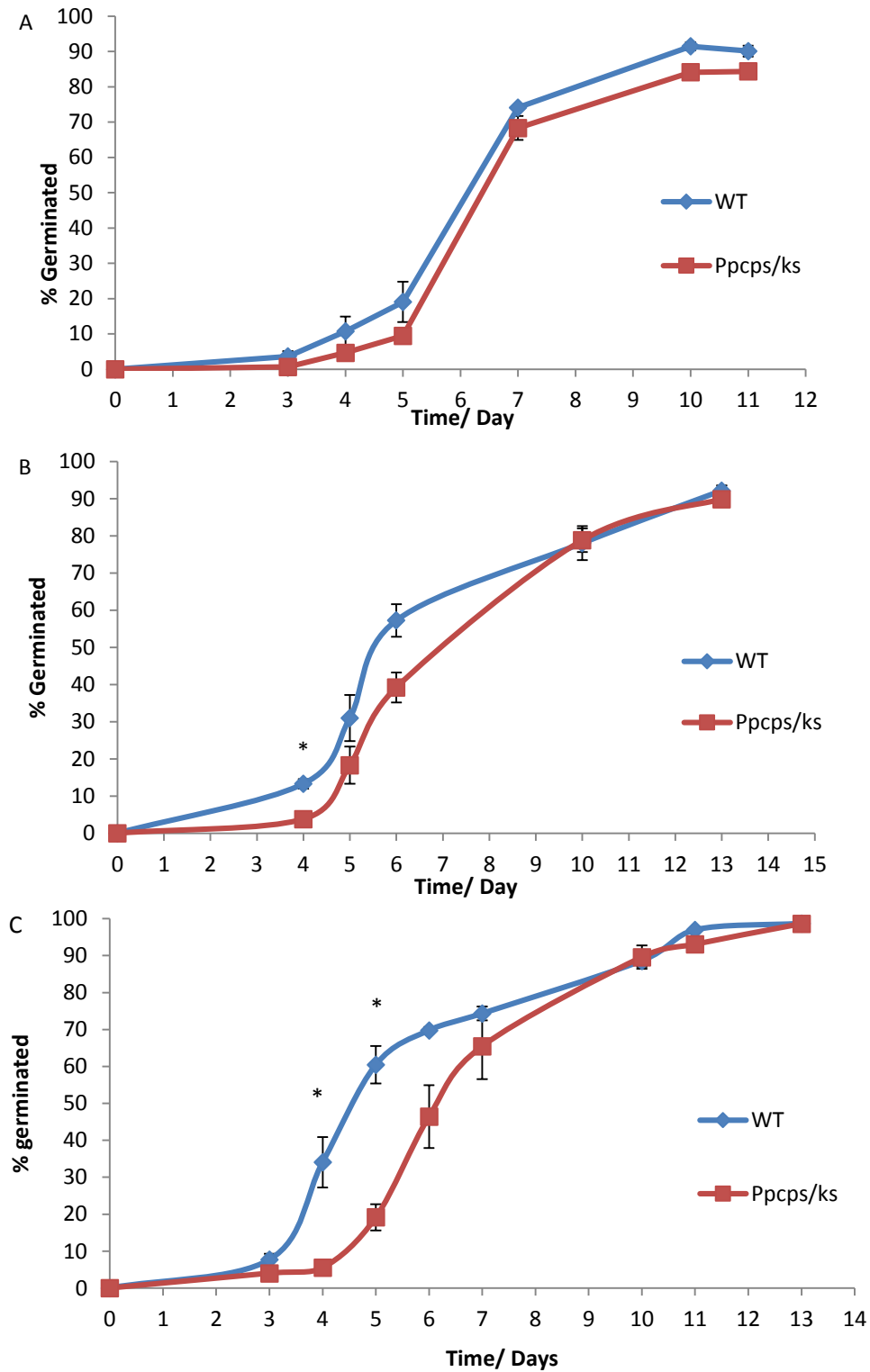
**Figure 4. The uncharacterised *Ppcps/ks* mutant has a smaller colony phenotype compared to the WT ‘Gransden2004’**

**A** and **B** showing *Ppcps/ks* KO and Wild type Gransden2004 colonies respectively at low magnification.

**C** and **D** shows *Ppcps/ks* KO and WT colonies at high magnification. Scale bars represent 500 μm, 1000 μm respectively. The averages of colony diameter of *Ppcps/ks* KO and wild type after 10 days growth from spores and ≥95% germination of both types. Error bars = ± the minimum and maximum colony sizes. A total of 150 randomly selected colonies on three different agar plates was analysed for each condition. ANOVA: Single factor was performed on the colony diameter data. \* indicates significance where the P value is < 0.05.

### 3.2 *Ppcps/ks* exhibits slower germination rates than wild type spores

Gibberellins are well known to be essential to germination in angiosperms. Using this knowledge we compared germination rates of a wild type and *cps/ks* KO. Hayashi *et al.* (2010) described no difference between germination 'rates' of WT and *cps/ks* KO at day 10. However, we propose that by looking at the germination percentages 10 days after sowing Hayashi *et al.* (2010) did not measure germination rates at all. To look into this further we measured the germination rates of WT and *cps/ks* knockout spores from when spores first start to germinate right through to > 85% germination. By measuring germination to at least 85% confirms a particular spore batch used in experiments is healthy and has the potential to germinate fully. Our results show that *Ppcps/ks* knockout mutant germinates more slowly than wild type spores (Figure 5). Looking at a range of different aged spores of the WT and *cps/ks* mutant we found the mutant was slower to germinate than the WT as shown in Figure 5 (Representative of three repeats of 15 conducted within the lab). Figure 5 shows that although the germination rate of *Ppcps/ks* is slower than the wild type, they maintain the same potential, the mutant population germinates to the same percentage as the wild type. This is why the rate must be measured. If on some of graphs the percentage germination was measured at Day 10 then no differences would be identified.

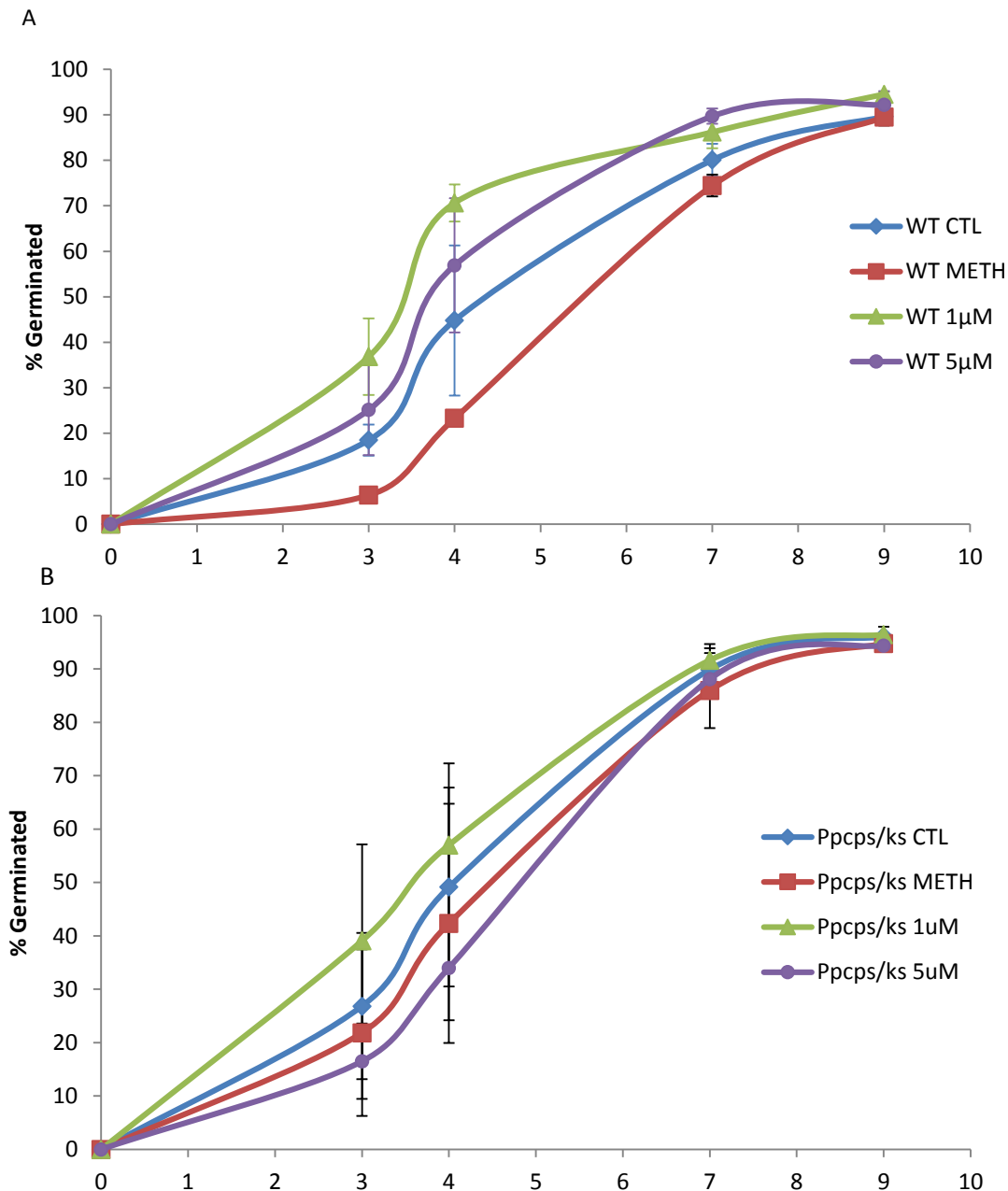


**Figure 5. Strains with the *ent*-kaurene synthase knockout germinate slower than the wild type but to the same potential.** A, B and C represent spores aged 1, 8 and 3 month old spores respectively.  $N \geq 200$  on each plate for at least 2 points at each time point. Student's T. Test was performed. \* indicates  $P < 0.05$ . Error bars =  $\pm$  SEM.

### 3.3 GA<sub>9</sub> methyl ester increases wild type and *Ppcps/ks* germination

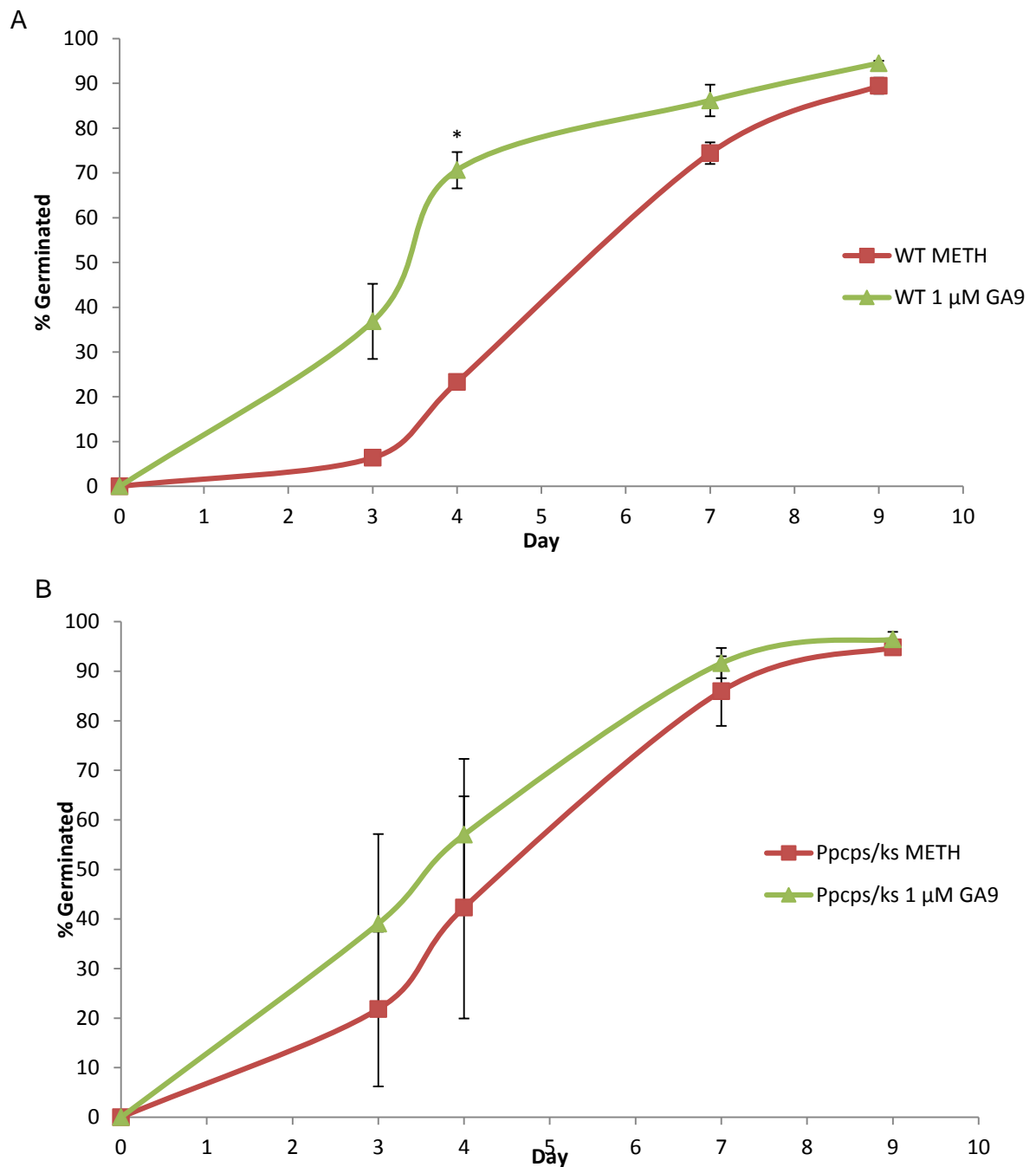
We have established that spores that lack *ent*-kaurene the germination rate are decreased. To further this finding we tested the effects of exogenously applied gibberellins that are known to have a bioactive effect on *Physcomitrella* on wild type and *Ppcps/ks* spores. GA<sub>9</sub> methyl ester is not endogenous to *Physcomitrella* (Hirano *et al.* 2007) but Hayashi *et al.* (2010) has reported it rescues the *cps/ks* knockout phenotype. GA<sub>9</sub> methyl ester showed significant promotion of germination in the WT (Figure 7A) in a reverse dose dependent manner with 1 μM concentrations having a greater effect than the 5 μM (Figure 6A) but the effect of GA<sub>9</sub> ME was not so clear cut in the mutant phenotype (Figure 6B). The *cps/ks* knockout phenotype showed a slight increase in germination rates at 1 μM levels but no difference at higher concentrations (Figure 7B).

In addition to this result, there is a clear decrease in rates of the methanol controls. Briefly, GA<sub>9</sub> ME is dissolved in methanol and this has an adverse effect on moss germination. During these experiments all the experimental conditions and the methanol control are solvent matched, meaning they all contain the same amount of methanol, whereas the CTL is plain BCD. It could be argued that GA<sub>9</sub> ME is having an even greater effect on spore germination as it is overcoming the detrimental effects of the methanol.



**Figure 6. GA<sub>9</sub> methyl ester promotes germination at 1  $\mu$ M on wild type and *Ppcps/ks* KO strains on 1 month old spores. (n>200). A and B. 1 and 5  $\mu$ M concentrations of GA<sub>9</sub> ME on wild type spores and *Ppcps/ks* respectively. Error bars =  $\pm$  SEM. Student's T-Test was performed on samples. P < 0.05. Each time point representative of the averages of 2 sets of a minimum of 200 spores.**

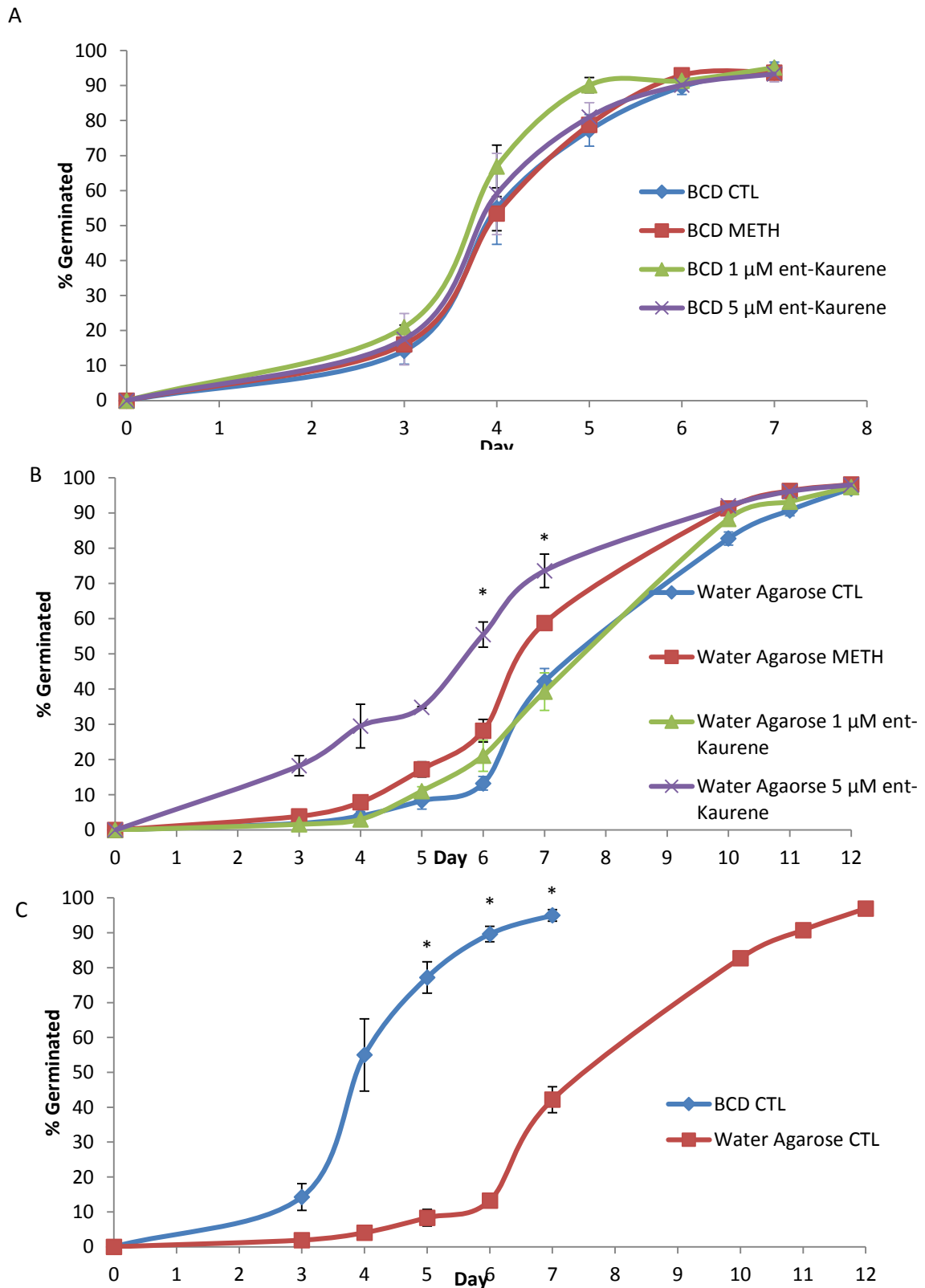




**Figure 7. GA<sub>9</sub> methyl ester promotes germination at 1  $\mu$ M on wild type and *Ppcps/ks* KO strains on 1 month old spores. (n>200) A and B. Methanol control and 1 $\mu$ M concentration of GA<sub>9</sub> ME on wild type and *Ppcps/ks* respectively. Error bars =  $\pm$  SEM. Student's T-Test was performed on samples. P < 0.05. Each time point representative of the averages of 2 sets of a minimum of 200 spores.**

### 3.4 *Ent*-kaurene promotes germination in wild type spores

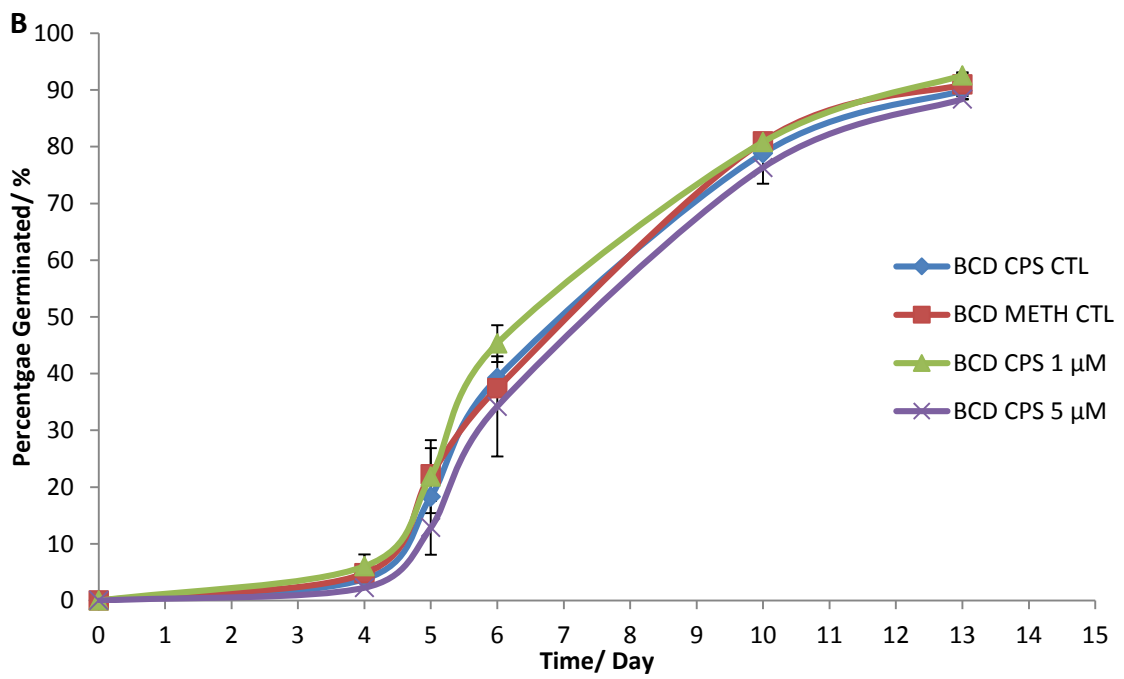
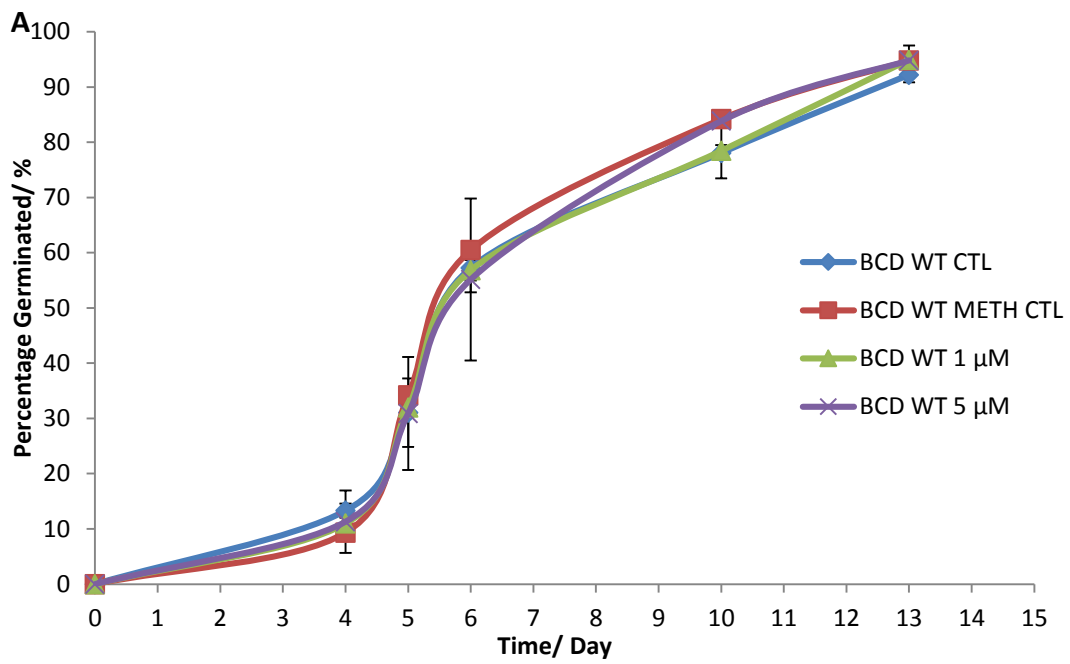
To expand upon these findings we analysed the effect of *ent*-kaurene, which is found endogenously in *Physcomitrella* and has a bioactive effect on colony formation, shown in Figure 4 (Hayashi *et al.* 2010), on wild type spores plated on different media. Normally spores are grown on a nutrient rich media called BCD with excess amounts of calcium for filamentous tip growth and high nutrient levels (Bushart and Roux. 2007). When *ent*-kaurene was added to BCD plates there was promotion in germination at 1  $\mu$ M levels of *ent*-kaurene but the effect was subtle (Figure 8a). This could be because the spores were already at their optimum growing conditions, they had all the nutrients they needed so did not react strongly to the added *ent*-kaurene. Furthermore, we already know wild type spores synthesise large amounts of *ent*-kaurene (Hayashi *et al.* 2010). By growing spores on water agarose, this media contains no nutrients at all and agarose is a purer form of agar without any other contaminants that may affect germination we found that spores germinated slower on water agarose which was to be expected (Figure 8C) but the effects of *ent*-kaurene were more dramatic when spores were grown on water agarose compared to BCD (Figure 8B). When grown on water agarose spores reacted more to the higher concentration of *ent*-kaurene (Figure 8B).

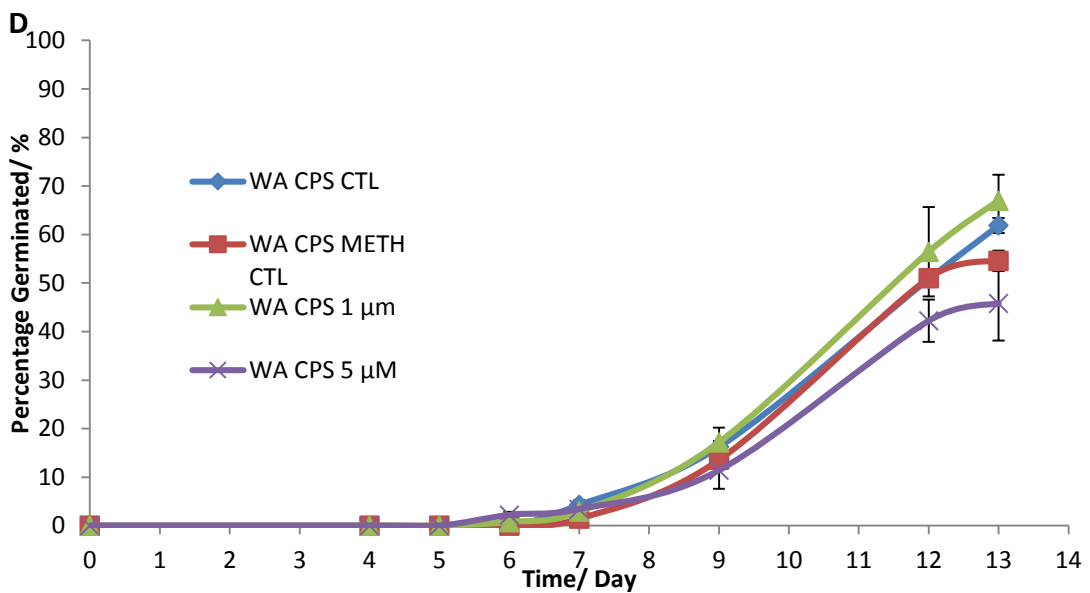
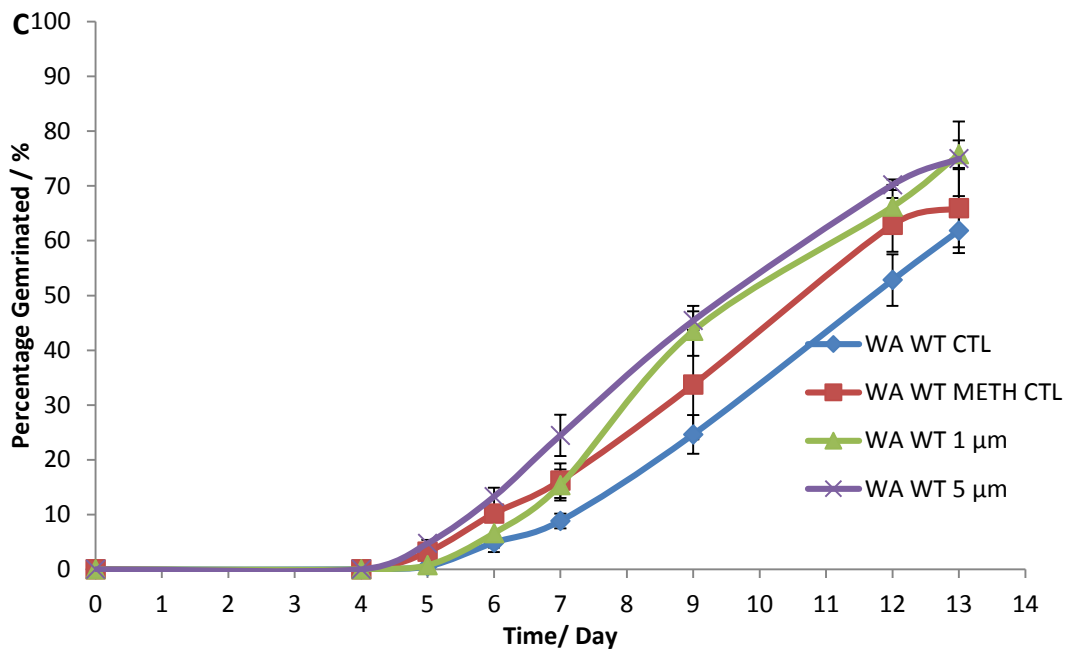


**Figure 8. *ent*-kaurene promotes wild type germination in a dose dependent manner when grown on water agarose but not BCD media. (n>200). Error bars =  $\pm$  SEM. Students T-Test was performed on samples. \* indicates P < 0.05. A, B, C. METH = solvent control matched to solvent levels in *ent*-kaurene conditions.**

### 3.5 *Ent*-Kaurene increases germination rate of WT and *Ppcps/ks* at 1 $\mu$ M concentration

As *ent*-kaurene showed promotion of germination in the WT spores in both BCD and water agarose media, albeit at different concentrations (Figure 8) we asked whether it could be used to rescue the *cps/ks* knockout germination phenotype better than the GA<sub>9</sub> methyl ester. As before, spores were plated on BCD and water agarose. Similarly to the previous experiment the BCD condition was not significant, either in the WT or the *Ppcps/ks* mutant, to the addition of *ent*-kaurene (Figure 9A and B). Effects of *ent*-kaurene were slightly more noticeable when spores were grown on water agarose (Figure 9C and D). Wild type showed promotion in germination at 5  $\mu$ M (Figure 9C) but little difference was seen with the *Ppcps/ks* knockout (Figure 9D). We do not know what the bioactive GA is in *Physcomitrella* and by adding large amounts of *ent*-kaurene could have different results. By adding large amounts of *ent*-kaurene at the  $\mu$ M level we may be overloading the system or this excess of *ent*-kaurene may be involved in a negative feedback system. This lack of effect could also be because it takes time to get the *ent*-kaurene into the cell and metabolise it and we see a greater effect in the WT because the WT already has its own supply of *ent*-kaurene and the higher concentrations boost its action a little.



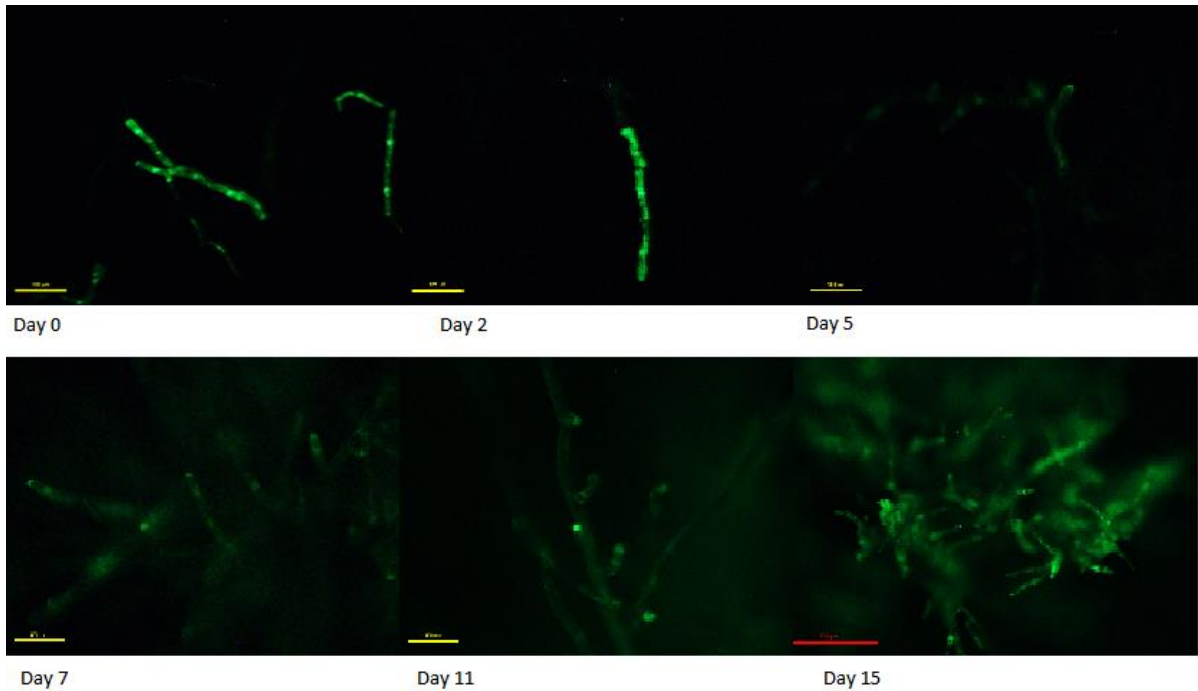


**Figure 9. Effect of ent-Kaurene and media type on germination rates of 8 month old wild type and *Ppcps/ks* spores.**

(n >200). Error bars show  $\pm$  SEM. A and B show the effect of *ent*-Kaurene on wild type and *cps/ks* mutant spores grown on BCD media. C and D show the effect of *ent*-kaurene on WT and *cps/ks* mutant spores grown on water agarose. Student's T-Test was performed on samples. P > 0.05. WT= wild type. CPS=*Ppcps/ks*. WA= Water agarose. METH= Methanol control.

### 3.6 PpCPS/KS promoter may be more active in tip cells

From the data already presented it is becoming clear *ent*-kaurene has an effect on moss germination and development. A transgenic line obtained from the Sorenesen lab where yellow fluorescent protein (YFP) was inserted in place of the *cps/ks* gene so that the promoter of the endogenous *cps/ks* gene produced YFP in the *Ppcps/ks* mutant background. Therefore we can study the CPS/KS activity levels in real time during development and ascertain where in the colony the YFP protein is most highly expressed. Homogenised tissue was plated onto BCD II agar plates and fluorescent images were taken from Day 0-15 of colony growth/regeneration, where Day 0 represented when the tissue was plated. Initially some filaments produced large amounts of YFP but overall production faded as colonies grew and seemed to be localised to tips and buds when observed under a dissecting microscope (Figure 10). Day 15 shows an image of a large colony before gametophyte development, showing production is still universal within the colony but the tips do look brighter (Figure 10). This may be due to the difficulty when focusing on a 3D structure. Colonies are not 2D so the background may appear duller because the lens is focussed on the tips of the colony.



**Figure 10. Fluorescent images showing production of YFP by the Ppcps/ks promoter of homogenised tissue over time in the Ppcps/ks KO background.** Scale bars for Day 0, 2, 5, 7, and 11 show 100  $\mu\text{m}$  and Day 15 500  $\mu\text{m}$ . Control homogenised WT tissue was also plated, no fluorescence was seen, data not shown.

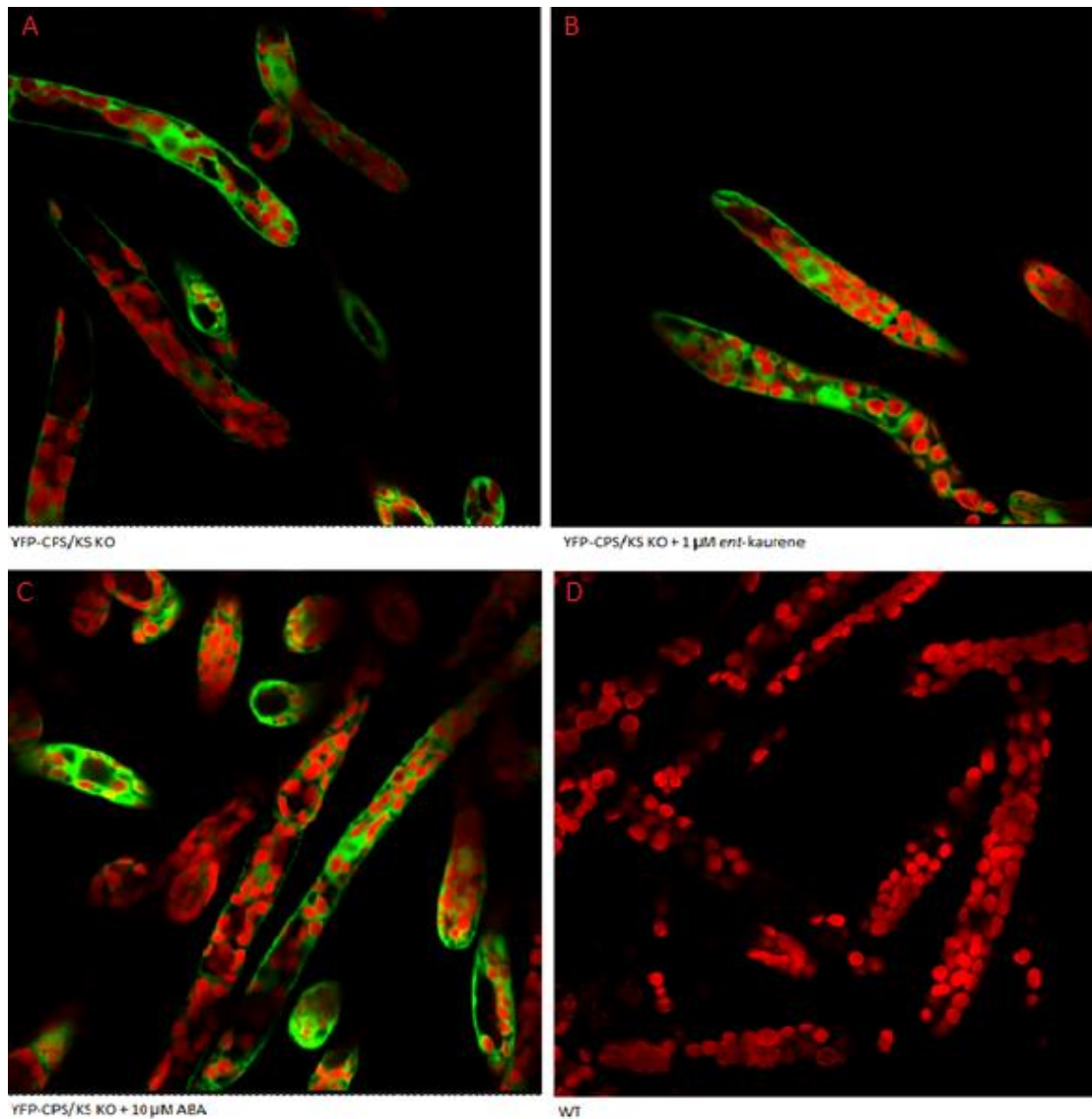
### 3.7 High Resolution imaging of YFP-CPS/KS promoter fusion

To explore CPS/KS promoter activity further, confocal images were taken of homogenised tissue at Day 6 after filamentous colonies have been formed but before gametophore development. By looking at the tissue at higher magnification we could study the promoter activity in different areas of the colony in much greater detail. From looking at the initial images in Figure 10 we looked at the difference in fluorescence between tip cells and intermediate filament cells at a higher magnification. However,



we did not identify any difference in fluorescence at the different positions of cells and YFP was expressed at high levels ubiquitously (Figure 11). Due to the colonies being of the *cps/ks* mutant phenotype 1  $\mu$ M *ent*-kaurene was added to the media to see if levels were different in these colonies (Figure 11b). As seen in figure 11 expression is of similar levels in both conditions. 10  $\mu$ M ABA was added in another condition; ABA inhibits germination and when added to moss in these levels results in a phenotype with decreased germination rates and shorter cells (E. Vesty. Unpublished raw data. 2013) and again no difference in CPS/KS promoter activity was observed (Figure 11C).

The YFP-*cps/ks* KO line obtained from the Sorenson lab had a different phenotype to that of the *cps/ks* KO. Although the knockout does not produce caulonema as readily as the WT gametophore production still occurs and colonies grow to maturity and produce gametophores and sporophytes in a normal manner. However, the YFP-*cps/ks* knockout colonies did not survive much longer than a month on agar plates before cell atrophy was seen and did not produce leafy tissue. The imaging suggests that the rapidly growing protonemal tissue produces massive amounts of YFP driven by the CPS/KS promoter and that this could prove toxic to the cells. From this it was concluded that in *cps/ks* is expressed in large amounts in rapidly growing tissue and any change in gibberellin signalling occurs via signalling in downstream events from *ent*-kaurene which is in line with conclusion from Hayashi *et al.* (2010).



**Figure 11. Representative confocal images of YFP-*cps/ks* knockout mutant. No difference was seen in CPS/KS activity in different parts of the colony or with added ABA or *ent*-kaurene.**

Grown on II BCD with added hormones (from left to right, top to bottom); no added hormones, 1 μM *ent*-kaurene, 10 μM ABA and wild type with no added hormones. Green represents the production of YFP linked to the *cps/ks* promoter, red represents chloroplasts.

### 3.8 Long chain AHLs promote germination

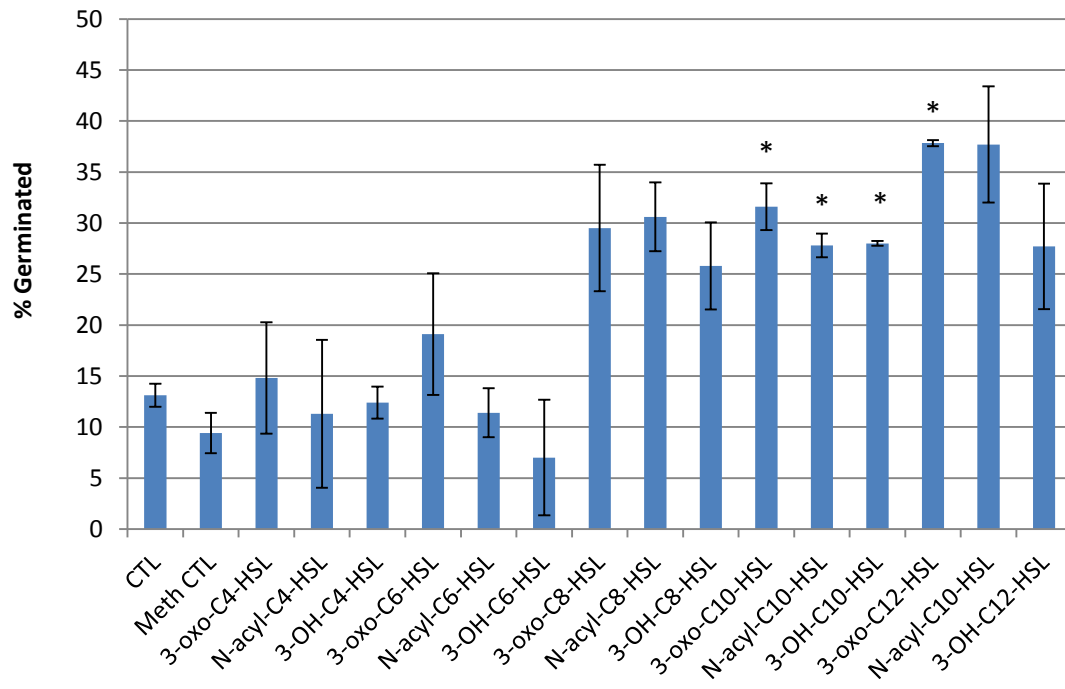
AHLs have been shown to have an effect on plant architecture (Williams *et al.* 2007. B; Ortiz-Castro *et al.* 2008) and algal spores (Twigg *et al.* 2014). Therefore we explored the idea that AHLs may affect moss spore germination. Preliminary testing looked at the effect of AHLs of different substitution states ranging from C4-C12 at 0.1  $\mu\text{M}$  concentrations on germination rates of *Physcomitrella*. 0.1  $\mu\text{M}$  was chosen as an initial test as it is close to physiological levels, similarly other experiments that have used mung beans used concentrations in the nM range (0-500 nM) and has seen a measurable effect (Ortiz-Castro *et al.* 2008). Other experiments have suggested that longer chain AHLs have the most bioactive effects on plants (Ortiz-Castro *et al.* 2008).

Testing the different AHLs on moss germination shows that long chain AHLs appear to promote moss spore germination (Figure 12). Overall, as chain length increases regardless of substitution state of C3, germination increases. There is a distinct jump in germination promotion when chain length reaches C8 compared with the control (averages of the germination rates for control at day 3 was 11% compared with 13%, 13%, for average of germination rates of C4 and C6 respectively and 29%, 29%, 34% of C8, C10 and C12 respectively). 3-OH-AHLs look to have the least activity out of substitution states in the AHLs (Figure 12A). AHLs with a chain length of 6 are under do not have a biological effect on germination. Student's T-Test was performed on the data and found that all the C10-AHLs showed a significant difference to the control as well as 3-oxo-C12-HSL at day 3. The difference between the long chain and short chain AHLs lessens by day 4 (Figure 12B). Figure 12C shows the averages of

the different substitution states for each AHL chain length. This shows the increase in germination between C8, C10 and C12 and the control, C4 and C6.

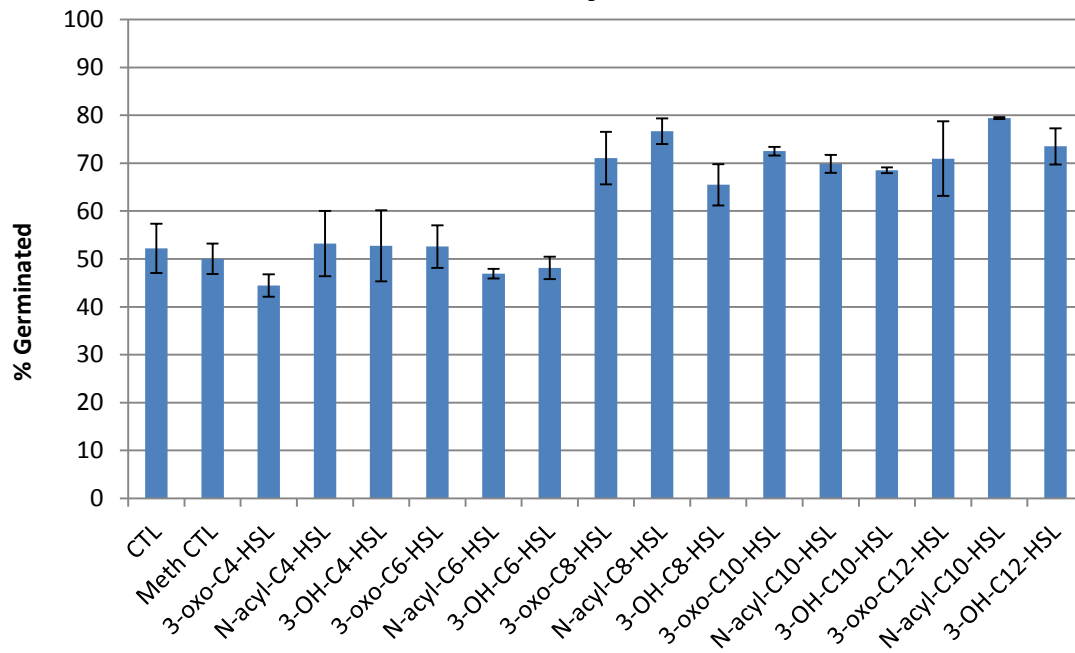
A

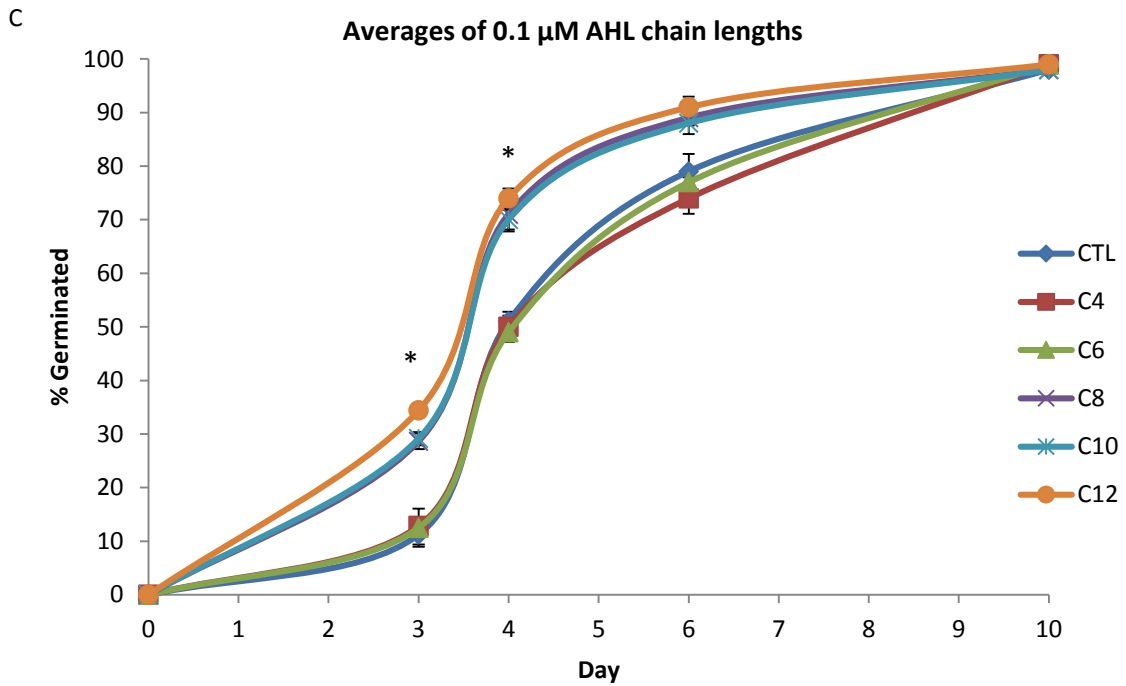
## Day 3



B

## Day 4



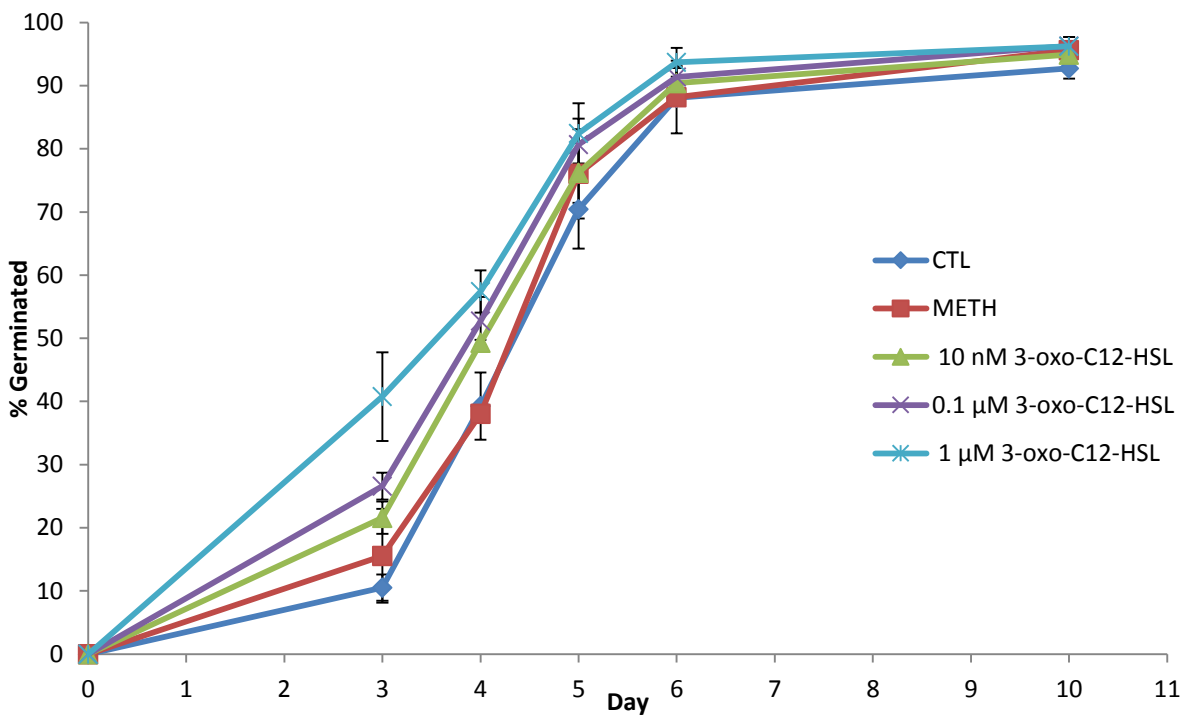
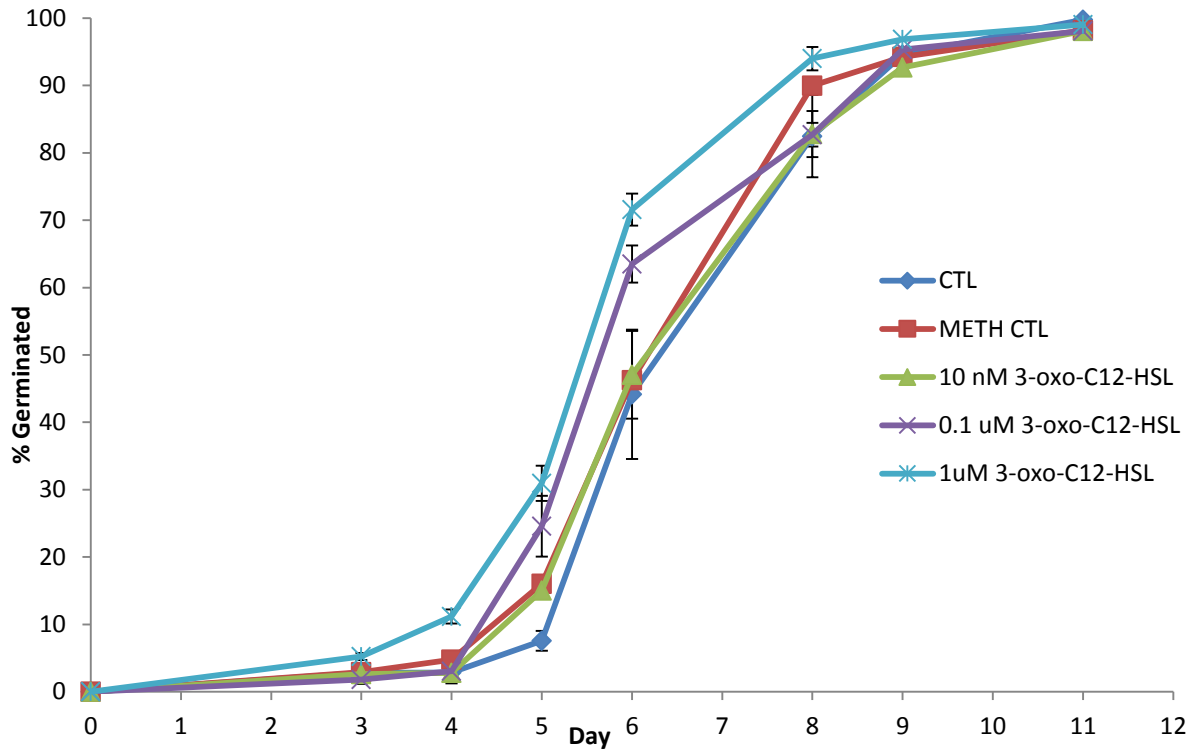


**Figure 12. A and B. Effect of AHLs at 0.1  $\mu\text{M}$  on germination of wild type spores on day 3 and 4 respectively. C shows the germination rates of the averages of the three substitution variations of each chain lengths.**

Error bars =  $\pm$  SEM. Students T-Test was performed on data. \* indicates  $P < 0.05$ . In the averages C8, C10 and C12-HSLs showed statistically significant difference to the control, C4-HSL and C6-HSL showed no difference.

### **3.9 3-oxo-C12-HSL exhibits dose dependent promotion**

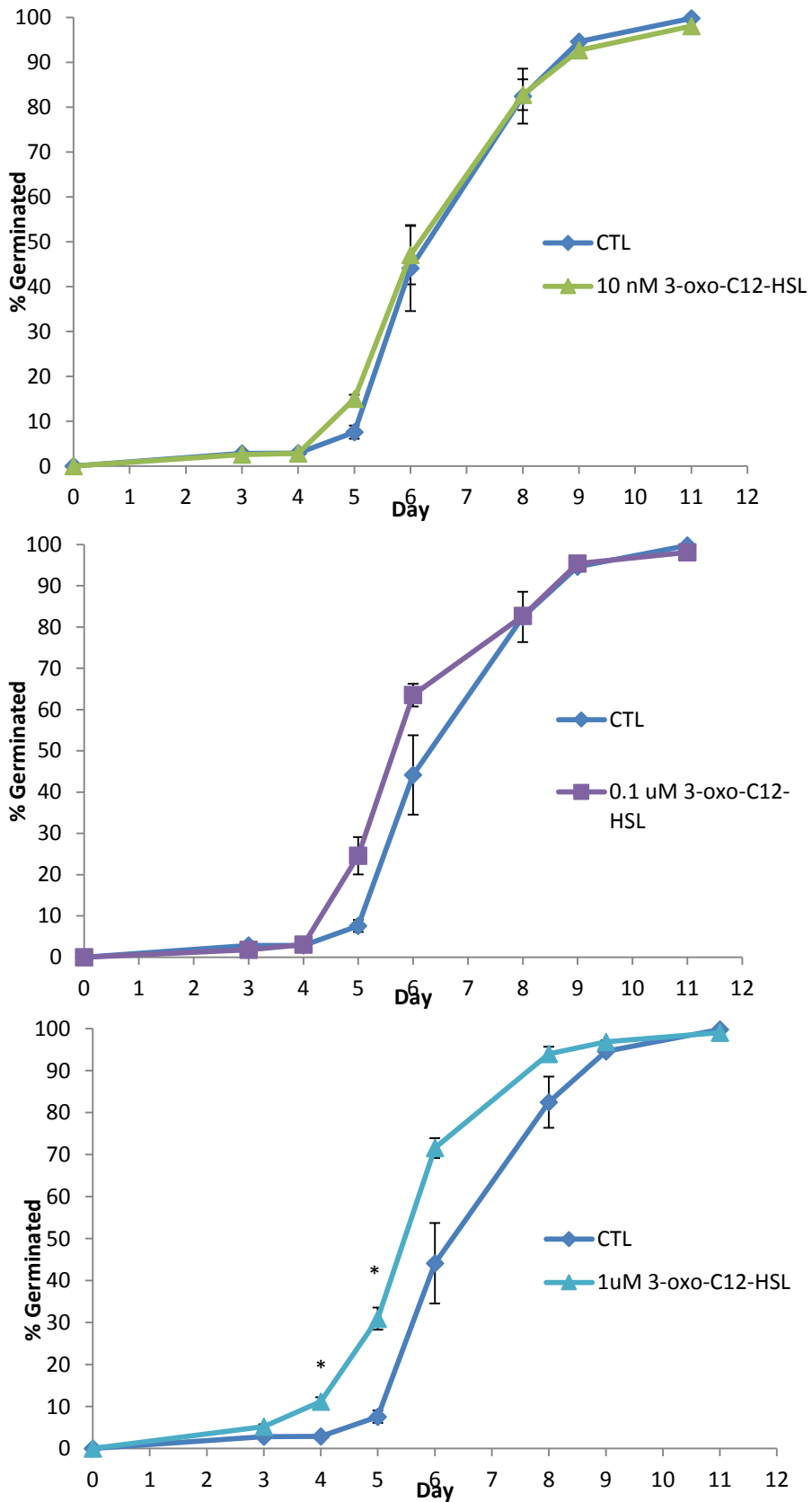
To extend the data collected in Figure 12, the effects of differing concentrations on the most biologically active AHLs, C12-HSLs, C10-HSLs and C8-HSLs. 3-oxo-C12-HSL consistently shows promotion of WT spore germination in a dose dependent manner, as levels increase so does the level of promotion it has on germination (Figure 13 and Figure 14). As shown in Figure 10 there is no difference between the Control and 10 nM of 3-oxo-C12-HSL and germination increases at concentrations 0.1  $\mu$ M and 1  $\mu$ M levels. Methanol controls were conducted on these samples and no difference was seen between the methanol control and control (data not shown). Methanol controls were removed for ease of demonstrating the promoting effects of 3-oxo-C12-HSLs.



**Figure 13. 3-oxo-C12-HSL promotes wild type spore germination in a dose dependent manner.**

(n≥400). Error bars = ± SEM. From top to bottom: 4 month old spores and 4.5 month old spores respectively.

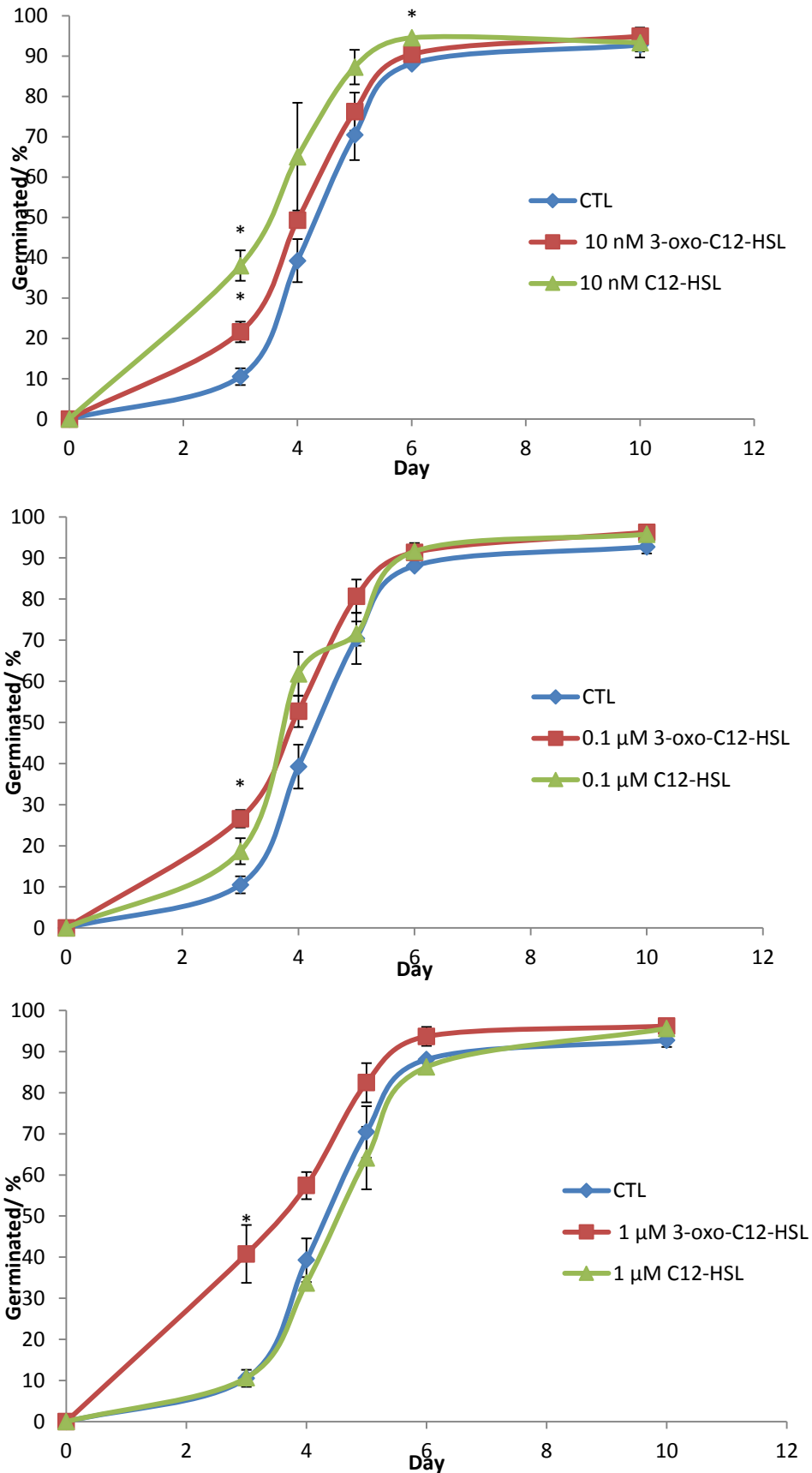




**Figure 14. 3-oxo-C12-HSL increases germination of WT spores in a dose dependent manner on 4 month old spores. Student's T-Test was performed on data. \* indicates results are P < 0.05. Error bars =  $\pm$  SEM.**

### 3.10 Dose Dependence of 3-oxo-C12-HSL and N-acyl-C12-HSL

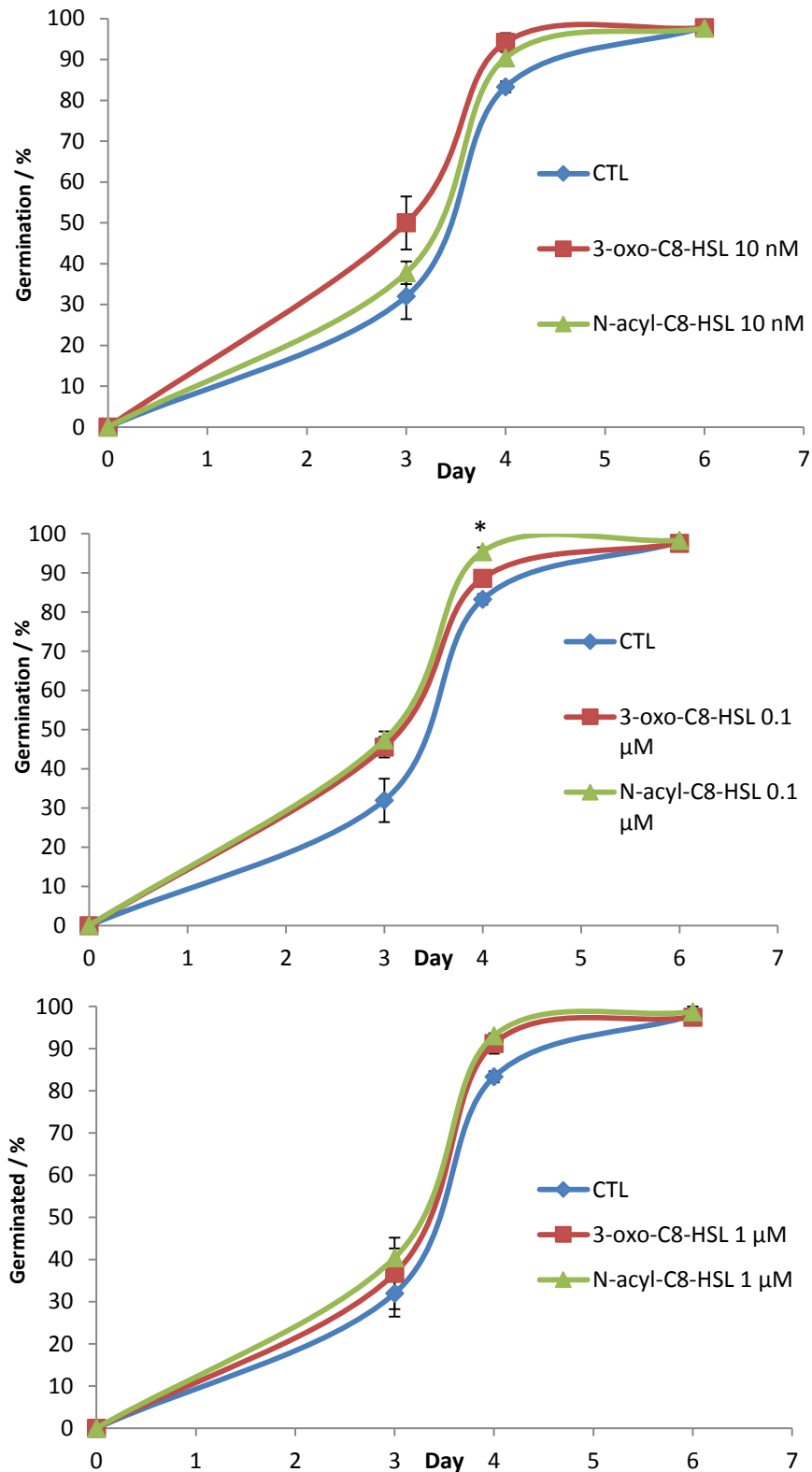
From the initial AHL experiments shown in Figure 12A showed N-acyl-HSLs had less of an effect on germination than the 3-oxo-HSLs we looked into the effects of the 3-oxy and the un-substituted form of C12. Interestingly *N*-acyl-C12-HSL acted in a reverse dose dependent manner compared to 3-oxo-C12-HSL which acts in a dose dependent manner with the greatest promotion seen at 1  $\mu$ M, compared to *N*-acyl-C12-HSL which showed the highest promotion at 10 nM range, as shown in Figure 15. This may suggest that *N*-acyl-C12-HSL is more potent than the 3-oxy form as it has greater effects at lower concentrations.



**Figure 15. Effect of 3-oxo-C12-HSL and N-acyl-C12-HSL on WT spore germination.** 4.5 month old spores at concentrations from 10 nM – 1 μM on BCD with 3 day cold treatment. Students T-Test was performed on data \* indicate results are significant where  $P < 0.05$ .

### **3.11 Dose dependence of 3-oxo-C8-HSL and *N*-acyl-C8-HSL**

3-oxo-C12-HSL acts in a dose dependent manner with *N*-acyl-C12-HSL acting in a reverse dose dependent manner. However, the opposite is true of C8-HSLs where the 3-oxo has the greatest effect at 10 nM concentrations as shown in Figure 16. The effects seen with C8-HSLs are not as great as the effects by C12-HSLs suggesting that longer chain HSLs have a greater bioactive effect on germination even at the 10 nM range. Similarly, methanol controls have been conducted on these experiments but have been removed for a more clear representation of germination rates. The methanol control showed no difference compared to the Control (Appendix 1).

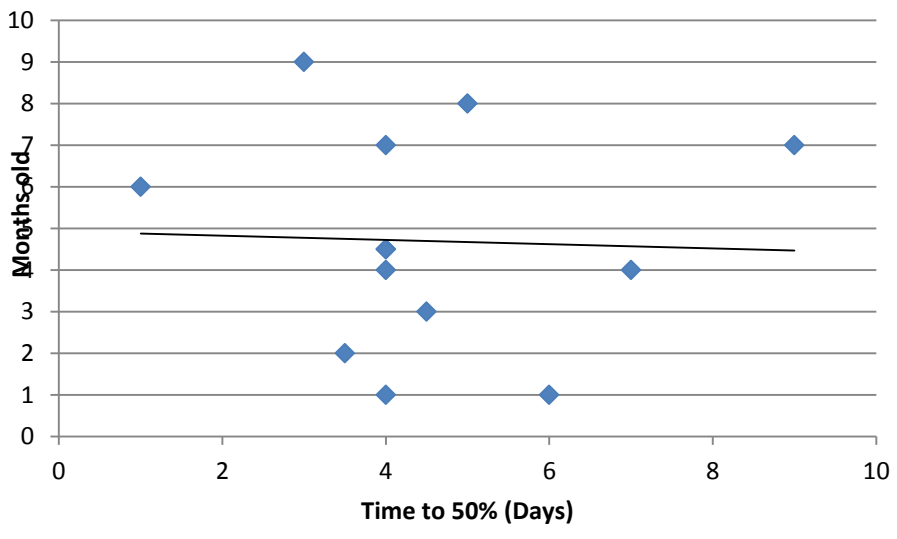
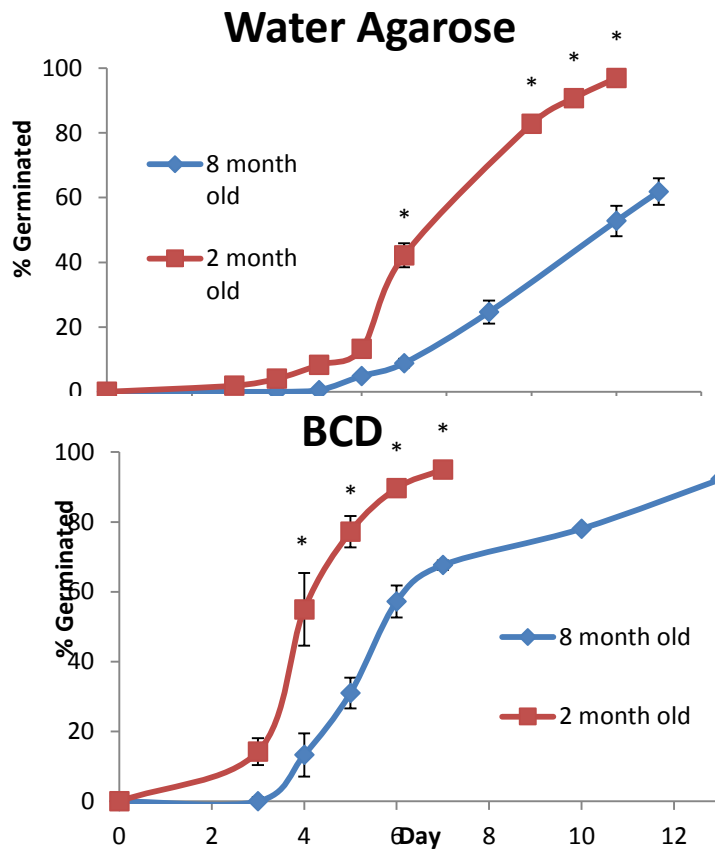


**Figure 16. Dose dependent response of 3-oxo-C8-HSL and N-acyl-C8-HSL. 9 month old WT spores. Students T-Test was performed on the data. \* indicates data is significant where P < 0.05. Error bars = ± SEM.**

### **3.12 Age of Wild type spores has no effect on germination rates**

Seeds undergo a period of primary dormancy, described as the inability of viable seeds to germinate even under positive conditions (Bewley 1997), which chilling breaks (Penfield *et al.* 2012). During most of these experiments the spores have been cold treated in the dark for 3 days before being moved to growth conditions. However, spores may not have a primary dormancy period that seeds have as they are developmentally different. The WT spores are assumed to all be healthy and germinate to greater than 85% and are grown on nutrient rich BCD media. However, when adding exogenous hormones or AHLs we are looking at promotion of germination. If the spores are already at the peak of fitness we may not see any great effects when adding molecules that aid in quick germination because the spores have all they need to germinate well. Growing spores on water agarose that has no added nutrients we have found that spores still germinate to the maximum potential but do so more slowly allowing us to catch any early effects more clearly as shown in figure 9.

As to whether spores have a primary dormancy period like seeds our results show this is unlikely. When two spore batches of 2 month and 8 month old spores were plated without chilling on BCD and water agarose we found the newer spores germinated faster than the older spores. If spores have a dormancy period we would expect the older spores to germinate faster. In figure 17E we looked at the different age spores used in these experiments and the time taken to germinate to 50%. We found no correlation between spore age and germination rates suggesting any difference in germination rate is purely due to spore variation.



**Figure 17. Effect of age and media on spore germination.**

A and B look at the effect of spores grown on either BCD or water agarose. Student's T. Test was performed on data.  $P < 0.05$ . C looks at the relationship between spore age and germination rates on BCD media with standard 3 days cold treatment. Linear trend-line fitted.

### 3.13 RNA Extraction

Bryophytes and algae are underrepresented in genome sequences, although with the increase in speed and the drop in price along with the greater interest in sustainable energy from algae biofuels and greater need for conservation has meant this is being improved. To study what proteins are expressed during germination we would like to compare the transcriptome of spores, newly imbibed spores and protonemal tissue of *Physcomitrella* and that of *Ulva sp.* Like *Physcomitrella*, *Ulva* produces spores. Using transcriptome studies can be important at looking at what proteins are expressed indifferent tissues at different developmental stages or conditions.

**Table 1. Nano drop analysis of RNA Extraction from *Physcomitrella* protonemal tissue:**

260/280	2.24
260/230	0.75
ng/ $\mu$ L	108.3
0.96 g wet protonemal tissue using Bioline Isolate II Plant RNA Kit.	

RNA extraction of *Physcomitrella* protonemal tissue worked well enough using a commercial kit. 260/280 ration should be around 2.1, any lower indicates protein contamination. However the 260/230 ratio should be around 2.0 is significantly lower at 0.75 indicating the presence of organic contamination probably from poor removal of reagents used in the isolation procedure (Farhat, Y. 2012). This may interfere with reverse transcription experiments downstream and further washes may be needed or a separate purification step. Protonemal tissue is rapidly growing filaments and we



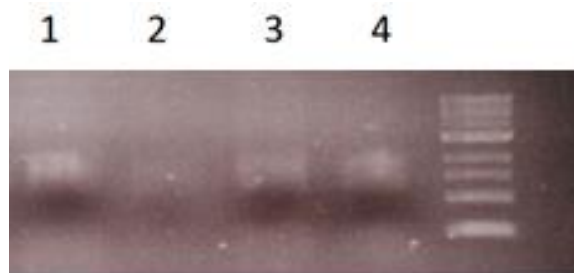
would expect there to be large amounts of RNA in this sample. The kit yielded  $108.3 \text{ ng}/\mu\text{L}^{-1}$ , around half the total binding capacity of the columns used.

### **3.14 *Ulva* sp. as a model system for *Physcomitrella***

Macroalgae like *Ulva* sp. produce spores similar to *Physcomitrella* and could be used as a model to study effects of *Physcomitrella*. Looking at mRNA in certain tissues gives us information about gene expression in these tissues under certain conditions. RNA extraction in plant tissues comes with several problems that need to be overcome. Although commercially available kits like the one used above work well on many plant species and tissues they can be ineffective on more troublesome tissues. The two main problems arising from RNA extraction in *Ulva* was physical cell lysis and polysaccharide and polyphenolic compound contamination. *Ulva* sp. contain high amounts of polysaccharides in the cell wall termed Ulvans (Robic *et al.* 2009). Polysaccharides can interact with nucleic acids irreversibly and render RNA useless in downstream molecular tests (Hilario and Mackay. 2007). Using TRIZOL method to extract RNA from *Ulva* yielded poor quality RNA as shown in Table 2. The results obtained from molecular studies of RNA depend entirely on the quality of sample obtained; mRNA sequencing studies require around  $100 \text{ ng}/\mu\text{l}$  of pure sample, higher than any samples produced by TRIZOL extraction.  $260/280$  and  $260/230$  are preferably greater than 2 but in this method they are significantly lower showing contaminations, most probably of polysaccharides and phenolic compounds making samples unlikely to be able to be used for sequencing studies.

**Table 2. TRIZOL Method: RNA extraction from *Ulva* blades**

<b>Table 2.</b>		
Sample 1	Ng/ $\mu$ L	106.7
	260/280	1.74
	260/230	0.34
Sample 2	Ng/ $\mu$ L	104.2
	260/280	1.76
	260/230	0.32
Sample 3	Ng/ $\mu$ L	82.8
	260/280	1.76
	260/230	0.31
Sample 4	Ng/ $\mu$ L	71.9
	260/280	1.71
	260/230	0.31



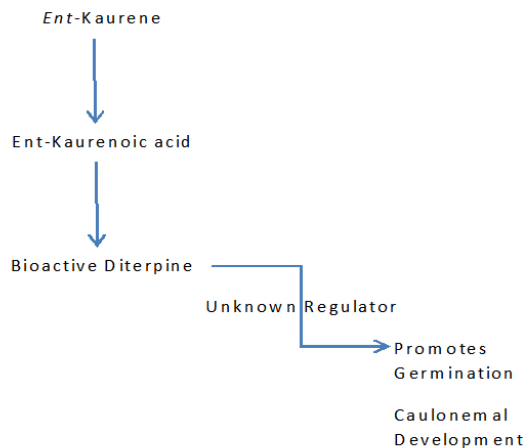
**Figure 18. 1% Agarose gel of RNA samples isolated from *Ulva* blades.**

4  $\mu$ l RNA, 2 $\mu$ l 6X loading dye was loaded into each well.

The amount of mRNA in the samples is not easily distinguishable; most of what the sample contains is ribosomal RNA which is more stable than mRNA. Other methods of RNA extraction from difficult tissues are available and will be discussed later.

## 4. Discussion

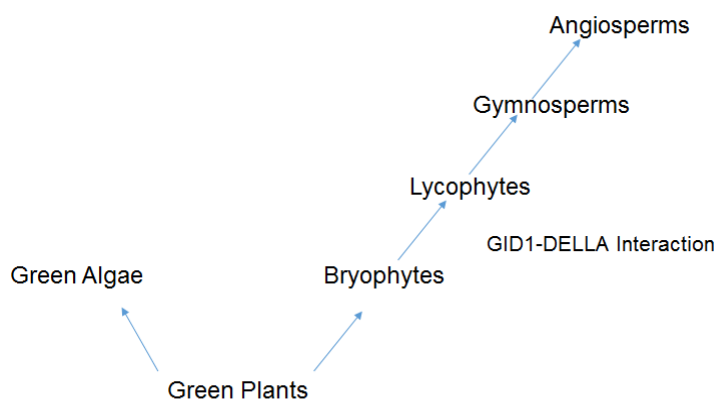
### 4.1 Gibberellin Signalling in Moss



**Figure 19.** Schematic diagram showing the role of ent-kaurene and the effect on *Physcomitrella* development. Adapted from Hayashi *et al.* 2010

The results show there is strong evidence that there is gibberellin signalling in moss that promotes germination with other studies support. When *ent*-kaurene is eliminated from the system we see reduced germination rates and inhibition of caulonemal development (Figure 19). Using a knockout of a gene is preferred than using inhibitory molecules. For instance Anterola *et al.* (2009) used the *cps* inhibitor AMO-1618. However, the phenotype was not completely rescued by addition of *ent*-kaurene leading Hayashi *et al.* (2010) to suspect other inhibitory activities of AMO-1618. The effect we see in the *cps/ks* KO we know not to be due to other factors. We propose a product downstream of *ent*-kaurene affects germination rates and caulonemal development. In studies using a gibberellin synthesis inhibitor, Paclobutrazol, was found to inhibit moss growth; however this substance does not inhibit *ent*-kaurene production (Ross and Reid. 2010) suggesting something downstream of *ent*-kaurene that is the bioactive gibberellin-like compound.

There are still many questions to be answered. One of the main hindrances in identifying homologues and analogues of these regulatory proteins is the lack of plant whole genomes at key nodes of the phylogenetic tree (Chen *et al.* 2013). Although GA regulatory proteins have been identified the classic interaction of GID1-DELLA proteins has not been observed in mosses but is present in lycophytes so the ability for GID1 to interact with DELLA proteins may have happened after lycophytes split from the



**Figure 20.** Simplified phylogenetic tree showing where the GID1-DELLA interaction may occur.

bryophytes (Figure 20; Yasumura *et al.* 2007). Two DELLA proteins have been identified in *Physcomitrella* but the functionally conserved sequences are less conserved (Chen *et al.* 2013). Yasumura *et al.*

(2007) looked at the effect of a double DELLA knockout mutant but found no effect on colony growth so they concluded DELLA has no involvement in gibberellin signalling in moss. Although moss are extremely resilient and are found in habitats where other plants cannot survive, perhaps like other effects like the *cps/ks* KO, changes are only seen early on during germination. From analysis of the knockout of PpDELLAb protein we have found a promotion in moss spore germination (E. Vesty. Unpublished raw data. 2013) suggesting DELLA proteins do have involvement in gibberellin signalling in moss.

Other papers have looked into gibberellin signalling in moss but no single conclusion has been drawn due to difficulties in experimental methods. These tend to be due to the fact they are using GAs that are bioactive in angiosperms. Many bioactive GAs in seed plants have no effect on mosses or ferns but other GAs that have no biological activity in seed plants have been observed to have an effect in mosses and ferns, like GA<sub>9</sub> methyl ester which we have also observed. Because the moss did not react to the GA used in the experiment does not mean gibberellin signalling does not exist but merely the GA used does not have a bioactive effect in moss. What is the signalling behind this and how is the system regulated?

#### **4.2 Long Chain AHLs promote moss spore germination**

Along with gibberellin signalling in moss we have found that AHLs with chain lengths of 8 or more promote germination in *Physcomitrella*. In nature moss are found in diverse habitats but are usually close to the ground. Do mosses even have contact with AHL producing bacteria? The answer is probably yes. Sampling of the rhizosphere associated with wild oats found that 24% bacteria had AHL producing capacity (DeAngelis *et al.* 2008). We know that AHL producing bacteria are in the soil but what concentrations can we expect? In this study we started with 0.1  $\mu\text{M}$  as other studies had found effects at the nM and  $\mu\text{M}$  range (Ortiz-Castro *et al.* 2008; Bai *et al.* 2012), however Rad *et al.* (2008) suggests AHL concentrations in biofilms can exceed 10  $\mu\text{M}$ . We started with the lower concentration so that if we saw an effect we could be confident this was close to physiological levels. For further testing we used a range of concentrations from 10 nM-1  $\mu\text{M}$  which had significant effect on germination of wild type spores, but are these physiologically accurate. If AHL-producing bacteria form

biofilms on moss the concentrations of AHLs that the plant would come into contact with would be quite high, however if the bacteria were a more diffuse population the local AHL concentration would probably be negligible. Moreover it is not just the AHL signals the bacteria would be in contact with but the products of quorum sensing gene regulation. These bacteria may be pathogenic or symbiotic or have no relationship at all.

The AHLs and other hormones used in these experiments are dissolved in methanol and stored at -20 °C. Solvents like methanol have an inhibitory effect of spore germination so as well as a control containing the largest amount of methanol used in the highest concentrations of the experimental conditions the other conditions were also matched so the same amount of methanol was present in all conditions. However, we would not expect to see inhibition of germination as the levels of methanol in different experiments were minimal, ranging from 53.6 µl to 9.4 µl in 75 ml of media.

How stable are the AHLs in the media and for how long? This affects the availability of AHLs for the moss spores. AHLs were first discovered in marine environments involved in the bioluminescence of *Vibrio fischeri* (García-Aljaro *et al.* 2011). In this environment AHLs are under the influence of abiotic degradation and quorum quenching activities of other bacterial species. AHL stability seems to be affected by chain length and oxo substitution as well as temperature and pH of the media (Hmelo and Van Mooy. 2009). Yates *et al.* (2002) found that from 22-37 °C degradation of AHLs increased rapidly and that more alkaline media aided degradation. C4-HSLs had the highest degradation rates and that C12-HSLs were more stable (Yates *et al.* 2002). On top of this, AHLs with an oxo substitution were more stable than unsubstituted AHLs (Hmelo and Van Mooy. 2009). It is unlikely that

the degradation rates of short-chain AHLs account for the effect we see; short-chain AHLs have no bioactive effect of moss germination. This is because we are only looking at the very early effects so the spores only need to have availability to the AHLs in the media for a short time for us to see the effects. Other degradatory effects of AHLs are possibly not applicable either as spores were grown at 22 °C and the media used was pH 7 and water used for addition of chemicals was on the acidic side of neutral.

We are confident that the effects shown by the AHLs as well as being statistically significant definitely in AHLs with chain lengths of C8 and greater are caused by the AHLs and no other experimental error. Anterola *et al.* (2009) used inhibitors of CPS/KS enzyme but this may have other more general effects (Hayashi *et al.* 2010).

We have identified that moss can react to AHLs but no LuxR-like proteins have been identified in *Physcomitrella*, however, this is not surprising. The low homology between LuxR proteins means they are difficult to identify. So what controls the effect AHLs have on moss germination? *Physcomitrella* contains certain uncharacterised enzymes that are capable of metabolising AHLs (cosmoss). The enzyme acylase cleaves the lactone ring from the fatty acid which can then be used for energy (Chong *et al.* 2012). Could it be that the moss is metabolising these molecules and using them for energy for germination and growth? Transcriptome studies to look at changes in proteins produced would be useful to look into this to see if metabolising proteins are upregulated. In the wild AHL producing bacteria also have links to nitrogen fixation (DeAngelis *et al.* 2008), could it be that the moss can perceive the AHLs and that gives information about the suitability of the environment and tells them to germinate? It

would also be interesting to look into what signalling pathways AHLs activate using 2<sup>nd</sup> messenger scavengers to look into the effects.

### 4.3 Spore Batch Variation

Using a plant that produces spores rather than seeds produced some problems. One of the main issues when looking at *Physcomitrella* was focussed on spore quality and variation. Although each moss plant is grown and kept in controlled conditions there are large variations between germination times in different spore populations. When sporophytes are harvested is largely subjective when we gage that sporophytes look large, round and go a dark brown colour, however we need to catch them before they burst so judgment about when to pick them is needed.

In our study we found that although the germination potential of different conditions might be the same the rate at which the germinated could differ dramatically at different time points in the experiment. Gibberellins are well characterised in seeds for controlling germination and it is its interaction with ABA that controls dormancy. Seeds go through a quiescent stage where even though environmental conditions could be favourable to germination, the seed will not germinate, termed primary dormancy. In *Arabidopsis* chilling is used to break primary dormancy (Penfield *et al.* 2012). However, primary dormancy has not been established in moss spores and may not even be present (Zanten *et al.* 1976). In this study we looked at the effect of chilling and not chilling spores before germination and found there was little difference between the two conditions and any difference seen was due to sporophyte batch variation. Therefore chilling or not chilling spores before germination makes little difference. Maybe a small difference is that when cold treated they are on the agar



plates, allowing longer imbibition time before being moved to growth conditions so that germination occurs faster. Time taken for spore populations to germinate to 50% and how long the spores were in storage was also looked into. We found no correlation between age of spores and germination rates. During germination assays spores were subjected to 24 hour light conditions. This is an artificial system in that in nature they would be under 16:8 light dark or similar conditions. This level of exposure may stress be a stress on the plant system, although spores grew normally and to mature levels under these conditions. Some studies grow spores under 16:8 light dark conditions.

#### **4.4 Fluorescence Microscopy**

For the germination assays spores were used but for the microscopy we used homogenised tissue. Using homogenised tissue is not ideal as it represents a somewhat artificial growth. Mature colonies are blended in sterile water and then plated on media plates; moss is very hardy and can regenerate from filaments. What is being grown here is already growing tissue so translation and transcription is already active and have been before the tissue was homogenised. For a more realistic look at the promotion of the *Ppcps/ks* it would be preferential to look at it from spore germination to see when the protein is first expressed. However, it does give a nice idea as the levels of production and a crude idea where the protein may be most active in the colony. However, there are issues with this strain. This strain does not produce gametophores. During transformation it is possible the restriction enzyme was not heat deactivated which may produce multiple inserts of YFP at different locations if the YFP insert had the same restriction site as the endonuclease that was used to linearize the

plasmid (Pers. Comms. Brian Christopher King. 2014). Therefore this needs to be repeated with another YFP-*cps/ks* knockout strain to compare results.

#### **4.5 RNA Extraction**

RNA extraction from plant tissue can pose many difficulties compared with that of animal cells and bacteria due to the tough cell walls and different components such as lignin and polysaccharides. In this study we looked at RNA extraction from *Ulva sp.* using TRIZOL with limited success. The first hurdle was to grind the plant material to a fine powder without the tissue thawing in the presence of liquid nitrogen. If the tissue is allowed to thaw when the cells are being lysed the cells die and RNA starts to degrade. Supporting eppendorfs in liquid nitrogen and grinding the tissue in this method was not effective and the tissue did not remain frozen, the most effective method required the tissue to be put into a mortar and liquid nitrogen added directly to it and ground in this way making sure to top up the liquid nitrogen as it evaporated. Although this allowed the tissue to remain frozen and looked to be fully homogenised when added to the TRIZOL and further ground it was clear this method would not result in the fine powder called for in the protocol and remained in relatively large pieces of tissue. Secondly, the samples produced via this method are not pure. Polysaccharides co-precipitate with RNA irreversibly rendering the RNA useless for downstream studies. One way of reducing this risk is to use a method that involves heating the sample, this destabilises the polysaccharides making them more soluble, and the TRIZOL method does not contain this step, possibly explaining the levels of contamination in the samples (Hialrio and Mackay. 2007). There are other methods

that can be used for particularly troublesome plant samples. Another commonly used protocol for RNA extractions is the CTAB and hot phenol acid methods.

The CTAB protocol uses different techniques to avoid organic contamination in nucleic acid samples. Buffers in these experiments contain polyvinylpyrrolidone (PVP), this binds to polyphenols and polysaccharides and can then be removed from lysis by centrifugation (Chan *et al.* 2007). Increased levels of sodium dodecyl sulfate (SDS) in buffers is used to remove proteins and stop activity of ribonucleases. SDS denatures proteins making them insoluble and again removed by centrifugation (Rio *et al.* 2010).

#### **4.6 Media – BCD or Water Agarose**

Most studies on moss use standard BCD media for growth (Hayashi *et al.* 2010; Anterola *et al.* 2009). This is perfect media for spore germination and growth but when looking at promotion is it the best choice? To study difference in germination you do not want the spores to germinate too quickly otherwise any subtle effects may be missed. Even without the nutrients from BCD media we know that moss can survive on water agarose.

#### **4.7 Statistical Choice**

In many germination studies the maximum level of statistical analysis done is that the error bars show  $\pm$  the standard error of mean and if the bars do not overlap the data is significant (Hunt *et al.* 2007; Browne *et al.* 2005; Hayashi *et al.* 2010; Rad *et al.* 2008). In seed germination assays the germination index is used, however this requires seeds to be measured at the same time point each day for seven days. In our germination assays spores were not always counted every day and it often took longer

than a week for the spores to reach > 85% germination. In this study we used an unpaired Student's T-Test to analyse the significance of the experimental condition against the control on each day. Although there is a clear difference in many of the conditions looking at the error bars in many cases the data was not statistically significant. This is probably due to the fact that although we are collecting large amounts of data (>200 spores per plate) this is then condensed into percentages and in most experiments only 2 plates are used. Although we have repeated the experiments we cannot use the repeats in statistical analysis due to spore batch variation. However, when we see only one day is statistically significant in the data, like in Figure 1, and the error bars do not overlap we are confident that all the differences seen are real.

#### **4.8 Further Study**

The gibberellin pathway is inescapably tied to ABA signalling. We would like to look at the effect of moss spore germination when spores grown on inhibitory ABA plates are given AHLs that promote germination.

To further study the effect of AHLs on spore germination would be to look at the effect of bacterial extracts that produce AHLs and see if this has any effect on germination. In nature systems are never monocultures, there is likely to be a large number of different AHLs being produced and this may have different effects on moss germination. It is very difficult to co-culture bacteria with moss. We spend time making sure our cultures are axenic, when introducing bacteria into the system we need to assure it is the right bacteria and not laboratory contamination.

## 4.9 Conclusion

After the draft genome sequence of *Physcomitrella patens* was published in 2008 (Zimmer *et al.* 2013) increased interest has been shown in this evolutionary important organism and has given information to allow comparative and evolutionary studies to take place. Studying *Physcomitrella* can give insights into signalling control and evolutionary adaptations in higher plants. In conclusion we have provided compelling evidence for the role of gibberellin signalling in moss with similar function to seed plants which was previously controversial. We have also provided evidence that long chain AHLs promote spore germination and that the most important factor is chain length and not substitution states.

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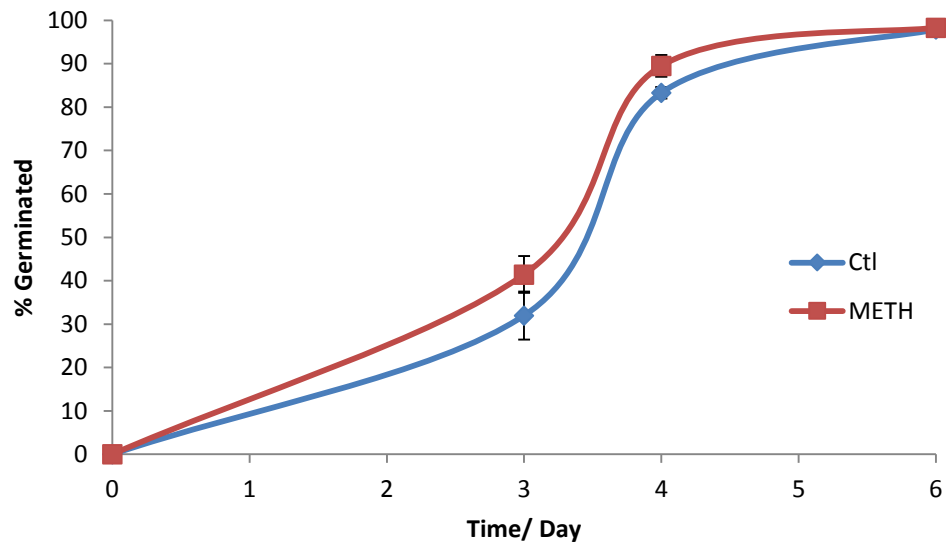
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## Appendices

### Appendix 1.



**Appendix 1.** Control and Methanol control for tests against C8-HSLs show no significant differences. 9 month old spores. Error bars =  $\pm$  SEM. Student's T-Test was performed as described.

## **Part Two: Investigation of the interaction between PGE<sub>2</sub> and phospholipase B1 in a *Cryptococcus*/macrophage infection model**

### **1.1 Overview**

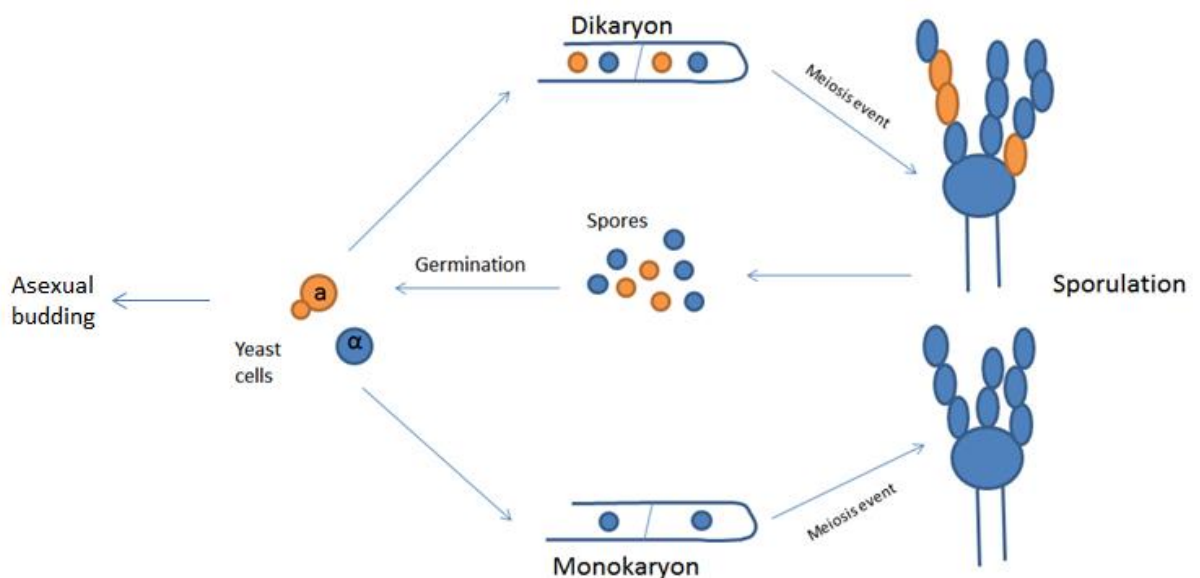
*Cryptococcus* is an opportunistic intracellular fungal pathogen of the basidiomycetes family that represents a life threatening disease in immunocompromised individuals such as those suffering from HIV/AIDS and organ transplant patients (Virtudazo *et al.* 2011). As such, incidences of this disease have increased in the last 30 years with the spread of HIV/AIDS pandemic with around 1 million cases documented each year (Alanio *et al.* 2011)(Giles *et al.* 2009) focused in sub-Saharan Africa (Alanio *et al.* 2011). In many of these cases the infection disseminates to the central nervous system (CNS) causing meningoencephalitis, the leading cause of mortality in individuals with HIV with a 30-60% mortality rate after 12 months (Cohen *et al.* 2010). *Cryptococcus* is rarely a problem for immunocompetent individuals and many infections are characterised by an asymptomatic pulmonary infection (Voelz and May 2010). The two medically relevant species of *Cryptococcus* are *neoformans* and *gattii* (Voelz and May 2010). This project focuses on *C. neoformans* and the effect of prostaglandins during infection in macrophages.

Although *Cryptococcus* usually only causes serious disease in immunocompromised individuals, new evidence is arising showing up to 25% of cases in the US are found in patients with no obvious immune deficiency. These are generally caused by the *C. gattii* strain (Olszewski *et al.* 2010). By understanding the biological interactions of *Cryptococcus* with cells of the innate immune system it may lead to improved, novel therapies.

## **1.2 *Cryptococcus* life cycle.**

*C. neoformans* is found globally and in diverse environments. This pathogen is highly adaptable and as well as living in environmental conditions is capable of surviving harsh physiological conditions. *Cryptococcus* does not just infect mammals but has a diverse range of hosts including plants and invertebrates (Olszewski *et al.* 2010) and is mostly reported to be associated with bird guano (Kronstad *et al.* 2011). Due to its abundance in nature, contact with this pathogen is common and many people exhibit antibodies against *Cryptococcus* at an early age. Goldman *et al.* (2001) showed that 56% of children aged between 2-5 years old possessed antibodies that recognised several *Cryptococcus* proteins which increased to 70% of children aged over 5 years (Goldman *et al.* 2001).

Unlike other fungi where the spores are usually the infectious agent, it appears that in *Cryptococcus sp.* both the spores and desiccated yeast form can cause disease when inhaled (Voelz and May. 2010). Asexual budding is the only type of reproduction that has been reported during infection, although *Cryptococcus* can also reproduce sexually (Cox and Perfect. 2013). Sexual reproduction results in the formation of basidiospores which then germinate to the yeast form (Ellis and Pfeiffer. 1992) as shown in the Figure 1, below.



**Figure 1. Simplified life cycle of *Cryptococcus* from the haploid spore through sporulation.** Showing the sexual life cycle from the yeast cells to sporulation and germination of the spores to yeast cells and asexual budding. (Modified from Idnurm *et al.* 2005)

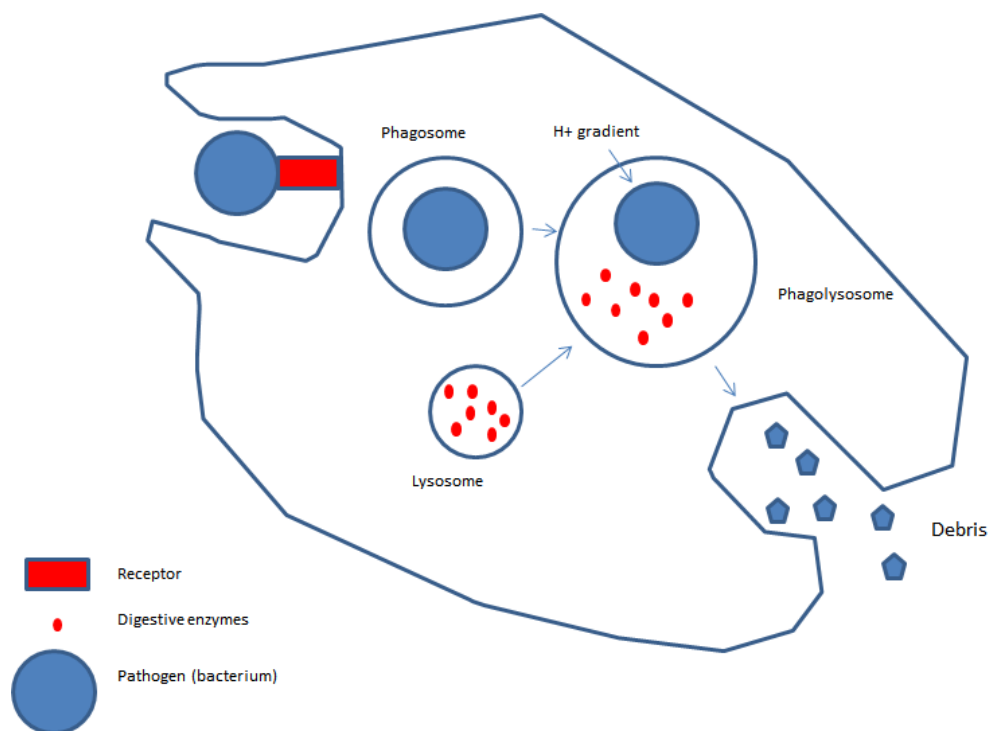
### 1.3 Interaction with the Immune System

Although individuals with AIDS have a working innate immune system the adaptive immune response is necessary for protection against the disease (Huffnagle *et al.* 1994). However, interactions with the innate immune system still has an impact on the clinical outcome of the disease, as will be discussed later (Sabiiti *et al.* 2014). The innate immune system is the first line of defence against pathogens and is involved in mediation of the adaptive immune response. One of the first cell types *Cryptococcus* comes up against are macrophages in the lungs. These phagocytic cells engulf and destroy foreign particles, apoptotic cells and cellular debris and use several defence mechanisms to kill pathogens.

The first step of phagocytosis is recognition of foreign particles using a number of receptor-ligand interactions; PAMP recognition receptors (PRRs) can recognise Pathogen-associated molecular patterns (PAMPs) on the bacteria (Poeta. 2004). Toll-like receptors (TLRs) bind cell wall components like mannose that bind to yeasts and  $\beta$ -glucan present in fungal cell walls (Bogdan. 2006). The complement system can bind covalently to pathogens, opsonising them which phagocytic cells can recognise via complement for engulfment. The complement system can also act as a chemo-attractant to recruit more phagocytic cells and activate these once they have reached the site of infection (Janeway. 2001). Activation of macrophages induces the production of cytokines that influence the adaptive immune system. Once the adaptive immune system has been activated, T-cells can produce antibodies that bind to the pathogen, and again, phagocytic cells can recognise these via Fc receptors. The C3 component of the complement system is the main opsonin for *Cryptococcus neoformans*. Although stated previously that we all have antibodies against *Cryptococcus* from an early age, these do not usually bind due to a large fungal capsule that surrounds the yeast cell. Macrophages have three C3 receptors, CD35, CD11b and CD11c, of which all three have been found to bind *Cryptococcus* (Levitz. 2002).

## 1.4 Macrophage killing

Once macrophages have recognised and engulfed the foreign particle they have different ways of killing them. When the particle is phagocytised it is taken into a phagosome, this fuses with a lysosome to form a highly acidic vesicle with a pH lower than 5.5 called the phagolysosome that inhibits microbial growth as shown in figure 2 (Voelz and May. 2010). Macrophages also produce reactive oxygen (ROS) and nitrogen (RNS) species, this causes oxidative damage to the microbes DNA and proteins as well as other important molecules. As well as the acidic environment and



**Figure 2. Simplified diagram of phagocytosis.** The pathogen is recognised by receptors on the macrophage cell surface, the pathogen is then engulfed and killed in the phagolysosome and debris is released.

oxidative stress, the vesicle in which the microbe is held in is a low nutrient environment for molecules like iron (Fe), arginine and tryptophan, inhibiting pathogen growth (Bogdan. 2006).

*Cryptococcus* is an intracellular pathogen. This means it is capable of living inside macrophages without being killed by these antimicrobial efforts. *Cryptococcus* exhibits a number of characteristics that help to protect it against macrophage killing.

### **1.5 Polysaccharide capsule**

One of the best studied virulence factors of *Cryptococcus* is its large polysaccharide capsule that inhibits phagocytosis by acting as a physical barrier between the cell wall and receptors and protects from oxidative damage if phagocytised. It has been shown that hypocapsular strains have an increased uptake by macrophages (Sabiiti *et al.* 2014). The capsule also inhibits the production of inflammatory cytokines and actively depletes complement components, as mentioned previously the main opsonising agent of *Cryptococcus* and results in reduced leukocyte recruitment to the site of infection (Bose *et al.* 2003) and dissemination.

The capsule is mainly made up of glucuronoxylomannan (GXM); this can compose up to 90% of the capsule. The complex structure of the capsule is composed of several proteins. Four of the genes that encode these proteins have already been identified (Steenbergen and Casadevall. 2003) and much research is going on to the elucidation of the function of these genes. In the environment the capsule is relatively small but after infection the capsule can increase in size massively. Several stress factors have been identified in the regulation of capsule enlargement including low iron environments and changes in the pH, which are present inside macrophages (Bose *et al.* 2003).

The capsule has also been identified as playing a role in macrophage damage; *Cryptococcus* releases vesicles filled with a polysaccharide into the macrophage

cytoplasm. This results in dysfunction and ultimately death of the macrophage (Steenbergen and Casadevall. 2003) which then releases any intracellular *Cryptococcus*. Furthermore, the capsule is regularly shed releasing GXM into the extracellular space; the shed GXM from this interferes with I-seletin on neutrophils, reducing extravasation to the site of infection (Olszewski et al. 2010). Virulence is not dependent on capsule size but the presence of one. Greater encapsulated yeast cells are not more virulent than ones with a smaller capsule (Olszewski et al. 2010).

*Cryptococcus* also secretes a number of proteins as virulence factors, such as antiphagocytic protein 1 (APP1) which binds complement receptors 2 and 3 to inhibit complement mediated phagocytosis (Stanos *et al.* 2009). Other enzymes are secreted to help deal with the harsh intracellular conditions, including superoxide dismutase which acts to detoxify reactive oxygen species and nitrogen radicals. (Voelz and May. 2010).

## **1.6 Urease**

Urease acts to convert urea to ammonia and carbamate and is produced in large quantities by *Cryptococcus*. In the ecological niche this is likely to be used to convert urea to a usable nitrogen source for metabolism but during infection is linked to increased virulence. Knocking out the single urease gene, *ure1*, results in lowered virulence in mouse models but no visible defects in capsule production or colony growth. Some pathogenic bacteria and pathogenic fungi also produce large amounts of urease. One such bacterium, *Helicobacter pylori* produces urease which results in a local increase in pH in the stomach allowing the bacteria to survive. Like laccase activity, urease is localised to the cell wall (Cox *et al.* 2000)(Kronstad *et al.* 2011).



## 1.7 Melanin and Laccase

Melanin is a negatively charged pigment that is localised to the cell wall that maintains its integrity. It is produced by polymerisation of phenolic compounds by laccase, also localised to the cell wall. Melanin is produced using various precursors including L-DOPA, dopamine, adrenaline and noradrenaline, since most of these are neurotransmitters found in the brain it may partially explain why *Cryptococcus* disseminates to the central nervous system (CNS) so readily (Steenbergen and Casadevall. 2003). Although, melanin protects *Cryptococcus* from phagocytosis, oxidative bursts and antifungal peptides, increased production of melanin has not been correlated to increased virulence, however increased laccase activity has, suggesting laccase has other roles in *Cryptococcus* virulence (Sabiiti *et al.* 2014).

## 1.8 Phagolysosome acidification

When a *Cryptococci* was taken up by a macrophage the phagosome fuse with a lysosome creating a highly acidic environment that *Cryptococcus* can survive in, however with evidence showing that artificially increasing the pH leads to reduced growth (Voelz and May. 2010). Unpublished research studying real time microscopy in conjunction with lysotracker has shown that the phagosome containing a *Cryptococci* does not become acidic. Lysotracker fluoresces red when a vesicle becomes acidic. However, when the experiment was repeated using heat killed *Cryptococci* the compartment does fluoresce red and is therefore acidic, suggesting the *Cryptococci* are having an effect on the macrophage to stop the acidification of the phagolysosome (Smith, L. 2014. Unpublished raw data).

## 1.9 Titan Cells

Another distinctive aspect of *Cryptococcus* infection is the emergence of titan cells, a subset of *Cryptococcus* cells that can reach 100  $\mu\text{m}$  in diameter, in contrast to the 5-7  $\mu\text{m}$  size of normal *Cryptococcus* cell. However, titan cells are arbitrarily defined as anything above 15  $\mu\text{m}$  in diameter (Zaragoza and Nielson. 2013) and are involved in increased survival, dissemination and latency (Zaragoza and Nielson. 2013). Commonly with cells of a larger size, titan cells have an enlarged vacuole and increased DNA content, being polyploidy whereas normal cells are haploid and represent around 20% of *Cryptococcus* cells present in the lungs during infection (Okagaki *et al.* 2010). Due to the large size of these cells they are not phagocytised, thus promoting persistence in the lungs as they are not cleared. However, the progeny produced by these cells are of a normal size. Many aspects of titan cells remain a mystery and anomalous. The presence of titan cells increases dissemination to the CNS by 300 fold. How this is achieved is unclear. Dissemination is thought to be facilitated by movement of macrophages with the fungal cells inside the macrophages, however, titan cells cannot be phagocytised. Furthermore, Okagaki *et al.* (2010) have shown the presence of titan cells also reduces the phagocytic uptake of the normal sized *Cryptococcus*.

The presence of titan cells has also been correlated with an increase number of eosinophils in the lungs in a murine model. This is typically seen in a Th-2 type response. It has been hypothesised that titan cells may modulate the immune response to result in a non-protective Th-2 type response but how this is achieved is still unknown (Zaragoza and Nielson. 2013). Production of significant numbers of titan cells has only been achieved in *in vivo* studies (Zaragoza and Nielson. 2013).

## 1.10 Dissemination to the Central nervous System

The innate immune system is the first line of defence against infection and consists of physical barriers and immune cells that are designed to halt dissemination, but these may not have the desired effect. One hypothesis as to how *Cryptococcus* disseminates to the brain is the 'Trojan horse' model. Since *Cryptococcus* can survive inside macrophages it may use these as transport to get to the brain (Olszewski *et al.* 2010). *Cryptococcus* can 'squeeze' through the endothelium of blood vessels or cross the blood-brain barrier in a transcellular manner meaning *Cryptococcus* has the ability to travel through the inside of cells to get to the other side (Olszewski *et al.* 2010). All methods of crossing blood-brain barrier have been observed.

*Cryptococcus* is not a natural pathogen of mammals and these virulence factors have evolved to deal with issues faced in its ecological environment, for instance the laccase breaks down lignin and *Cryptococcus* is naturally associated with some trees (Waterman *et al.* 2007) and any anti-phagocytic properties probably evolved via evasion of amoebic predators found in the soil. It is interesting that these factors that allow for its survival in nature make it so adept at evasion of mammalian immune responses. Interestingly many of these factors are anti-phagocytic, yet greater phagocytosis in the body is correlated with increased virulence (Sabiiti *et al.* 2014).

Another interesting characteristic of *Cryptococcus* is a method of escaping host cells without causing death to either cell termed vomocytosis. Escaping from cells without causing damage may aid in dissemination without causing an inflammatory response caused by apoptotic cells. Vomocytosis occurs more often in the presence of cytokines from a Th-1 response compared to Th-2 (Olszewski *et al.* 2010). This may

promote long term persistence. During a non-protective Th2 response the *Cryptococci* can survive within macrophages for a long time with no immune action against them.

### **1.11 Phospholipase B**

Phospholipase B (PLB) is part of a large family of enzymes that cleave fatty acids from phospholipids in the cell membranes by hydrolysing ester bonds (Steenbergen and Casadevall. 2003) and has been identified as a virulence factor in *Cryptococcus* and other fungal pathogens including *Candida albicans* (Noverr *et al.* 2003). PLB1 has been identified in maintaining cell wall integrity, dissemination and survival in macrophages among others (Siafakas *et al.* 2007)(Santangelo *et al.* 2004). Strains lacking phospholipase B have no defects in growth or capsule production but do exhibit increased phagocytic uptake, lower intracellular budding, do not disseminate from the lungs to the brain (Djordjevic *et al.* 2005) and are less virulent (Cox *et al.* 2001) and so PLB1 is thought to be involved in host immune evasion.

Mammalian cells have several phospholipases to cleave fatty acids at different points but PLB1 has PLB activity, lysophospholipase hydrolase and lysophospholipase transacylase activities (Noverr *et al.* 2003), meaning PLB1 can cleave fatty acids from a phospholipid at both the sn-1 and sn-2 points and reattach fatty acids to a free phospholipid head group to remake a functional phospholipid within one active site (Jones *et al.* 2007).

PLB1 has also been identified as being involved in cell wall maintenance. PLB1 is targeted to lipid rafts in the membrane by a glycosylphosphatidylinositol (GPI) anchor at its C-terminus. Inhibition of the GPI anchor inhibits PLB1 secretion and deletion of *plb1* results in a loss of cell wall integrity compared to the wild type strain, H99.

Furthermore, during heat stress PLB1 is secreted less and is targeted to the cryptococcal cell wall (Siafakas *et al.* 2007).

Additionally, PLB1 secretion is Sec14 dependent (Chayakulkeeree *et al.* 2011). *Cryptococcus* has two Sec14 homologues Sec14-1p and Sec14-2p. In contrast to *S. cerevisiae* where Sec14 is essential to viability, Sec14 knockout in *Cryptococcus* is reported to inhibit PLB1 secretion but shows no defects in capsule production and melanisation. Sec14-1p KO exhibits similar characteristics to the plb1 KO and exhibits decreased virulence, vomocytosis and dissemination in murine models (Chayakulkeeree *et al.* 2011) but no obvious defects when grown in a monoculture.

As PLB1 acts on phospholipids in the plasma membrane it is proposed to be involved in the lytic escape by destabilising the host cell membrane as well as the release of lipid signalling precursors (Cox *et al.* 2001).

In mammalian cells phospholipases are used to release lipid precursors like arachidonic acid from phospholipids and glycerophospholipids (Fernandez *et al.* 2010). Arachidonic acid is a major precursor in eicosanoid biosynthesis, which is involved in immune regulation. Studies have shown that *Cryptococcus* and other fungal pathogens produce prostaglandins endogenously (Noverr *et al.* 2003). As well as *Cryptococcus neoformans* and *Candida albicans* a study tested prostaglandin production in a selection of dermatophytes, subcutaneous and systemic fungal pathogens in the presence of arachidonic acid and without. Noverr *et al.* (2002) found that prostaglandins were produced in both cases meaning fungi can produce their own PG and synthesise it from host arachidonic acid (Noverr *et al.* 2002). Noverr *et al.* have shown that *Cryptococcus* mutants lacking PLB1 produce significantly less

prostaglandins than WT strains and reconstituted strains (Noverr *et al.* 2003). From this it is hypothesised that prostaglandins are involved in virulence.

### **1.12 Treatment**

Until around 60 years ago disseminated *Cryptococcal* infection was consistently fatal (Saag *et al.* 2010). But the production of antifungal drugs including fluconazole in the 80's has improved prognosis, yet mortality rates remain excessively high. Treatment schedule depends on the site of infection and the status of the host's immune system. Treatment of pulmonary infections usually involves fluconazole for several months with severe infections treated with the addition of amphotericin B (Saag *et al.* 2010). HIV-associated infection always requires treatment and continued lifelong therapy with fluconazole being recommended.

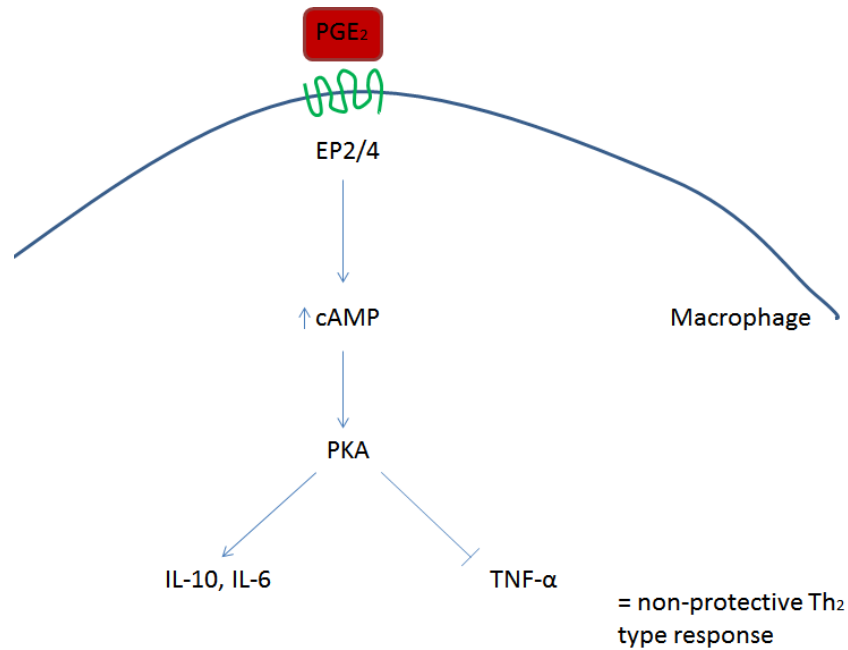
By 1999 fluconazole was used to treat 16 million people in the US alone (Ghannoum and Rice. 1999). This drug acts by decreasing the stability of the cell wall by inhibiting the synthesis of ergosterol, an important component of the fungal cell wall (Abe *et al.* 2009). This increases permeability leading to a reduction in fungal viability. However, the exact effects on the cell membrane are not entirely clear (Abe *et al.* 2009). As well as this, fluconazole resistant *Cryptococcus* has been reported more than a decade ago (Xu *et al.* 2001).

### **1.13 Effects of Prostaglandins**

PGE<sub>2</sub> is an important determinant during infections and is present in high levels during inflammation (Harizi *et al.* 2008). PGE<sub>2</sub> is associated with the classic inflammatory response and induces such responses including redness and swelling. PGE<sub>2</sub> is usually seen as starting the inflammatory response but also has anti-

inflammatory effects (Ricciotti and FitzGerald 2011) such as suppressing lymphocyte proliferation and inducing the production of anti-inflammatory lipoxins (Harizi *et al.* 2008). This identifies the complexities of this system *in vivo* as although starting the inflammatory response it may also have a role in its eventual resolution.

Overproduction of PGE<sub>2</sub> is associated with an increase susceptibility to bacterial infections (Aronoff *et al.* 2007). As already known, PGE<sub>2</sub> is involved in down regulating the killing functions of macrophages. PGE<sub>2</sub> acts via G-protein coupled E prostanoid receptors, EP1- EP4. Different receptors are associated with different outcomes. Binding EP1 results in an increase in intracellular calcium levels whereas EP3 results in lower levels of cAMP via inositol signalling cascades (Legler *et al.* 2010). Using alveolar macrophages, Aronoff *et al.* (2007) has looked into bacterial killing activation. Macrophages present in the lung are the first point of contact for *Cryptococcus*. In alveolar macrophages EP2 and EP4 bind PGE<sub>2</sub> which results in activation of stimulatory G-proteins. This increases levels of intracellular cAMP which activates PKA, which in turn phosphorylates CREB to inhibit the production of TNF- $\alpha$  release and increases IL-6 and IL-10 production (Harizi *et al.* 2008). TNF- $\alpha$  is released in response to bacterial signals including LPS. Inhibiting TNF- $\alpha$  results in lower microbicidal activity by reducing production of ROI (Aronoff *et al.* 2007).



**Figure 3. The intracellular effects of PGE<sub>2</sub> binding EP2/4.** Binding of PGE<sub>2</sub> to the G-Protein coupled receptors EP2 or EP4 results in an increase in intracellular cAMP which activates PKA pathway. This results in an increase in IL6 and IL-10 production and a inhibition of TNF- $\alpha$  production

Immune responses are controlled by T-lymphocytes which PGE<sub>2</sub> interacts with. The immune system has a polarised response; Th-1 and Th-2. T-helper type 1 response produces IFN- $\gamma$ , IL-2 and TNF- $\beta$ . This activates macrophages to help kill pathogens and is marked by an increase in ROS (Romagnani. 1999). In contrast, T-helper type 2 response is marked by production of IL-4, 5, 10 and 13 (Romagnani. 1999) which results in a down-regulation of macrophage functions but also antibody production and eosinophil recruitment, leading to a non-protective response. *Cryptococcus* infection is marked by a shift from Th-1 to Th-2 type response, PGE<sub>2</sub> produced by *Cryptococcus* may be involved in this shift. PGE<sub>2</sub> inhibits T-cell and dendritic cell activation and proliferation (Kuroda and Yamashita. 2003). This results in a Th-1 type response (Obermajer *et al.* 2011).



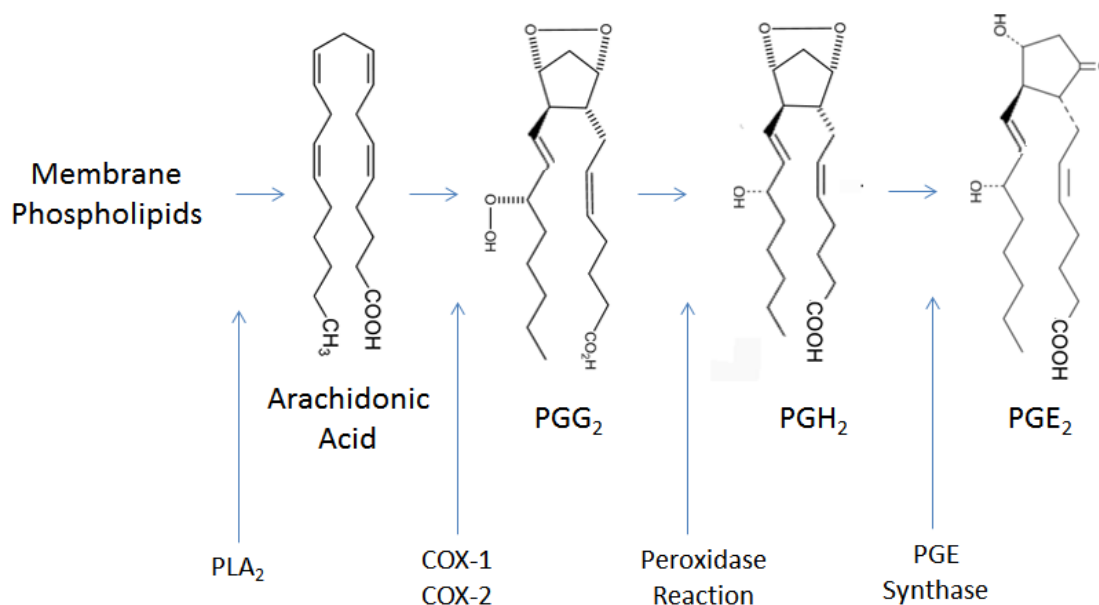
Mackenzie *et al.* (2013) found that PGE<sub>2</sub> in conjunction with LPS activation of macrophages increased IL-10 production via PKC. IL-10 has several effects on macrophages. Increased IL-10 is linked to anti-inflammatory phenotype and it also down regulates MHC Class II molecules that present antigens to immune cells. This acts to reduce activation of the adaptive immunity (Gordon. 2003). PGE<sub>2</sub> has been found to increase IL-10 production and inhibit TNF- $\alpha$  at 0.65 nM levels in mitogen-stimulated splenocytes (Noverr *et al.* 2001).

### **1.14 Eicosanoid Synthesis**

Eicosanoids are ubiquitous immune regulatory molecules in mammals that have diverse effects on the body including regulation of inflammation, allergies and venom toxicity (Fernandez *et al.* 2010)(Harizi *et al.* 2008). Eicosanoids encompass a range of molecules including prostaglandins, thromboxanes, prostacyclins, leukotrienes, lipoxins and epoxyeicosatrienoic acids (EETs) (Fernandez *et al.* 2010). Prostanoid nomenclature is denoted with a letter to identify the oxygen substitution on the ring structure and a number to identify the *cis* double bonds on the fatty acid chain. Those denoted with a 1 are synthesised from dihomo- $\gamma$ -linolenic acid, 2 are synthesised from arachidonic acid and those denoted with a 3 are synthesised from eicosapentaenoic acid (Harizi *et al.* 2008).

*In vivo* these are mostly metabolised from arachidonic acid which is freed from phospholipids in the cell membrane. The presence of arachidonate is dependent on the essential fatty acid, linoleic acid which comes from the diet, but in tissue culture experiments is present in foetal bovine serum (FBS) which is supplemented in the media. Eicosanoids are only synthesised when needed and have a short life, as they

are metabolised quickly, of less than 5 minutes (Legler *et al.* 2010). Humans make around 1 mg of eicosanoids a day which mostly act in an autocrine fashion (Fernandez *et al.* 2010). These are critical molecules with around 25% of all drug targets acting on eicosanoid mediated processes (Fernandez *et al.* 2010). This study focuses on prostaglandins, specifically PGE<sub>2</sub> which is synthesised via cyclooxygenase (COX) activity. Two COX enzymes have been identified in mammals. COX-1 is constitutively active in most cells in the body and is generally regarded as the 'housekeeping' production of PGE<sub>2</sub> (Parente and Perretti. 2003)(Barbieri *et al.* 2003) COX-2 is the



**Figure 4. Schematic diagram showing PGE<sub>2</sub> synthesis in mammalian cells.** Free arachidonic acid is converted to PGG<sub>2</sub> via oxygenation reactions by either COX-1 or the inducible COX-2. PGG<sub>2</sub> is converted to PGH<sub>2</sub> by peroxidase reactions and converted to specific prostaglandins by specific prostaglandin synthases, in this case PGE synthase.

inducible form. COX-2 can be swiftly induced by inflammatory signals including cytokines like TNF- $\alpha$  (Kuwano *et al.* 2004)(Ricciotti and FitzGerald 2011) in cells involved in inflammation such as monocytes and dendritic cells (Parente and Perretti. 2003). COX-2 produces more prostanoids than COX-1 and is thought to be the main enzyme involved in inflammatory response (Parente and Perretti. 2003).

No COX homolog has been identified in *Cryptococcus*, or in any other fungal pathogens. Initial studies used COX-inhibitors to look at prostaglandin production by fungal pathogens. Noverr *et al.* (2001) looked at the COX inhibitor, indomethacin. Although this reduced PGE<sub>x</sub> production by 65% in *C. neoformans* it additionally reduced the viability of the cells; after 24 hours 99.9% of the cells were not viable. Similar effects were also seen in other COX inhibitors including etodolac and piroxicam (Noverr *et al.* 2001).

Evidence provided by Erb-Downward *et al.* 2008 has shown that the mechanism of PG synthesis in *Cryptococcus* involves a unique pathway requiring a laccase enzyme.

*Cryptococcus* possesses two laccase genes, Lac1 and Lac2 (Erb-Downward *et al.* 2008). Generating knockouts of each of the genes PG production was analysed for each strain.  $\Delta$ Lac1 produced significantly less prostaglandins whereas  $\Delta$ Lac2 exhibited no significant difference in PG production compared to the wild type. It was identified that Lac1 played a key role in the synthesis of prostaglandins. Furthermore, it was investigated at what point in the pathway the laccase enzyme was active. In the mammalian COX system, arachidonic acid is converted to PGH<sub>2</sub> by COX enzymes then converted to various PGs by specific prostaglandin synthases. PGH<sub>2</sub>, PGG<sub>2</sub> and AA were incubated with the enzyme. It was shown that PGE<sub>2</sub> was only able to be produced when the enzyme was incubated with PGG<sub>2</sub>. Meaning that the laccase is unable to convert the free arachidonic acid to PGG<sub>2</sub> or PGH<sub>2</sub> to PGE<sub>2</sub> suggesting Lac1 does not have a COX or PG synthase activity but that this is new pathway to synthesis PGs (Erb-Downward *et al.* 2008).

### 1.15 Study Objective

*Cryptococcus* secretes PLB1 which has been implicated in *cryptococcal* virulence. *Cryptococcus* has been shown to produce prostaglandins endogenously and from exogenous arachidonic acid. One pool of available arachidonic acid is from the cell membranes of the *Cryptococcus* cell itself but during infection the host cell membranes of phagocytic cells may also be used as a source of arachidonic acid.

Using a *Cryptococcus plb1* mutant in the H99 background that has been identified to produce significantly lower levels of prostaglandins than the H99 strain, we looked at the intracellular proliferation rates inside the mouse macrophage-like cell line, J774 *in vitro*. From previous work done within the lab *plb1* has been reported to have a lower intracellular proliferation rate compared to the wild type and reconstructed strain. Using a standard infection model we looked at the effect PGE<sub>2</sub> has on H99, *plb1* and *plb1<sup>rec</sup>* intracellular proliferation rates to identify whether the reduced intracellular proliferation rate of *plb1* mutant was due to the lower levels of prostaglandins produced.

As identified above, *Cryptococcus* lacks the mammalian enzymes COX-1 and COX-2 responsible for synthesising prostaglandins. However, laccase has been described as a key enzyme in prostaglandin synthesis in fungi as well as melanin synthesis (Erb-Downward *et al.* 2008). Using clinical isolates that have been shown to have high and low levels of laccase activity due to the amount of melanin produced and therefore high and low levels of prostaglandins, we will study the intracellular

proliferation rates of these isolates to see whether any effects we see in the H99 and *plb1* mutant are observed here.

Intracellular proliferation rate is not the only characteristic of macrophage interactions that can be studied. Initial phagocytic uptake has also been implicated in virulence studies. Using imaging techniques, the phagocytic uptake was calculated for H99, *plb1* and *plb1<sup>rec</sup>* strains along with the clinical isolates to identify any trends observed in this area.

Previously observed in the lab is the increase in cell size of *plb1* during infection. Although not reaching the same size as titan cells it has been hypothesised that PLB1 may be involved in titan cell generation. Using live cell microscopy the cell size of *Cryptococcus* strains at 0 hours of infection and after 18 hours with and without the addition of PGE<sub>2</sub> in the growth media was calculated to look at the effect this has on cell size during infection. From this study we hope to identify some of the roles prostaglandin production, specifically PGE<sub>2</sub>, has during *Cryptococcus* infection.

## 2. Methods and Materials

### 2.1 Strains

Murine Macrophage J774.A1

**C. neoformans** var. *grubii*: H99,  $\Delta plb1$  knockout mutant,  $\Delta plb1:PLB1$  reconstituted ( $plb1^{rec}$ ) was created in the H99 background as described in Cox *et al.* 2001. CTP30 and IFN2(402) strains was also used. Stocks were stored at -80 °C and streaked onto YPD (1% yeast extract, 2% peptone and 2% dextrose with 15 g agar per 1 L) agar plates. These were incubated for 48 hours at 25°C allowing *Cryptococcus* colonies to grow before being moved to 4°C for later use. Liquid cultures of *C. neoformans* were grown overnight on a rotator at 25°C in 2 ml of liquid YPD media (1% yeast extract, 2% peptone and 2% dextrose). J774.A1 macrophages were cultured in warmed DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml Penicillin and 100 mg/ml streptomycin, at 37 °C at 5 % CO<sub>2</sub> and was used at passage number of 4 to 15. DMEM was warmed for at least 20 minutes in a 37 °C water bath.

### 2.2 J774 Stocks

Eight flasks of confluent J774's were used for approximately ten 1 ml stocks. The old media was discarded and new warmed (37 °C water bath) complete DMEM (5 ml) was added (2 mM glutamine, 100 units/ml streptomycin, 100 units/ml penicillin). The cells were removed from the flask by scraping. The eight flasks were divided into two 50 ml falcon tubes and centrifuged at 1000 g for 7 minutes. The supernatant was discarded and freezing media was added (50% FBS, 40% complete DMEM and 10% DMSO). Each pellet was re-suspended in 10 mls of freezing media and aliquoted into 1 ml cryovials. These were frozen at -20 °C for 1 hour then -80 °C overnight before being moved to liquid nitrogen for long term storage.

### 2.3 Thawing J774 Stocks

The cryovials were defrosted for approximately 1 minute in a 37 °C water bath. The J774 stock was diluted in 9 ml of complete DMEM media and centrifuged at 1000 xg for 7 minutes. The supernatant was discarded and the pellet re-suspended in 15 ml of complete DMEM media to remove the DMSO present in the freezing media. This was then transferred to a T75 cell culture flask and kept overnight at 37 °C at 5 % CO<sub>2</sub>. Cells were split the next day and were continued to be passaged until passage four was reached.

### 2.3 Intracellular Proliferation Rate Assay

J774 cells were cultured as described in T75 cell culture flasks. Once cells reached confluence the old media was discarded and 15 ml new, warm media was added. Macrophages are scraped from the flask and the number of cells was counted using a haemocytometer. Cells are diluted with warm complete DMEM media so each well of a 24 well plate contained 10<sup>5</sup> macrophages. The plate was incubated overnight at 37 °C and 5 % CO<sub>2</sub>. At the same time *Cryptococcus neoformans* was inoculated into 2 ml liquid YPD media and incubated overnight at 25 °C. 1 ml of *C. neoformans* culture was washed. The culture was centrifuged at room temperature at 6500 rpm for 2.5 minutes, the supernatant discarded in virkon solution and re-suspended in 1 ml of warm sterile PBS. This was repeated three times. After the final wash *C. neoformans* is re-suspended in 1 ml PBS and a 1/100 dilution was taken in PBS. The diluted culture was counted using a haemocytometer and the correct volume for 10<sup>6</sup> *Cryptococcus* per well was calculated and diluted in PBS.

## **Opsonisation and Activation**

0.1 µl per 100 µl of 10 µg/ml of the monoclonal 18B7 antibody was added to the dilute volume of *Cryptococcus* culture. This was incubated for 1 h at room temperature on a rotator. At the same time the macrophages were activated using either LPS or 150 ng/ml PMA. PMA was kept in 5 ml aliquots at -20 °C and was diluted with 495 µl of sterile PBS. 15 µl of this dilution was added per ml of serum free DMEM for enough media for 1 ml per well. The old complete media was removed by an aspirator and 1 ml PMA serum free DMEM was added and incubated for 1 hour at 37 °C at 5 % CO<sub>2</sub>. LPS activation as completed overnight for 24 hours in complete media. 1 µl of 1 mg/ml *E. coli* LPS per 1 ml of DMEM was added and incubated with macrophages overnight. After the incubation time is complete the media containing the method of activation was removed by aspiration and replaced with 1 ml per well warm serum free DMEM media (alone or with appropriate experimental condition such as PGE<sub>2</sub> or ethanol control).

## **Infection**

100 µl of the opsonised *Cryptococcus* culture was added to each well and incubated at 37 °C at 5 % CO<sub>2</sub> for 2 hours. The media was removed by pipetting into virkon solution and the macrophages were washed at least 3 times with warm PBS to remove any un-phagocytised *Cryptococcus*. The end of washing represented the 0 hour time point. The media in the wells for the 0 time point were replaced with 200 µl of sterile water and were left to lyse for 20-30 minutes at 37 °C at 5 % CO<sub>2</sub>. Fresh serum free media (1 ml per well) was replaced on the 18 hour time point wells. When the macrophages had lysed the bottom of the wells were scraped using a 1000 µl pipette tip and was pipetted into a 1.5 ml eppendorf tube. 200 µl of PBS was added to the



empty wells to aid removal of *Cryptococcus* and was pipetted into the previous 1.5 ml eppenderof. 15 µl of this was counted on a haemocytometer. This was repeated at 18 hours to obtain the intracellular proliferation rates of the different strains of *Cryptococcus*.

IPR with addition of PGE<sub>2</sub> or ethanol

For prostaglandin IPR PGE<sub>2</sub> was stored at – 20 °C at 1 mg/mL in filter sterilised ethanol. A 1 in 1000 dilution was made using serum free DMEM and was used as a stock. The ethanol control was conducted by matching the amount of ethanol in the experimental procedure.

During pre-treatment of macrophages and *Cryptococcus* cultures 0.6 ng/ml PGE<sub>2</sub> were co-incubated for 24 hours. This was removed in both cases by washing with PBS before infection. PGE<sub>2</sub> was not re-added to any media during or after infection.

## **2.4 CFU – Viability**

IPR experiments were set up and lysed as before. *Cryptococcus* was counted in the neat lysate using a haemocytometer. The lysate was diluted 1/10 then using this as a stock diluted to get 200 cells in 100 µl. 100 µl of this was spread onto YPD agar (50g YPD; 20g agar per L) and incubated at 25 °C for 48 hours. CFUs were counted. If all cells counted are alive 200 colonies will be present on the plate at 0 hours. This was repeated at 18 hours.

## **2.5 Flow Cytometry**

IPRs were completed as before. 2 µl of 1 mg/ml propidium iodide (PI) was added to 400 µl lysate to give a final concentration of 5 µg/ml of propidium iodide. This was loaded onto the flow cytometer and 30 000 events counted.

**Propidium iodide used in conjunction with Calcofluor.** Propidium Iodide was added as before. 0.8 µl of 1 mg/ml calcofluor was added to the lysate give a final concentration of 2 µg/ml in 400 µl lysate. Lysate was stored in the dark at room temperature for 10 minutes before being loaded for tube measurements on the flow cytometer.

## **2.6 Microscopy**

Live cell movies were taken using bright field microscopy on Nikon Eclipse TE2000 U using a 20X objective at 5 minute intervals for 18 hours in a controlled atmosphere at 37 °C and 5% CO<sub>2</sub>. 3 positions were imaged per well and a minimum of 100 *Cryptococcus* were analysed at 0 and 18 hours per frame. Cell diameter and phagocytic uptake was measured using Nikon Software NIS Elements. AR 3.0. Cell diameter was measured using a circle function, three points were attached to the edge of the *Cryptococcus* yeast cells and a circle was automatically drawn. The diameter was measured from this circle. Phagocytic uptake was measured by counting the number of macrophages in each frame, excluding macrophages that were partially in the frame then counting the number of macrophages that contained one or more *Cryptococci*. From this a percentage was calculated. The average number of *Cryptococci* in each macrophage was also calculated. For this the number of *Cryptococci* in each macrophage was counted and the average calculated. Macrophages with no intracellular *Cryptococci* were excluded.

### **Stills stained with propidium iodide.**

Neat lysate from IPR assays were undertaken as before and stained for propidium iodide at 5 µg/ml. This was pipetted (100 µl) onto microscope slides and covered with

a cover slip. Images were taken using NIKON Eclipse Ti microscope with a QImaging QICAM-B mono camera.

### 3. RESULTS

#### 3.1 Intracellular Proliferation Assays

##### Macrophage Activation: PMA vs LPS

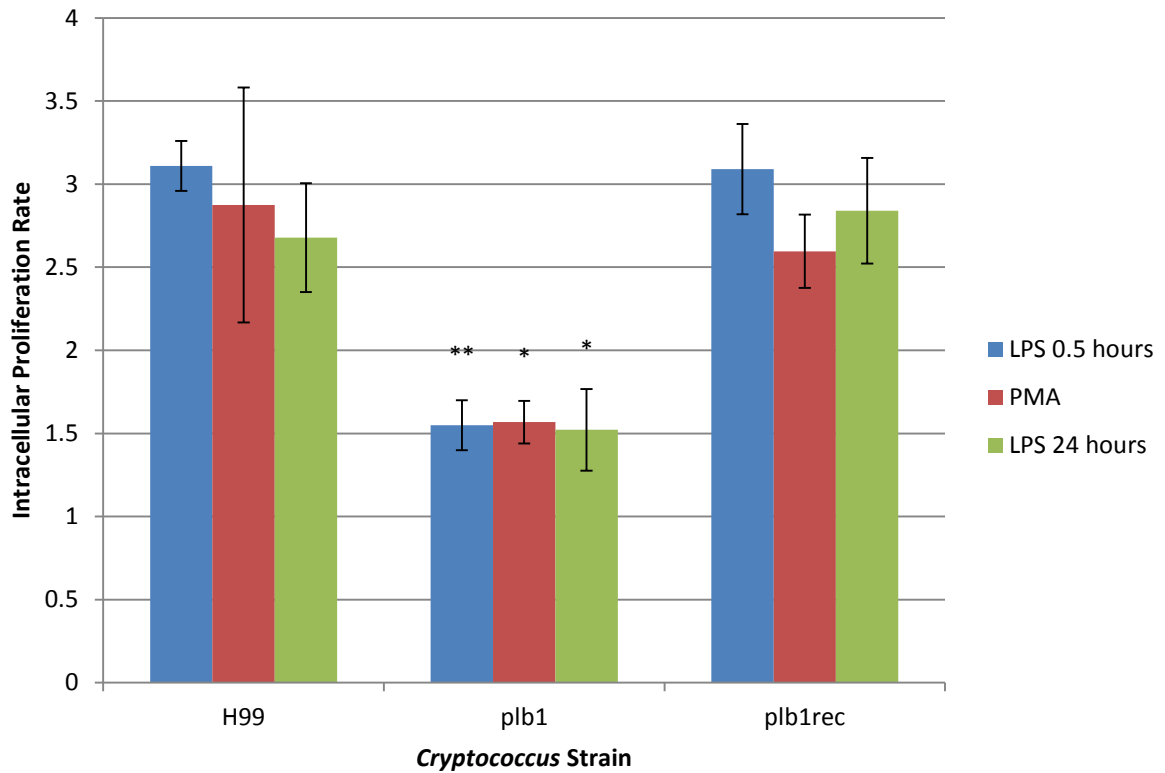
When macrophages are activated by a T helper 1 type response, IFN- $\gamma$  primes the macrophage, but this alone cannot fully activate the cell. The macrophage requires the presence of a foreign signal like bacterial LPS which binds Toll-Like receptors on the macrophage surface, this leads to the production of TNF- $\beta$ . Activation results in the macrophages becoming competent at killing, for example the production of NO for oxidative bursts (Mosser 2003). This response has different roles in protection, and evidence has shown there are at least three different groups of activated macrophages (Mosser 2003). Classically activated, alternatively activated macrophages which have a regulatory role and a clear difference in cytokine profile produced compared to classically activated macrophages. A third subset, Type II activated macrophages has also been identified that again has a different cytokine profile to the two subsets above (Mosser 2003).

Depending on the method of macrophage activation and therefore the type of pathogen it perceives, a different response will be elicited. Firstly we compared a mouse macrophage like cell line activation by bacterial (*E. coli*) lipopolysaccharide and phorbol myristate acetate (PMA) on the effect of *Cryptococcus* intracellular proliferation. PMA is a Protein kinase C activator (PKC) which acts to increase phagocytosis and cytokine production (Basta *et al.* 2001). Briefly, the macrophage like cells were activated with either LPS for 0.5 hour or 24 hours or PMA. *Cryptococcus neoformans* H99, *plb1* and *plb1<sup>rec</sup>* were then added to the macrophages and incubated for 2 hours together. After this, any unphagocytised *Cryptococcus* were removed and

the macrophages were lysed in distilled water at 0 and 18 hours after infection. To calculate the rate of intracellular proliferation (IPR) the number of *Cryptococci* seen at 18 hours is divided by the number seen at 0 hours. An IPR of two corresponds to a doubling of intracellular *Cryptococcus* over the 18 hours. Due to *Cryptococcus*'s large capsule the fungal cell is not very immunogenic, therefore we have opsonised the *Cryptococcal* cells using 18B7 monoclonal antibody.

The *plb1* strain has already been identified in the lab to have a lower intracellular proliferation rate compared to that of the H99 and *plb1<sup>rec</sup>*, indicating its inability to proliferate successfully when inside macrophages.

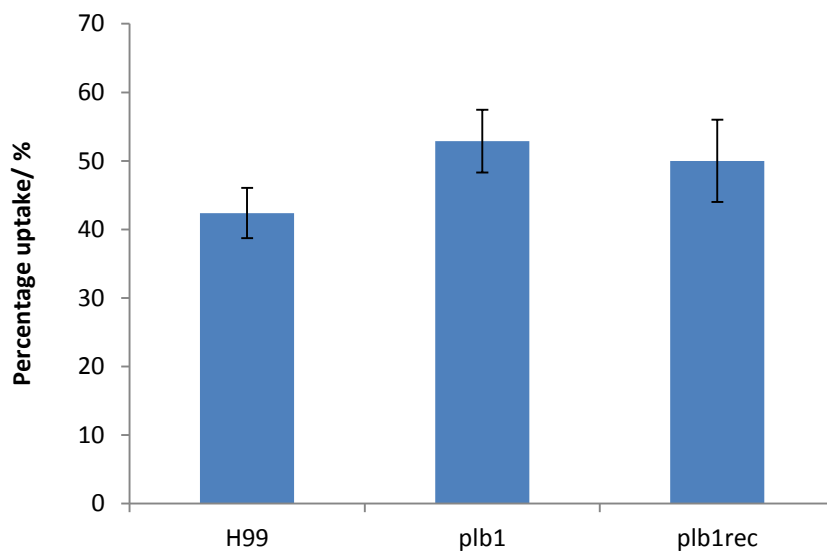
For all subsequent experiments macrophages were activated over 24 hours with LPS. Mosser and Zhang (2008) state 24-72 hour activation with LPS is required for looking at intracellular pathogen killing, and several hours is needed to result in a change in RNA. However, we found no difference in IPR between an incubation time of 30 minutes or 24 hours, as shown in Figure 5. This was also compared to PMA activated macrophages; again there was no difference between PMA and LPS activated macrophages. Therefore, we are confident that the subsequent results are not due to a phenotype produced by specific activation type.



**Figure 5. Activation of macrophages with either LPS or PMA does not shift the intracellular proliferation rates of the H99, *plb1* and *plb1*<sup>rec</sup> Cryptococcal strains. *Plb1* mutant has a significantly lower intracellular proliferation rate to that of the wild type and *plb1*<sup>rec</sup> strain. Student's T-Test was performed on strains compared to the wild type H99. \* = P < 0.05. \*\* = P < 0.001. n≥3. Error bars = ±SEM.**

### Confirmation of *Cryptococcal* strain characteristics

A characteristic of the *plb1* is increased phagocytic uptake by macrophages compared to the *H99* and *plb1<sup>rec</sup>* strains. The initial phagocytic uptake by macrophages with one or more intracellular *Cryptococci* at 0 hours after infection was calculated. As expected, uptake of the *H99* wild type strain was lower than the *plb1*. However, the *plb1<sup>rec</sup>* phagocytic uptake was almost as high as the *plb1* mutant; this could be because the phenotype is only partially rescued as uptake is still lower compared to *plb1* and is unlikely to be of any significance, as shown in Figure 6.



**Figure 6. *plb1* and *plb1<sup>rec</sup>* are phagocytised by macrophages more readily than H99.** Graph shows the percentage of macrophages containing one or more *Cryptococci*. Student's T-Test was performed. No significant differences were identified. N=3. Error bars =  $\pm$ SEM

## Effect of PGE<sub>2</sub> on *Cryptococcal* Intracellular Proliferation Rate

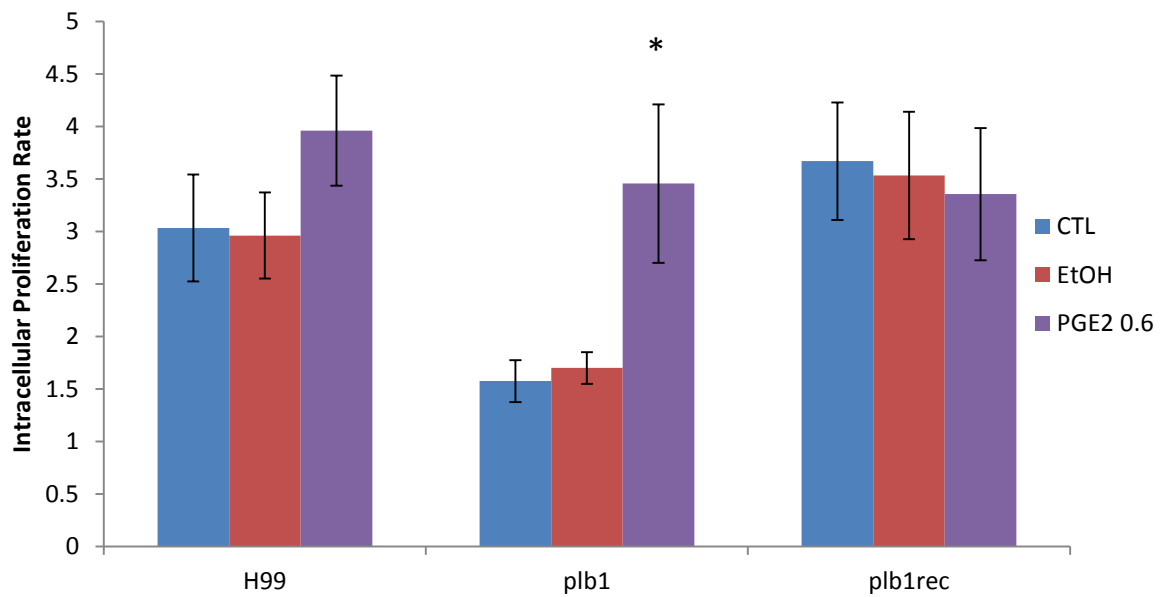
As mentioned previously, PGE<sub>2</sub> is an important lipid immune mediator involved in inflammatory responses in mammals. PGE<sub>2</sub> is also naturally produced by *Cryptococcus neoformans* and other fungal pathogens. PLB1 cleaves arachidonic acid from phospholipids in the cellular membrane. This results in an increase in the precursor for prostaglandin synthesis, including PGE<sub>2</sub>. Noverr *et al.* (2001) estimates the amount of prostaglandins in tissues at the site of infection is around 0.65 nM. The addition of PGE<sub>2</sub> to splenocytes at this level has produced a measurable change in cytokines produced (Noverr *et al.* 2001). In the following experiments 0.6 ng/ml (1.7 nM) of PGE<sub>2</sub> was used to look at the effect on intracellular proliferation rate of *Cryptococcus*.

With the addition of 0.6 ng/ml PGE<sub>2</sub> during infection the intracellular proliferation rates were calculated for H99, *plb1* and *plb1*<sup>rec</sup>. As seen in Figure 3, in the presence of additional PGE<sub>2</sub> the H99 strain of *Cryptococcus* exhibited a slight increase in IPR. More dramatically, when PGE<sub>2</sub> was added to the *plb1* mutant infection model the IPR was significantly higher compared to the control and was rescued back to the level of the wild type H99 strain. This suggests PGE<sub>2</sub> is vital to intracellular proliferation of *Cryptococcus*, however, whether this is due to prostaglandin acting on the macrophages or the *Cryptococcus* is unknown.

PGE<sub>2</sub> is dissolved in 100% ethanol and stored at -20 °C. Although the amount of ethanol added with the PGE<sub>2</sub> is slight (13 nM) it is important to rule out that the ethanol in the sample is having this effect on the IPR. As presented in Figure 7, there is no difference between the ethanol controls compared to the control with no additives.



From this, we are confident the result seen is due solely to the prostaglandin in the sample and not the solvent used.

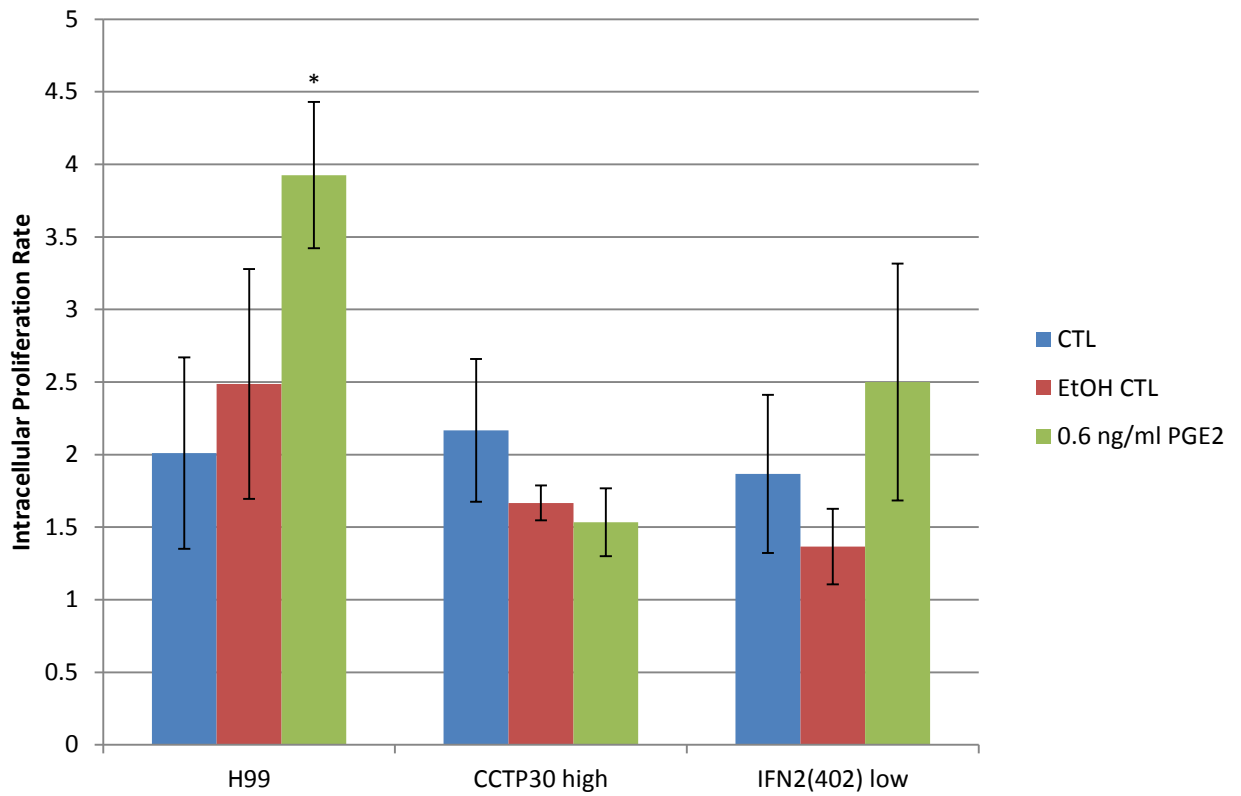


**Figure 7. PGE<sub>2</sub> rescues the intracellular proliferation rate of *plb1* to the level of the wild type strain, H99.** Student's T.TEST was performed using a two-tailed, un-paired test compared to the control condition. \* = P<0.05. n≥7. Error bars = ±SEM

## Effect of PGE<sub>2</sub> on *Cryptococcus* expressing high and low laccase activity

As mentioned previously, laccase has been implicated in prostaglandin synthesis in *Cryptococcus*. Using two clinical strains that have been previously identified to have high and low melanin production, CCTP30 and IFN2(402) respectively and therefore high and low laccase activity as laccase also synthesises melanin (W. Sabiiti. 2013. Raw data). These two strains have IPRs of around two, similar to that of the H99 strain in *in vitro* experiments (W. Sabiiti.2013. Raw data).

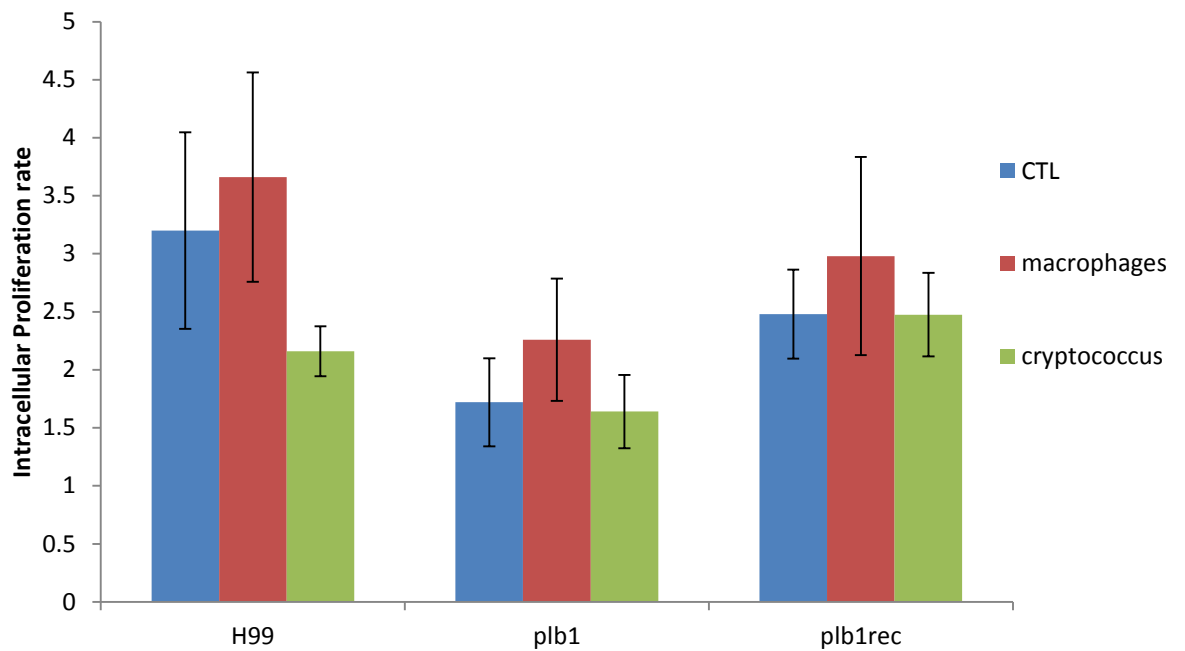
These strains were treated with 0.6 ng/ml PGE<sub>2</sub> during infection, as before. As shown in Figure 4 we see an increase in the IPR in of the H99 strain when treated with PGE<sub>2</sub>, as seen before. Interestingly, we find that the strain that exhibits low melanin production (IFN2(402)) shows an increased IPR when treated with PGE<sub>2</sub>. However, the strain that has high laccase activity displays no difference between the control and PGE<sub>2</sub> treated. This suggests that IFN2(402) has a greater increase in IPR compared to CCTP30 because of the low melanin production and therefore low laccase activity. This shows similar results to that seen with the H99 and *plb1* strains; with H99 with normal levels of prostaglandins produced and *plb1* mutant exhibiting low levels of prostaglandin production. However, the changes seen in Figure 8 are not as substantial as those seen in H99 and *plb1* experiments.



**Figure 8. PGE<sub>2</sub> increases intracellular proliferation rates of the wild type *Cryptococcus* and *Cryptococcus* with low laccase activity.** Intracellular proliferation rates of *H99* wild type strain compared with two clinical isolates which exhibit high and low melanin production. CCTP30 has a high melanin production and IFN2(402) has low melanin production rate. N=3. Error bars =  $\pm$  SEM. Student's T-test was performed. \* =  $P < 0.05$ .

### **PGE<sub>2</sub> affects both the macrophages and the *Cryptococcus***

To determine whether the effect of prostaglandins was solely limited to effects on the macrophages, both macrophages and *Cryptococcus* were pre-treated with 0.6 ng/ml PGE<sub>2</sub> for 24 hours before infection. The PGE<sub>2</sub> was removed during and post infection. It was found that macrophages that had been pre-treated with PGE<sub>2</sub> before infection and incubated with non-pre-treated *Cryptococci* had a slight increase in IPR, similar to the results seen when PGE<sub>2</sub> was added during infection, though not as high, possibly due to the effect of the PGE<sub>2</sub> 'wearing off' during infection. Interestingly, when the *Cryptococci* were pre-treated with PGE<sub>2</sub> a marked decrease in the IPR of the *H99* strain was observed, although no difference in the *plb1* or *plb1<sup>rec</sup>*. Looking at the effect of the same amount of ethanol pre-treatment it was found this had no effect on the IPR compared with the control, as shown below (Appendix 1).

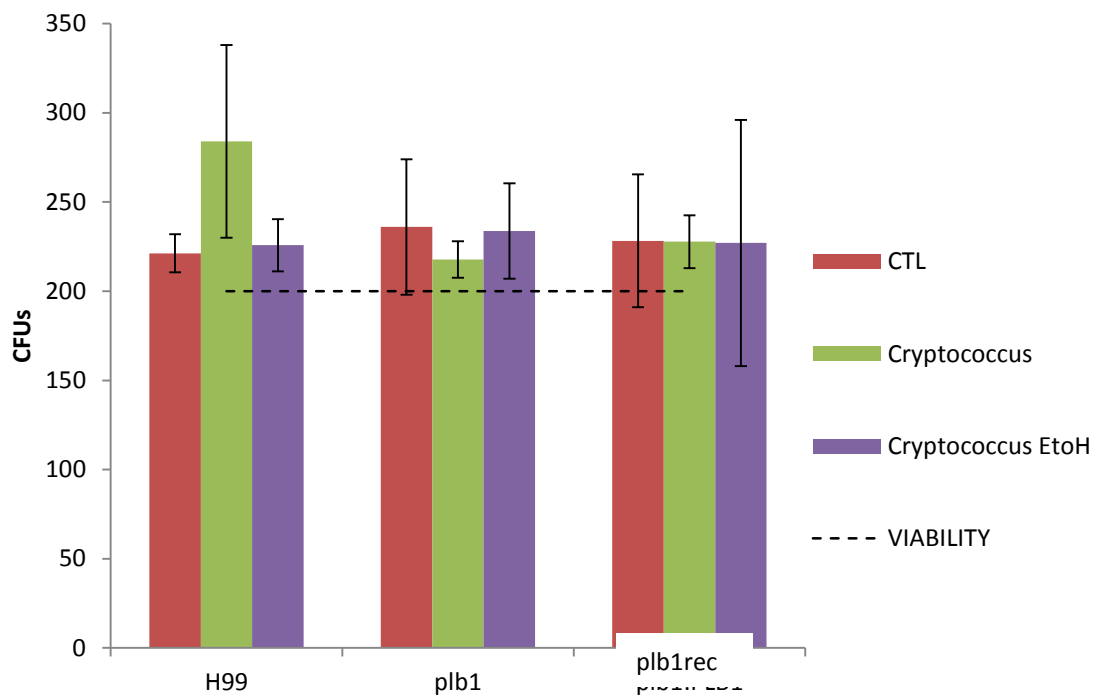


**Figure 9. Pre-treatment of macrophages increases IPR in all strains; whereas, pre-treatment of *Cryptococcus* decreases IPR of H99 strain, but not others.** The effect of pre-treatment with 0.6 ng/ml PGE<sub>2</sub> on macrophages and *Cryptococcus*. Ethanol has no effect on IPR in either case (Appendix 1). ANOVA: single factor was performed; no significant differences were found. N=2 error bars =  $\pm$ SEM.

### **Pre-treatment of *Cryptococcus* does not affect viability**

The decrease in IPR seen in Figure 9 in the H99 strain may have been due to *Cryptococci* death. To identify *Cryptococcal* death after 24 hours incubation with ethanol viability counts were undertaken. Briefly, the number of 200 cells per 100  $\mu$ l of lysate was counted on a haemocytometer. This was plated on YPD agar plates. If less than 200 colonies were present on the plate all cells that were counted were viable. It was found that there was no cell death during the pre-treating condition of the *Cryptococci* at 0 hours as shown in Figure 10. This showed that the 24 hour pre-treatment with the PGE<sub>2</sub> or the ethanol was not having a detrimental effect on the *Cryptococci* viability. Further to this, during macrophage pre-treatment the macrophages were observed to identify any unusual amounts of cell death and no cell death was identified. However, it would also be advantageous to study the viability at 18 hours infection to see if the effects were more detrimental over a longer time period.

Although the amount, per ml, of PGE<sub>2</sub> in the cultures is the same as before, the local concentration may be higher during the *Cryptococcal* pre-treatment due to the fact that during infection the *Cryptococci* are within macrophages and contact with the prostaglandins may be partially buffered by the molecules having to diffuse through the macrophage membrane.



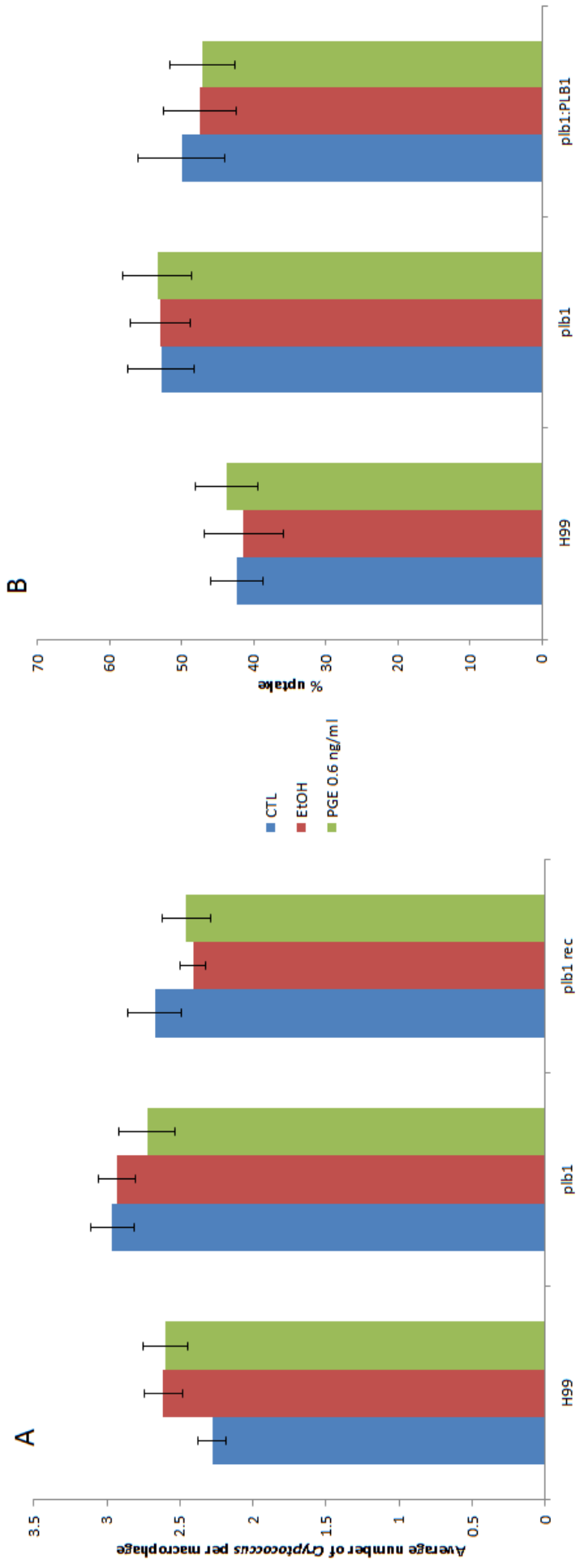
**Figure 10. PGE<sub>2</sub> and Ethanol does not cause *Cryptococcus* cell death after 24 hour incubation. N=2. Error bars = ± SEM. ANOVA: single factor was performed; no significant differences were found.**

### 3.2 Initial Phagocytic Uptake

#### **PGE<sub>2</sub> does not affect initial phagocytic uptake of *Cryptococcus***

PGE<sub>2</sub> has been identified in down-regulating killing functions and phagocytic action of macrophages. To further investigate the role of PGE<sub>2</sub> during *Cryptococcal* infection, the percentage uptake by macrophages containing one or more *Cryptococci* and the average number of *Cryptococci* per macrophage was calculated. As already shown, we know that *plb1* has a slightly increased phagocytic uptake than *H99* shown in Figure 6. Figure 11 shows there is no significant difference between any of the control and experimental conditions and that the ethanol solvent or the PGE<sub>2</sub> has no effect on the phagocytic uptake of the *Cryptococcus*.

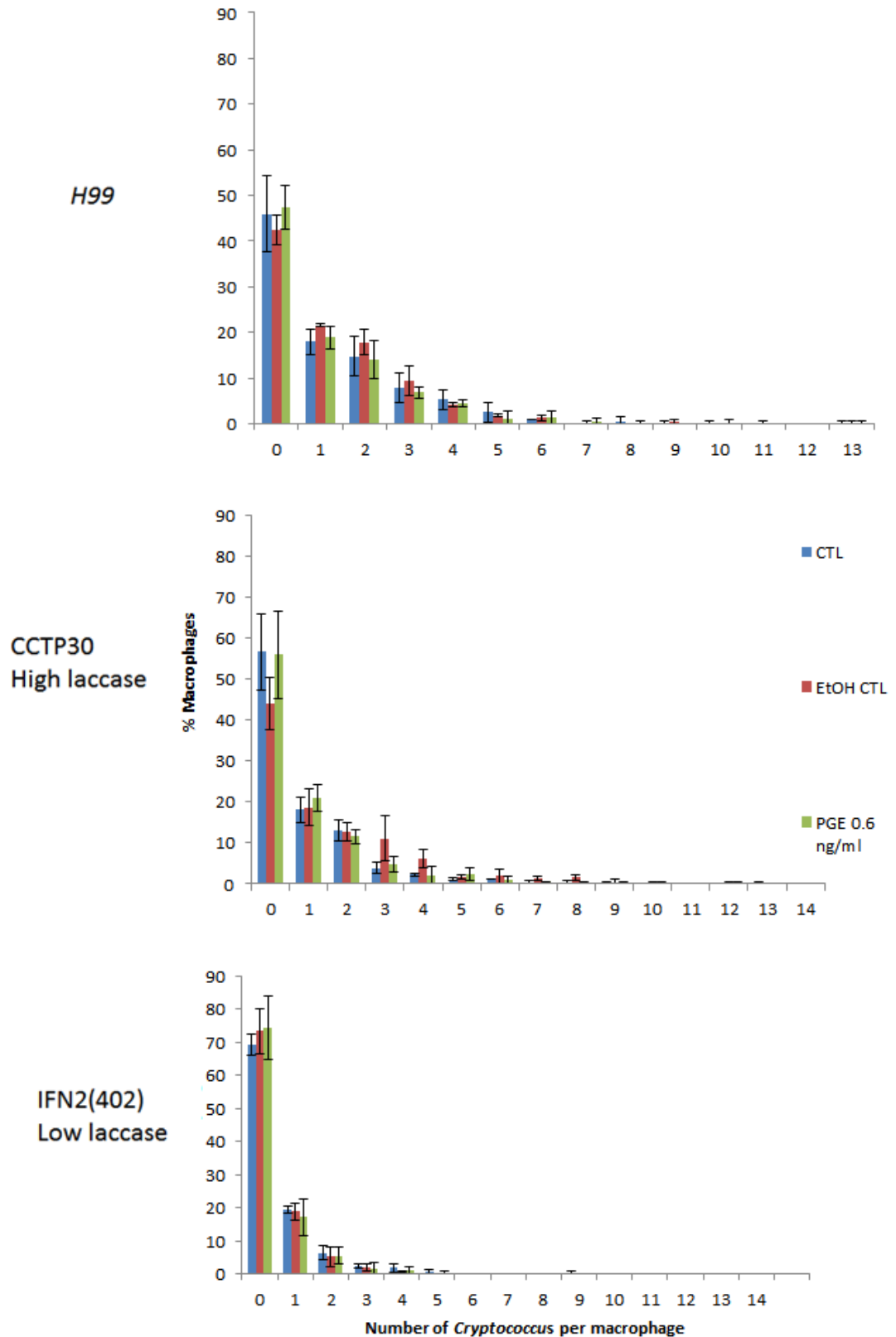




**Figure 11. PGE<sub>2</sub> has no effect on the uptake of *Cryptococcus* by macrophages.** A. The average number of *Cryptococcus* taken up by macrophages. B. The percentage of macrophages with one or more *Cryptococcus* inside. N=3. Error bars = ± SEM. Anova: single factor performed, no significant differences were found.

## **PGE<sub>2</sub> has no effect on phagocytic uptake in strains with high and low laccase activity**

Although we have shown that PGE<sub>2</sub> increases IPR in certain conditions, recent evidence has found increased IPR does not correlate to increased virulence in *C. neoformans*, and increased phagocytic uptake is more important in relation to virulence (Sabiiti *et al.* 2014). This is probably due to the increased burden associated with increased phagocytic uptake rather than proliferation after the *Cryptococcus* has been phagocytised (Sabiiti *et al.* 2014). However, we found that although there were differences in uptake between the strains as shown in Figure 8, for instance H99 strain has a greater uptake in terms of percentage and average number of *Cryptococci* per macrophage than both clinical isolates, PGE<sub>2</sub> had no effect on the individual strains.



**Figure 12. PGE<sub>2</sub> does not influence phagocytic uptake of *Cryptococcus* in strains with low and high laccase activity.** n=1. Representative of one experiment in which 3 images per condition were analysed. Error bars = ±SD. ANOVA: single factor was used; no significant differences were found between the controls and the experimental conditions for any of the strains.

### 3.3 Viability

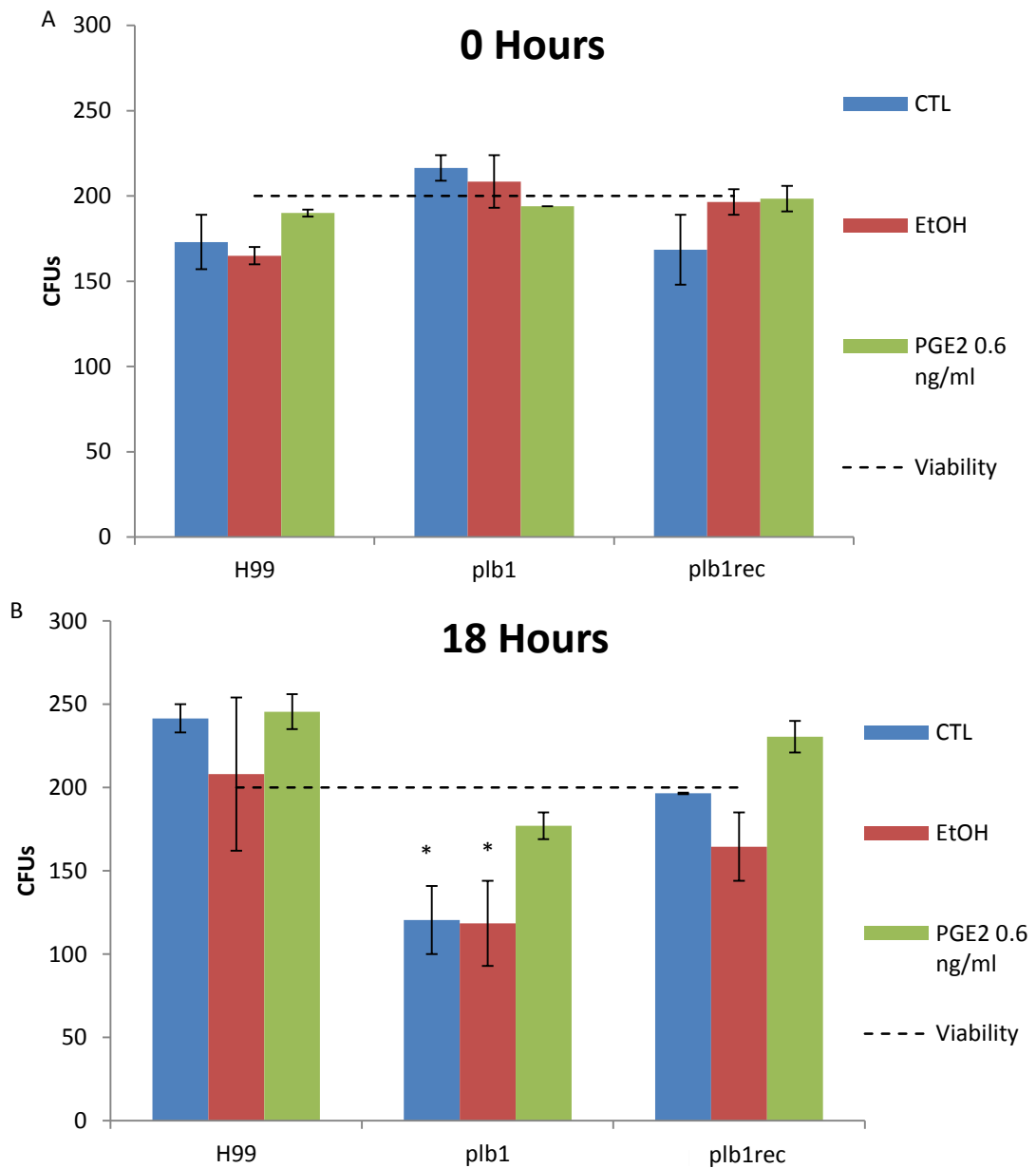
#### **PGE<sub>2</sub> increases the long-term viability of *plb1***

Using live cell imaging we have not seen any compelling evidence for macrophage cell death over 18 hours during exposure to PGE<sub>2</sub>. To identify whether long-term exposure to PGE<sub>2</sub> has an effect on *Cryptococcus* viability during infection, colony forming units were undertaken as before and viability was measured at 0 and 18 hours after infection. Briefly, if 200 colonies was present at each time point it represented that all cells counted by used of a haemocytometer were viable, any less indicated *Cryptococci* cell death.

At 0 hours the colony forming units of all strains including with the addition of PGE<sub>2</sub> and ethanol are around 200 and therefore viable as shown in Figure 13A. Interestingly, at 18 hours the viability of *plb1* is drastically reduced by 40% meaning only 60% of the cells identified by the haemocytometer are viable. This tells us the intracellular proliferation rates for *plb1* are significantly over estimated and should be near halved to include the presence of non-viable cells. Nonetheless, the *H99* and *plb1<sup>rec</sup>* strains remain viable at 18 hours post infection.

Interestingly, when PGE<sub>2</sub> is added to the *plb1* infection cell death decreases compared to the control measure with viability being decreased by 40% and 41% in the control and ethanol control respectively and only 12% with the addition of PGE<sub>2</sub>. However, viability is not increased to that of the wild type or the reconstructed strain and cell death is still present. Be that as it may this result continues to support the evidence that PGE<sub>2</sub> production is critical for intracellular *Cryptococcus* survival and proliferation as shown in the proliferation rate studies. Although, it must be stated this

is representative of only one experiment of duplicate plates and must be repeated to validate the results and the differences seen are not statistically significant.



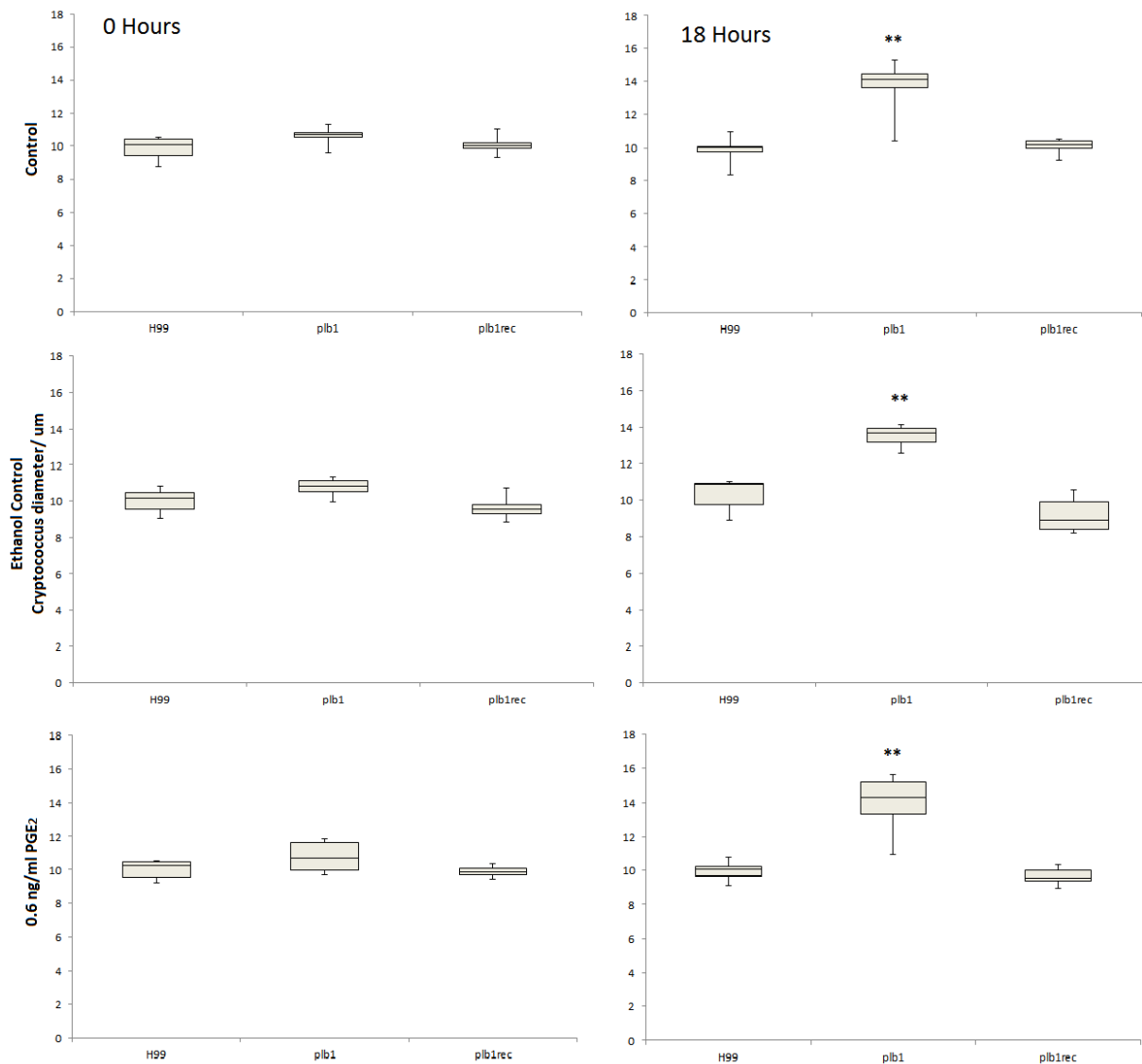
**Figure 13. PGE2 increases viability of plb1 over 18 hours.** Cell viability at (A) 0 and (B) 18 hours. *plb1* shows significantly decreased cell viability at 18 hours compared to *H99* and *plb1<sup>rec</sup>*. Representative of one experiment using two plates. Counts below 200 shows cell death. N=1. Error bars = ± SEM. ANOVA: single factor and Student's T-Test was performed. \* = P < 0.05.

### 3.4 Cell size

#### ***plb1* increases in cell size during infection**

Above we have found the addition of PGE<sub>2</sub> rescues some of the adverse characteristics associated with the *plb1* mutant including the intracellular proliferation rate and long-term viability. However, to further investigate the effect of PGE<sub>2</sub> on other characteristics of *plb1* live cell imaging was used to capture the *Cryptococcus*/macrophage interactions and allowed measurement of the change in cell diameter of the *Cryptococci* over 18 hours of the *H99*, *plb1* and *plb1*<sup>rec</sup> strains.

Previously discovered, *plb1* increases in cell size in the intracellular environment during infection (R. Evans. Unpublished data. 2014). As shown in Figure 14, the cell size of each strain is around the same at 0 hours between 4 and 6 µm in diameter. However, at 18 hours we get an approximately 30-40% cell diameter increase in the *plb1* strain in all conditions, including with the addition of PGE<sub>2</sub>. As shown in the box plots below the range of the cell sizes of *plb1* in the controls and experimental condition are the roughly same with the range of the minimum cell sizes of the *plb1* conditions at 18 hours being 4.5 – 5.8 µm and the maximum cell size ranging from 6.5 – 7.2 µm. Moreover, the skew of the data is fairly symmetrical in all cases. These shows there are the same proportion of the larger and the normal sized cells in each condition.

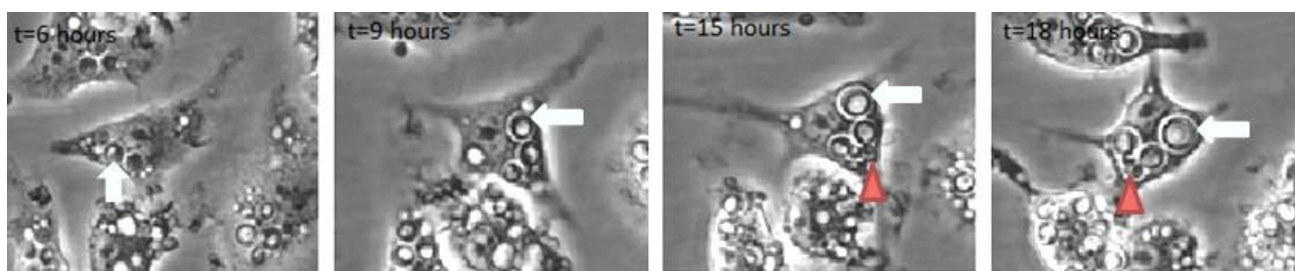


**Figure 14. Addition of PGE<sub>2</sub> does not change the cell diameter seen in *plb1*.** Representative of 3 experiments, 3 points of each condition were viewed and analysed. ANOVA: single factor was conducted at the two time points. Differences were found at 18 hours. Student's T-Test was then conducted using two-tailed hypothesis type 2. \*\* =  $p < 0.001$ . Middle line represents median. Error bars represent minimum and maximum cell diameter. N=3.



### Cell size increase of *plb1* and proliferation

Furthermore, it has been observed that the *Cryptococci* that increases in size tend not to bud and form daughter cells (R. Evans. Unpublished data. 2014). Using live cell imaging, macrophages were infected with the wild type, *plb1* mutant and *plb1<sup>rec</sup>* strains. Similar results were seen with the cells that clearly increased in size during in the infection did not tend to bud (data not shown). Correspondingly, during infection with the addition of exogenous PGE<sub>2</sub> comparable results were found in that the clearly larger *Cryptococcus* cells did not tend to form daughter cells. Figure 14.1 represents an example of this phenomenon. In figure 14.1 the grey arrow follows a *Cryptococci* cell increasing size by 35% over 12 hours but not budding and two smaller *Cryptococci* within the same macrophage budding to produce two daughter cells.



### 3.5 Flow Cytometry

**Figure 14.1. Representative image showing that *plb1* cells that increase in size do not proliferate; this is unchanged with the addition of PGE<sub>2</sub>.** Grey arrow shows the 35% cell size increase of *plb1* in the presence of PGE<sub>2</sub> and does not bud to form daughter cells. Red triangles show two smaller *Cryptococci* that bud to form a daughter cell each. This is also seen in control conditions.

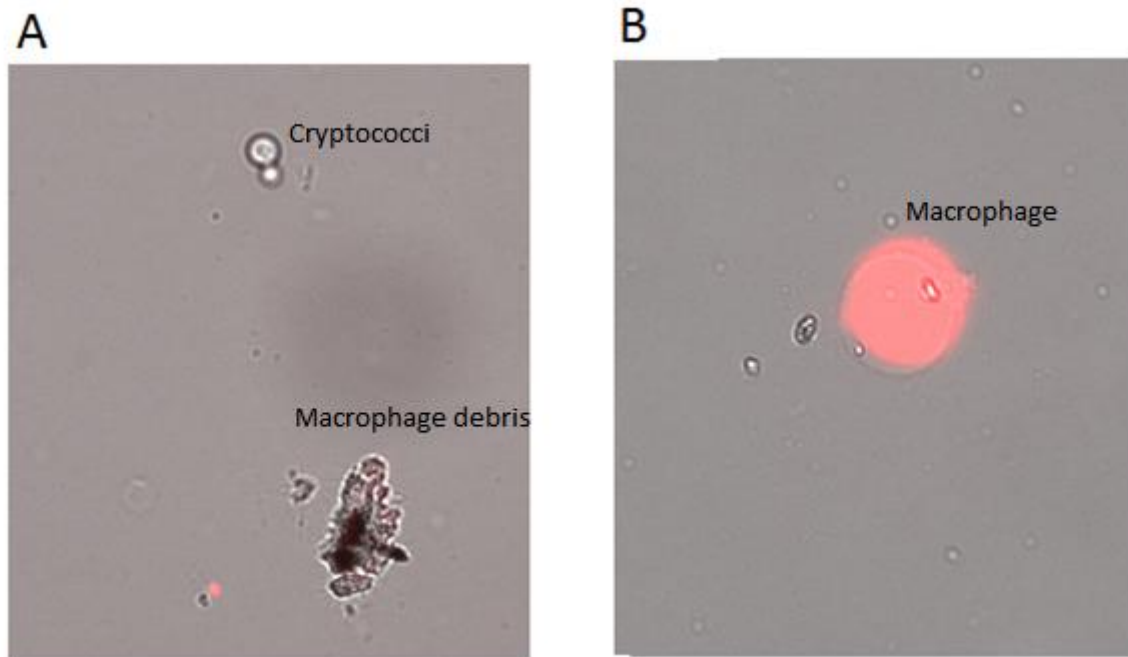
#### Rapid method of IPR calculation

The main limiting factor of these experiments is the time taken to count the number of *Cryptococcus* using a haemocytometer. Each condition is counted twice at both 0 and 18 hours; this can take an appreciable amount of time for a simple experiment. Therefore, a more efficient method to count cells using flow cytometry was investigated. Our lysate samples contain the *Cryptococcus* and the macrophage debris during macrophage lysis. To be able to count the *Cryptococcus* cells accurately a method to identify the yeast cells from the macrophage debris was needed. Initially, propidium iodide (PI) was used. PI is a red fluorescent dye that binds double stranded DNA in non-viable cells, and is excluded from live cells, and should bind to the macrophage debris and any dead *Cryptococcus* cells to act as a viability detector as well.

However, there was no clear divide between to the two populations (Appendix 2) and PI does not bind all the macrophage debris as shown in Figure 15. From GFP

tagged *H99* experiments (R. Evans. 2014. Unpublished raw data) the rough area *Cryptococcus* yeast cells are present have been identified (Appendix 2). In spite of this another issue needed addressing. As shown in Figure 14, *plb1* mutant increases in cell size over the 18 hours infection. This means we cannot be as sure of the location of the *Cryptococcus* at 18 hours, as shown in Appendix 3 we do see a slight shift in the scatter to the right, but cannot gate accurately around it.

Another problem faced with this method was that the signal from the propidium iodide was not as fluorescent at 18 hours. This could be due to the macrophages being older and slightly more damaged. Further, the *H99* and *plb1<sup>rec</sup>* are continuing to produce PLB1 which damages the host cell's membranes and could potentially increase cell lysis making the cells in this condition more fragmented which does not bind propidium iodide. It is only the more intact bloated cells that really take up the dye well shown in Figure 15B but does not bind viable *Cryptococcus* shown in Figure 15A.



**Figure 15. Propidium binding of macrophage debris and not *Cryptococcus* cells.** 5 µg/ml final concentration of propidium iodide was added to neat lysate samples. A shows propidium iodide does not bind viable yeast cells but only binds partially to fragmented macrophage debris. B shows propidium iodide binding a bloated macrophage cell. During lysis with distilled water some macrophages do not completely fragment and stay largely intact.

### Flow cytometry using propidium iodide and calcofluor

In view of the partial success of propidium iodide binding macrophage debris we further investigated the use of PI in conjunction with Calcofluor White. Calcofluor White is a dye that binds chitin found in the fungal cell wall. Using this method we were able to separate the two populations much more effectively than before (Appendix 3).

However we find three separate populations not just *Cryptococcus* yeast cells and macrophage debris. This may be because during cell lysis not all the yeast cells are fully released from the macrophages and are still 'held' by the macrophage, so this population is stained highly for both propidium iodide and Calcofluor. Another

possibility is that these are non-viable *Cryptococcus* cells that have become permeable to propidium iodide and therefore is highly stained for both dyes but it is unknown which population this is and may be a mixed population of both (Raw data shown in Appendix 4) making it a problem as to whether to include this population in the counts of yeast cells.

Using gating of only the population we are confident is only live *Cryptococcus* cells we have seen small increases in IPR in conditions treated with PGE<sub>2</sub> but none as considerable as results obtained by haemocytometer counts (data not shown).

## 4. Discussion

### 4.1 Overall conclusions

#### PGE<sub>2</sub>

This project explores the importance of prostaglandins in the interaction between *Cryptococcus neoformans* and macrophages *in vitro*. Prostaglandins *in vivo* are synthesised mainly from arachidonic acid, amongst other fatty acid precursors. These precursors are released from phospholipids in the plasma membranes by phospholipases. The *Cryptococcal plb1* mutant lacks PLB1 and has previously been shown to produce significantly less prostaglandins than the wild type strain, H99 and the reconstructed strain *plb1<sup>rec</sup>*, specifically PGEs (Noverr *et al.* 2001). Although the *plb1* mutant has no defects in growth or capsule production when grown in media, it does not proliferate inside macrophages to the same extent of the wild type, as shown in Figure 5. By adding exogenous PGE<sub>2</sub> back into the system during infection the intracellular proliferation rate was rescued to that seen by H99. Furthermore, as shown by the viability of the *plb1* mutant at 18 hours, survival is approximately 60%. This shows that the IPR rates for *plb1* are much higher than in actual fact. However, survival of *plb1* in the presence of PGE<sub>2</sub> is much higher at around 88%. This confirms that the rescue in intracellular proliferation rates we see in the *plb1* with PGE<sub>2</sub> is legitimate. From this it was concluded that PLB1 and prostaglandins play an important role in the *Cryptococcus* cell cycle and the ability to proliferate, and survive, after phagocytosis by macrophages.

Although PGE<sub>2</sub> rescues the effect of decreased IPR in the *plb1* mutant we do not see a rescue in other characteristics. After 18 hours it has been documented that *plb1* mutant increases in cell size. This is possibly due to a dysregulation in the cell

cycle in a stress environment; the cells get bigger and duplicate the genome but are never able to bud. However, it is unknown how PLB1 is involved in this eventuality. Even with the addition of PGE<sub>2</sub> there was no difference in cell size compared to the control conditions.

Studying the images produced by live cell imaging over 18 hours suggests that the cells that did increase in size also did not bud to form daughter cells; however, there were also a number of average sized cells which continued to bud. This would only occur in a stressful environment, such as, inside macrophages, as no cell growth defects (Noverr et al. 2001) have been seen when *plb1* is grown in media. This suggests that the increase of IPR we see is due to the increased survival of the *plb1* mutant. Alternatively, this could be the result of faster proliferation of the normal sized *Cryptococci*.

### **Laccase**

The experiments involving strains with a low and high laccase activity support the evidence that shows PGE<sub>2</sub> is important in intracellular proliferation for *Cryptococcus neoformans*. *H99* has been reported to make PGE<sub>2</sub> endogenously. When extra exogenous PGE<sub>2</sub> is added (0.6 ng/ml) an increase in its intracellular proliferation rate is observed. Using two clinical isolates of *C. neoformans* with a high and low melanin production, and therefore assumed high and low laccase activity respectively, no increase in intracellular proliferation is observed in the strain with high melanin production when exogenous PGE<sub>2</sub> is added during infection. However, the strain with low melanin production does exhibit a slight increase in IPR when PGE<sub>2</sub> is added during infection.

This could be due to the high laccase activity strain already producing a high amount of prostaglandins and adding extra PGE<sub>2</sub> will not make a considerable difference to the prostaglandin levels. However, when we look at the strain with low laccase activity we see a small increase in IPR when PGE<sub>2</sub> is added. This could be a similar effect we see in the *plb1* mutant. The strain with low laccase activity is hypothesised to produce less prostaglandin than the strain with high laccase activity and the exogenous PGE<sub>2</sub> added boosts the amount of PG more. Although this is not as considerable as that seen by *plb1* mutant as this strain has been shown to produce approximately 100 pg/ml during culture in SDB (Noverr *et al.* 2003).

The two laccase strains were picked because they have similar IPRs as each other and the H99 strain. This was done so any low IPRs were not mistaken for an effect by the laccase, as previously mentioned IPR is not the best method to use to test virulence. These strains also have similar growth rates (Sabiiti. Unpublished data. 2013). The aim was to get two strains with as many similarities except the amount of melanin produced and therefore high or low laccase activity so any effects seen were not due to any other characteristics the individual strains possessed.

However, it must be stated that the actual activity of laccase and PG production in these strains is unknown and the assumption on laccase activity is based on melanin production (as measured by W. Sabiiti). The actual prostaglandin production would need to be confirmed to validate these results.

We also wanted to investigate whether PGE<sub>2</sub> has an effect on the *Cryptococcus* as well. We found that pre-treatment of the H99 strain reduced the IPR, although not killing the *Cryptococcus* after 24 hours incubation with PGE<sub>2</sub>, the effect on long term survival also needs to be investigated. Although the concentration was the same per ml as that



during infection, 0.6 ng/ml, the concentration perceived may be higher. During infection, *Cryptococcus* is phagocytised by the macrophages. This means PGE<sub>2</sub> is required to diffuse through another membrane before it gets to the yeast cells. But the effect seen could be due to *Cryptococcus* being able to sense the concentration of PGE<sub>2</sub> and therefore not producing more. Further experiments could include the use of PG receptor inhibitor. If the receptors are inhibited on the macrophages then the IPR should go back to the control level, unless PG is having an effect on the action of the *Cryptococcus*.

## 4.2 EXPERIMENTAL PROCEDURES

### Macrophages *in vitro*, the best model?

*In vivo* macrophages are very dynamic and have a plastic phenotype having several different activated phenotypes. However, *in vitro* much of this plasticity is lost. It would be interesting to see whether experiments conducted using primary human monocytes produced similar results to those seen in mouse macrophage like cell line used here.

Along these lines we have studied the intracellular proliferation rates of different strains using different methods of macrophage activation. Depending on what macrophages are activated with can result in a different set of cytokines released. We compared the IPR of H99, *plb1* and *plb1*<sup>rec</sup> when activated with PMA and LPS. Since no significant difference was seen we are confident that any of the effects seen are not just specific to LPS or PMA phenotype.

## **Haemocytometer versus flow cytometry**

During this research project a method for rapid cell counting using flow cytometry was investigated. Although the haemocytometer is more time-consuming than flow cytometry it is probably more accurate than our current flow cytometry protocol for counting non-fluorescently tagged yeast cells. Several issues have been identified with this, despite using propidium iodide and Calcofluor to separate the two populations of yeast cells and macrophage debris, the results obtained from this have been significantly different from those obtained from haemocytometer counts. This is probably due to inexact population separating and errors in gating.

As well as a third population has been identified exhibiting a high level of staining for both propidium iodide and Calcofluor. This could be a population of non-viable yeast cells that have become permeable to propidium iodide. Alternatively, as sometimes seen during haemocytometer counts, the macrophages do not completely release the intracellular yeast cells during lysis and remain 'trapped' in a clump inside the macrophage. It is unknown from the data collected which population this might be and could be a mixed population of either.

Furthermore, different results have been obtained depending on how the samples are being measured, either by the plate reader high throughput analysis or the tube reader (data not shown). From this it was concluded that the haemocytometer reads were the most reliable method of cell counting for non-tagged yeast cells.

## **Prostaglandin Stability**

Prostaglandins are metabolised quickly in vivo with PGE<sub>2</sub> having a half-life of around 30 seconds when in the blood stream (www.Uscn. 2009). However, in vitro

PGE<sub>2</sub> is present for a much longer time. Maddipati *et al.* (2011) quantified the stability of prostaglandins in common cell culture media including PBS and RPMI at different temperatures. PGE<sub>2</sub> is fairly stable at 4 °C over 3 days but at 37 °C the levels of PGE<sub>2</sub> halved over 24 hours. Since the IPR assay takes 18 hours it is certain that there will be a decrease in the concentration of PGE<sub>2</sub> over this time.

It is estimated that the levels of PGs in tissue during infection is around 0.65 nM (Noverr *et al.* 2001) compared to around 0.008-0.034 pM concentrations normally in plasma (www.uscn. 2009). During the experiments above a concentration of 1.7 nM was used. This is slightly above double that estimated to be present during infection but from stability studies of PGE<sub>2</sub> it is likely that over the 18 hours during the IPR experiments the level of PGE<sub>2</sub> will halve.

To identify what concentration of PGE<sub>2</sub> should be used an IPR assay was completed using different concentrations of PGE<sub>2</sub> ranging from 0.4 ng/ml to 1.8 ng/ml to study the effect of PGE<sub>2</sub> on the macrophages and H99 strain. These concentrations were chosen from estimations of the difference between the amount of prostaglandins produced by H99 and *plb1* strain as quantified by Noverr *et al.* (2003) and higher concentrations to study if high quantities damaged the cells. During IPR assays containing 0.9 ng/ml PGE<sub>2</sub> and above significant macrophage cell death was recorded (data not shown) and no change in IPR was recorded at 0.4 ng/ml. For the above experiments 0.6 ng/ml was used as it was identified as enough to produce a change in IPR but low enough to remain physiologically relevant.

## Intracellular Proliferation Rate on Clinical Outcome

Recently, evidence has been published that shows that the intracellular proliferation rates of *Cryptococcus neoformans* is not correlated with virulence. Testing IPR against clinical outcome it was found IPR was not correlated with negative clinical outcomes in *C. neoformans* but is for *C. gattii* (Sabiiti *et al.* 2014). However, phagocytic uptake is correlated with virulence for *C. neoformans*. Sabiiti *et al.* (2014) suggests that increased phagocytic uptake is associated with increased burden in the cerebrospinal fluid (CSF) and poor clinical outcome, independent of whether the *Cryptococcus* was opsonised with mAb 18B7. Although a lot of experiments into *Cryptococcal* virulence have been conducted in rodent studies, Sabiiti *et al.* (2014) has looked into the virulence factors of *C. neoformans* and the resulting clinical outcomes of patients with HIV-associated *Cryptococcosis*. This is an interesting point as greater phagocytic uptake, while more virulent in a clinical sense would not be advantage to *Cryptococcus* in the environment. Sabiiti *et al.* (2014) suggest this might show a subset of hypocapsular *Cryptococci* that are more readily taken up but exhibit an increased intracellular survival.

Although our data shows an increase in IPR with PGE<sub>2</sub>, the addition of the prostaglandin has had no effect on percentage phagocytic uptake or the average number of *Cryptococci* per macrophage suggesting PGE<sub>2</sub> may not have a direct role in virulence but is more important for *Cryptococcal* survival and correct cell cycle progression after phagocytosis.

Sabiiti *et al.* (2014) went on to find that laccase activity is associated with increased phagocytic uptake and *ex vivo* survival of *Cryptococcus* from cerebrospinal

fluid and lowered rate of clearance after the starting treatment. This process is melanin independent suggesting it must be another process laccase controls. Although we cannot say it is prostaglandin mediated, it does suggest this. Our results confirm that the low laccase strain, IFN2 (402) had significantly lower percentage uptake than the high laccase strain, Figure 12, and therefore likely to be less virulent. But again, PGE<sub>2</sub> had no effect on the phagocytic uptake but did increase the IPR of the low laccase strain suggesting PGE<sub>2</sub> is somehow increasing the rate of budding or cell survival.

An important outcome of this paper is that it confirms that *Cryptococcal* and phagocytic interactions of the innate immune system has a meaningful role in the clinical outcome of patients, underpinning the importance of research in this area.

### **4.3 Further Experiments**

#### **Biomarkers**

Currently diagnosis for cryptococcal infections is testing of the cerebrospinal fluid and blood samples (Galanis *et al.* 2009) and even this is unable to tell the difference between *C. neoformans* and *C. gattii* for which further testing must be done. It would be advantageous to create simpler and faster methods of testing. For this, since eicosanoid synthesis plays a key role in cryptococcal virulence it would be interesting if eicosanoid profiles could be used as biomarkers for *Cryptococcal* infections.

Additionally, if PGE<sub>2</sub> is in fact pushing the immune response towards a Th-2 type response, eicosanoids could be a potential target for therapies. With less prostaglandin in the system it may allow the host to better fight of the infection.

## **Mouse models**

Currently there are mouse models that exist that lack the different type of eicosanoid receptors (Legler *et al.* 2010). It would be compelling to study *Cryptococcal* infections in mice that lack the EP2/4 receptors to see changes in the outcome of the infection.

## **Laccase**

It would be interesting to see the laccase strain CFUs were similar to that if *plb1*, in that viability decreased over 18 hours in the low laccase activity and if this was increased with the addition of PGE<sub>2</sub>.

Furthermore, it would be interesting to study the IPR of the wild type strain in the presence of a laccase inhibitor such as sodium azide (Johannes and Majcherozyk. 2000) to see if this had a similar effect as the *plb1* mutant at lowering the IPR. From this we could support the evidence that a loss of a laccase controlled product was what was causing the characteristics we see in the *plb1* mutant.

## **Shift to Th-2 response**

After T cells are activated they differentiate into either Th-1 or Th-2 type effector cells that differ in cytokines produced or effects on immune regulation. It is recognised that prostaglandins affect macrophages and that PGE<sub>2</sub> is generally seen as a pro-inflammatory agent that down regulates macrophage killing functions. PGE<sub>2</sub> also promotes the production of cytokines associated with a non-protective Th-2 type response, such as IL-10 and inhibits differentiation and proliferation of dendritic cells that activate the Th1 branch of the immune system. There is a positive feedback system associated with PGE<sub>2</sub>. As PGE<sub>2</sub> is produced, this stimulates COX-2 activity that produces more. It is possible that PGE<sub>2</sub> produced by the *Cryptococci* can then

stimulate the production of more PGE<sub>2</sub> in mammalian hosts and therefore cause this shift in the immune system towards the Th2 response. However, to study this would require animal studies.

#### **4.4 Conclusion**

As mentioned previously, macrophage/*Cryptococcus* interactions have important implications on the clinical outcome of the patient and study into this area is important for a better understanding of the disease. Although IPR is not directly associated with virulence it does give an insight into the role of prostaglandins in the cell cycle and intracellular survival of *Cryptococcus*. PGE<sub>2</sub> is required for *Cryptococcus* to survive intracellularly in macrophages and proliferate and may play a role in pushing the immune system towards a Th-2 type response by increasing production of IL-10 and suppressing TNF- $\alpha$ .

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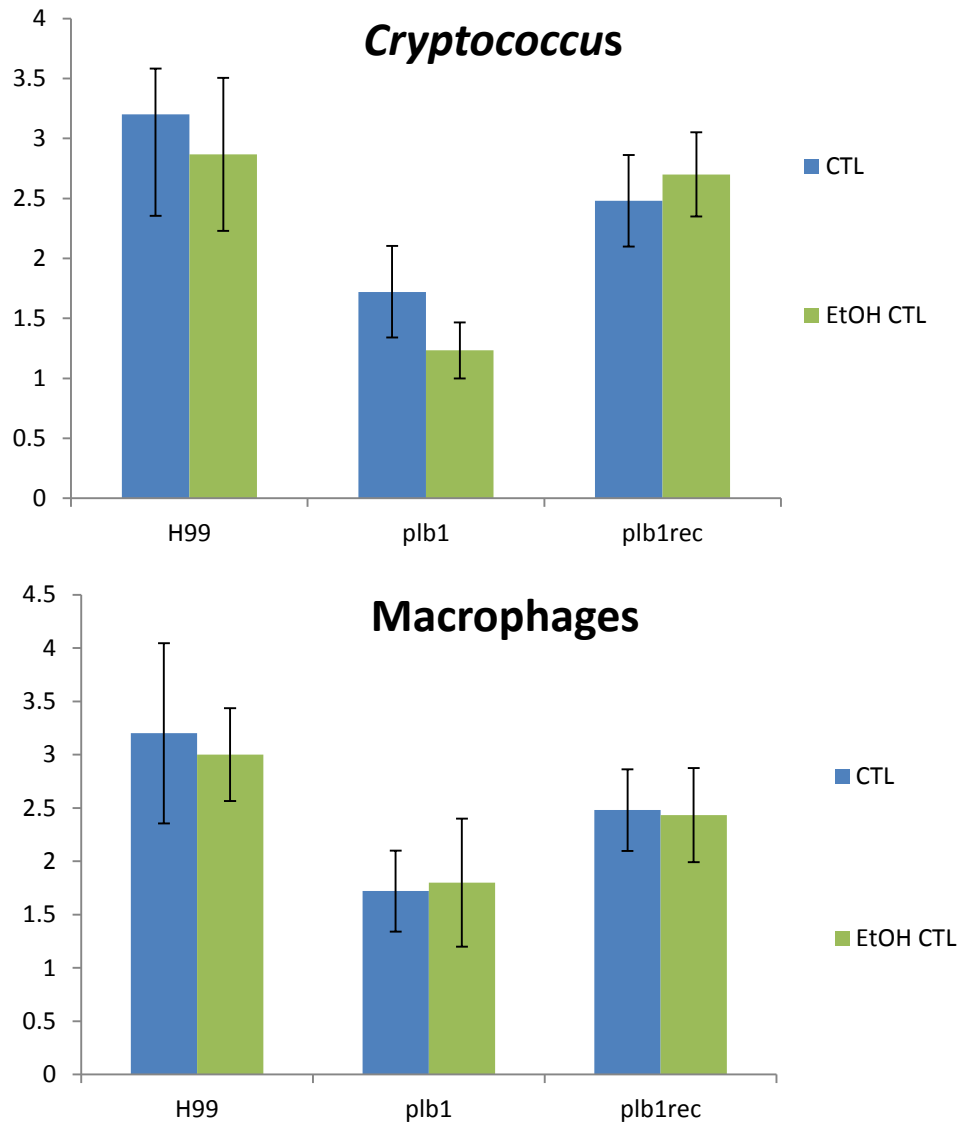
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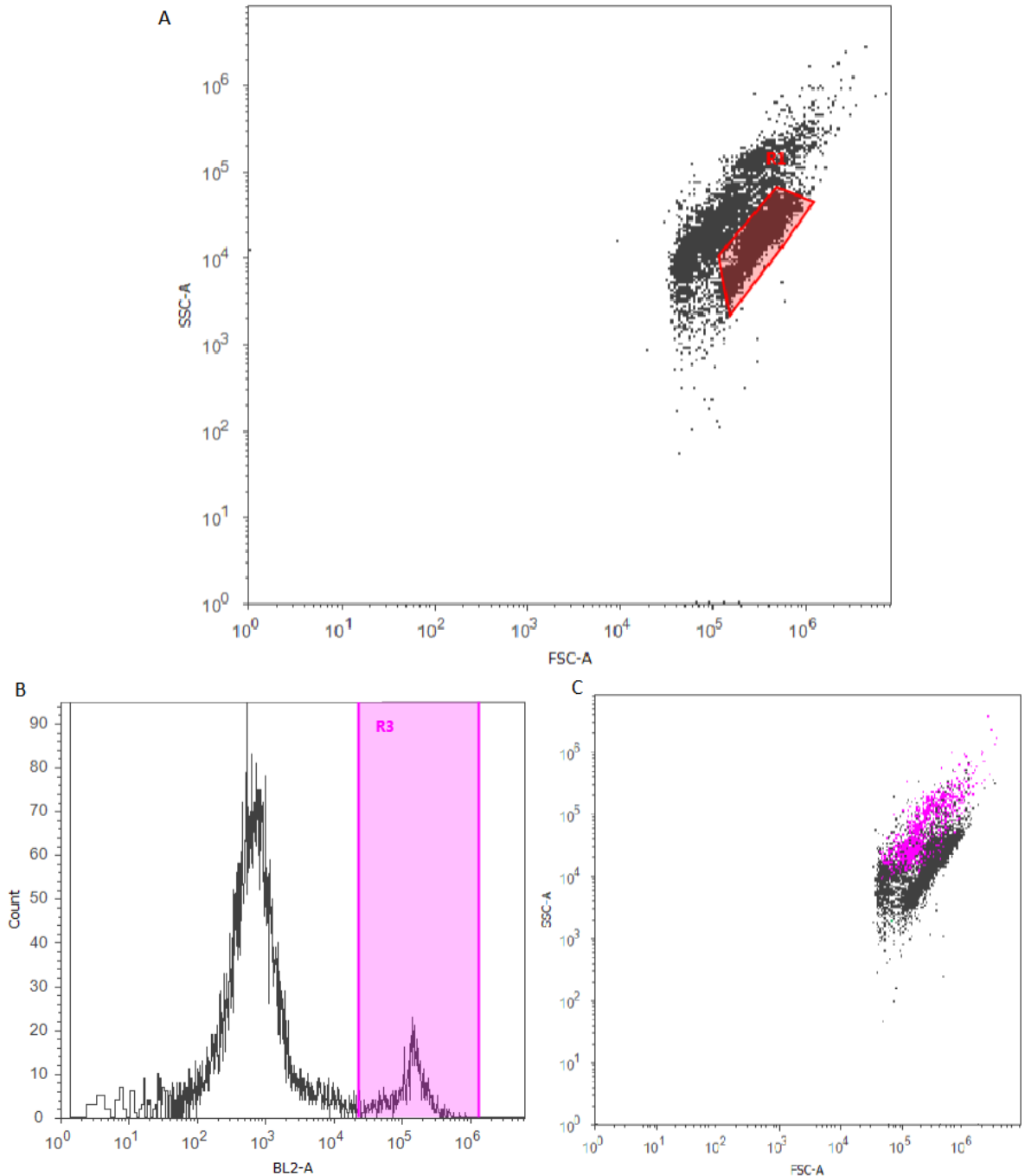
## Appendices

**Appendix 1.** Ethanol does not have an effect on the pre-treatment of macrophages or *Cryptococcus*.



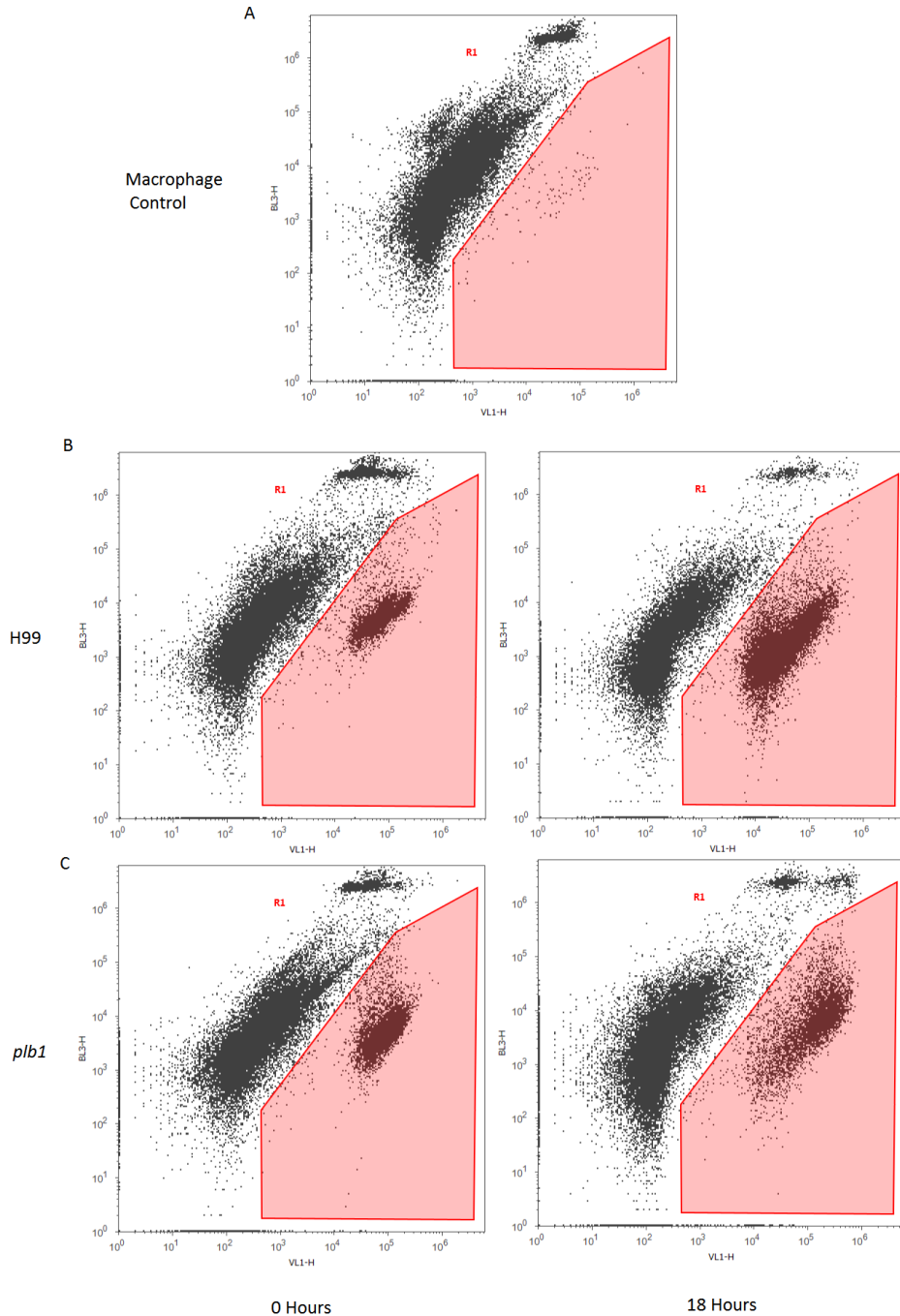
**Appendix 1. Ethanol controls during PGE<sub>2</sub> pre-treatment of macrophages and *Cryptococcus* cultures.** ANOVA: Single factor was undertaken and no significant differences were found between the control and the ethanol control.

## Appendix 2. Propidium Iodide does not stain all macrophage debris



**Appendix 2. Propidium iodide is not enough to distinguish between macrophage debris and yeast cells.** A. From previous GFP-tagged studies this shows the gating of where *Cryptococcal* yeast cells are located in the whole lysate sample. B. Histogram plot with R3 gating the propidium iodide peak of the whole lysate. C. Previous dot plot showing R3 gate, this shows R3 peak does not include all the macrophage debris and there is a significant portion still unstained.

**Appendix 3. Propidium iodide and Calcofluor separate macrophage debris and yeast cells better than propidium iodide alone.**



**Appendix 3. Propidium iodide and Calcofluor separates *Cryptococcus* yeast cells and macrophage debris. A.** Macrophage lysate with no fungal yeast cells. **B.** H99 macrophage lysate at 0 and 18 hours post infection. **C.** *plb1* macrophage lysate at 0 and 18 hours post infection in which an increase in size can be seen at 18 hours compared to the H99 sample. Red gate indicates *Cryptococcus* population used for IPR analysis.